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Spectrophotometric Determination of Vitamin A by Means of Chromatography

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At the time being, three methods are available for the chemical determination of vitamin A: (1) the spectrophotometric method in which the absorption spectrum of vitamin A is measured and the concentration calculated from the height of the maximum in the 325—328 mμ range; and two colorimetric methods, (2) the Carr-Price reaction with antimony trichloride which gives a blue colour, and (3) the reaction with glycerol dichlorhydrin giving a red colour.

For the determination of vitamin A in substances containing very small amounts of light absorbing substances besides vitamin A, the spectrophotometric method will generally be preferred mainly for the following reason. When measuring the absorption curve in a wave length region around the absorption maximum the specificity of the measurement can be checked by comparison of the curve obtained with one for pure vitamin A. A control of this type cannot be made when using the colorimetric methods; an additional drawback of the Carr-Price reaction is the fact that the measurement cannot be performed with as high an accuracy as can the direct absorption measurement on vitamin A: The reaction with antimony trichloride is very unstable — in the course of 10 seconds the colour has reached its maximum and begins to fade. The reaction with dichlorhydrin does not show this defect but, on the other hand, it has not yet been tested sufficiently on different materials to allow the conclusion in which cases it can be regarded as satisfactorily specific.

Obviously, certain requirements must be put forth as to the course of the absorption curve in order that the spectrophotometric method can be used without correction. Unfortunately, however, none of the substances containing vitamin A usually available can completely satisfy our requirements in this respect. Nevertheless, for many years this method has been used and regarded

as the most satisfactory determination of vitamin A in fish liver oil. At the Second International Conference on Vitamin Standardization in June 1934, it was decided to acknowledge the application of this method, and to calculate the amount of vitamin A in international units per gram from $E^{1\%}_{1\,cm}$ at 328 mμ after multiplication by 1 600. As is well known, at that time the international unit of vitamin A was the biological effect of 0.6 μg β-carotene on rats; thus, the value of the conversion factor had to be based on comparisons between the biological effect on rats and the measured extinction for different vitamin-oils. In the course of time, the correctness of the factor 1600 has been under dispute, but it can now be regarded as well established that application of the factor 1600 is justified. In reality, the absorption spectrum of very few fish liver oils corresponds completely to the spectrum of pure vitamin A, a slight unspecific absorption is observed. Therefore, a correction of the measured extinction is always required, in accordance with the fact that the factor chosen is somewhat below the conversion factor for pure vitamin A found in recent years, namely ca. 1800.

The situation is somewhat changed since August 1949, when WHO has accepted the new international unit of vitamin A[1] as the biological effect of 0.344 μg vitamin A acetate, corresponding to 0.3 μg vitamin A alcohol. In fact, the conversion factor for spectrophotometric measurements can now be given disregarding the great uncertainty of biological determinations, because it is possible from the extinction coefficient of pure vitamin A to calculate the conversion factor to be 1894. It was decided to apply the factor 1900 which thus holds for absolutely pure vitamin A, contrary to the factor applied earlier which was used when working with fish liver oil and based on comparisons between biological and spectrographic investigations on this material. The new factor can therefore be applied only when the measured curve deviates but slightly from the accurately determined curve of pure vitamin A. The limits for permissible deviations at different wavelengths were established. When the deviations are greater than permissible a correction is required. If the deviations are not too great, *i. e.* if the measured curve reaches its maximum at the right wave length of 325—328 mμ, a correction is introduced according to the calculation suggested by Morton and Stubbs [2]. When the deviations from the vitamin A curve are considerable, Morton and Stubbs' method of correction will not lead to the correct result in most cases. This means that a spectrophotometric determination cannot be applied directly after saponification. A separation of vitamin A from interfering impurities has to be carried out. This separation can be performed by means of chromatography.

In recent years, chromatography has been applied rather frequently to a partial isolation of vitamin A, mainly in connection with determinations according to the Carr-Price method. Very recently, chromatography has been used in connection with the spectrophotometric determination of vitamin A. Gridgemann, Gibson and Savage [3] have published such a method for the investigation of whale liver oil; the same technique was applied by Barua and Morton [4].

However, the method of chromatographic-spectrophotometric determination of vitamin A described below, which for some time has been used in a great number of investigations, seems to have several advantages over the method outlined by Gridgemann and coworkers. Partly, it was found applicable to most substances containing vitamin A, partly much smaller quantities of vitamin A are required and, finally, it is more convenient as a routine method. In the investigations discussed here, calcium diphosphate has always served as an adsorber in the chromatography of fish liver oil. In the determination of substances containing less vitamin A it has been necessary sometimes to use the stronger adsorber aluminum oxide which was also applied by Gridgemann. In general the adsorbent applied should be as weak as possible, since this leads to the most effective separation.

EXPERIMENTAL

Prior to the chromatographic determination proper, the vitamin A must of course be brought into solution, *i. e.* it must be extracted from the present material; since, moreover, in the method here described it was considered preferable to avoid working with both vitamin A ester and vitamin A alcohol, saponification is necessary so that all of the vitamin A is present as alcohol.

When investigating fatty substances such as fish liver oil, vitamin A concentrates or alike as well as butter, saponification is performed directly with about twice the amount of alcoholic potassium hydroxide (ca. 2 *N* KOH in 80 % alcohol) subsequent addition of water and three times extraction with ether in the separatory funnel.

Solid material such as foddermixtures containing vitamin A and different vitaminized food products are also saponified with alcoholic potassium hydroxide; subsequently, the insoluble component is filtered off by means of a glass filter, boiled again twice with alcoholic posassium hydroxide until complete extraction of vitamin A is obtained.

The collected alcohol extracts are diluted with water and then extracted three times with ether in a separatory funnel.

To investigate the vitamin A content in milk equal parts of alcohol are added and the fat soluble constituents are extracted with ether in a separatory funnel. The ether is evaporated and, subsequently, saponification is performed with alcoholic potassium hydroxide and followed by renewed ether extraction in the separatory funnel.

In all materials investigated it was found of importance for the chromatography to remove as completely as possible fatty acids from the ether extract. Washing of the extract with water, as is usual in vitamin A determinations, was not sufficient. Instead, the following procedure is recommended: The extract is first washed once with water, then once or twice with $N/2$ KOH in 30 % alcohol and, finally, washed twice with water.

Subsequently, the washed ether solution is dried by filtration through a layer of dehydrated sodium sulphate and evaporated to dryness in a CO_2-atmosphere, the last traces of ether being removed at room temperature by means of a CO_2-stream.

The evaporation residue is dissolved in a suitable volume of petroleum ether and this solution is then used for chromatography.

Chromatography

Apparatus:

1. Glass tubing, for the adsorption column upper part 12 mm inner ø, 100 mm long, lower part ca. 8 mm outer ø, 50 mm long. Inserted with rubber stopper onto an ordinary suction flask.

2. U.-V. lamp, Philips HPW 120, emitting ca. 90 % of the light at 365—66 mμ and only ca. 1 % visible light.

Reagents:

1. Petroleum ether; boiling point below 70°.

2. Ethyl ether, peroxide free: peroxides are removed by 1—2 days storage with KOH and subsequent distillation.

3. Di-calcium phosphate. In chosing this reagent two properties have to be taken into consideration, *viz.* its adsorption capacity and grain size.

With regard to the adsorption capacity the following will be convenient. 300—400 μg pure vitamin A adsorbed in a column of the tube, dimensions given above, should move only slightly when 100 ml pure petroleum ether are poured through the column, while 100 ml of a petroleum ether — ethyl ether mixture (50 : 1) causes complete eluation.

With respect to the grain size it must be required that the adsorbent is not packed so tightly in the column that it is difficult to filter the solutions through in the course of a reasonable time. In a column of the above dimensions a sample of calcium phosphate which during suction with a suction

pump allows ca. 100 ml petroleum ether to pass in the course of 5—10 minutes can be used; it is possible, however, to obtain a preparation allowing a higher speed.

4. Aluminum oxide. When using commercial preparations meant for chromatography, an excellent filtration rate will always be obtained; therefore it is only necessary to specify requirements as to adsorption capacity. When pouring 25 ml of a petroleum ether-ethyl ether mixture (4 : 1) through the column of the above dimensions, 300—400 μg vitamin A should move only a few cm, while 25 ml petroleum ether-ethyl ether mixture (1 : 1) should eluate the vitamin A completely.

The adsorption capacity of both dicalcium phosphate and aluminum oxide can be increased by heating for some time to 100—110° C, it can be decreased by exposure to air at room temperature.

Procedure:

The column used for chromatography is prepared in the following way.

As a support of the adsorbent some scoured cotton wool is placed at the transition from the broader to the narrower part of the adsorption tube. Then the adsorbent is introduced into the tube in small portions suspended in petroleum ether. Each time the powder is allowed to settle and then pressed together by suction. However, the tube must never be sucked dry, some petroleum ether must always remain in the tube. The tube is filled with the adsorbent to 1—1 ½ cm from the top and then the column is ready for chromatography.

A convenient quantity of the extract in petroleum ether prepared as described above and corresponding to 150—600 μg vitamin A (in exceptional cases down to 50 μg) is sucked through the column and then washed with 25—50 ml pure petroleum ether. The column is inspected in U—V light where the vitamin A should be visible as a yellowish green fluorescent zone in the column. If such a zone is not visible, the reason may be that the solution contained too little or no vitamin A or that the adsorption capacity of the adsorbent used is too poor so that the vitamin A has run through the column. In the last mentioned case a more efficient adsorbent has to be applied.

If the vitamin A is adsorbed satisfactorily to the dicalcium phosphate, the chromatogram is developed by continued washing with a petroleum ether — ethyl ether mixture (50 : 1) until the yellowish green fluorescent vitamin A zone appears on the very bottom of the column, however, without any vitamin A having escaped. Then a clean suction flask is inserted and a mixture of petroleum ether-ethyl ether (50 : 1) is added until all vitamin A is eluated. This fraction is transferred to a volumetric flask of suitable size which is filled up with petroleum ether so that the solution contains 2—4 μg vitamin

A per ml. This solution can be used directly for the determination of the absorption curve in a Beckmann spectrophotometer. Evaporation of the chromatographed solution should be avoided, since it has been found that in a solution of vitamin A in petroleum ether unspecifically absorbing substances may be formed on evaporation which cause a deviation of the absorption curve from that of pure vitamin A.

If, however, vitamin A from the solution available for chromatography cannot be adsorbed on dicalcium phosphate, aluminum oxide must be used. Then, development of the chromatogram and eluation of vitamin A is performed, using other mixtures of petroleum ether-ethyl ether, starting, for example, with 25 ml 20 : 1, then 5 : 1, 3 : 1, 1 : 1 and changing the mixing ratio in the given sequence until the vitamin A moves at a suitable rate. When the vitamin A zone appears at the very bottom of the column, the suction flask is changed and eluation is continued with the last employed mixture until all vitamin A is eluated.

If dicalcium phosphate is found not to adsorb the vitamin A while aluminum oxide appears to be too strongly adsorbing, it is suggested to apply dicalcium phosphate which has been heat treated more efficiently than normal. In this case, a higher content of ethyl ether than the normally used 1/50 should be tried in the eluation mixture. In all cases, however, the solution must be brought to a volume giving — without evaporation — a concentration of vitamin A suitable for the measurements.

EXPERIMENTAL RESULTS

It is the aim of the experimental work presented here to determine the applicability of the method to the determination of vitamin A. The investigations were made partly on pure vitamin A, in order to detect the loss during chromatography, partly to demonstrate the applicability of chromatography to the purification of vitamin A from solutions containing unspecific, absorbing substances.

Experiments with pure vitamin A*

A prerequisite for the application of the method outlined above is the knowledge of the absorption curve of pure vitamin A in the employed mixture of petroleum ether and ethyl ether. Therefore, measurements were performed

* The vitamin A applied was produced synthetically and kindly put at the disposal of the State Vitamin Laboratory by Messrs. Hoffmann—la Roche, Basel, via Messrs. Wørum and Co., Copenhagen.

of solutions of known quantities of pure vitamin A in petroleum ether, ethyl ether, and absolute alcohol, respectively, the latter being the generally used solvent in vitamin A spectrophotometry. In Table 1 the results of such measurements are shown together with the measurements on pure vitamin A in absolute alcohol reported by Morton and Stubbs [5].

Table 1. Absorption curve for vitamin A in different solvents.

Wave length mμ	Petroleum ether		Ethyl ether		Abs. alcohol		Morton and Stubbs $\frac{E}{E_{325}}$ in abs. alcohol
	$E_1^{1\%}{}_{cm}$	$\frac{E}{E_{325}}$	$E_1^{1\%}{}_{cm}$	$\frac{E}{E_{325}}$	$E_1^{1\%}{}_{cm}$	$\frac{E}{E_{325}}$	
280	420	0.230	260	0.141	404	0.224	0.224
290	687	0.376	616	0.333	662	0.368	0.414
300	1 077	0.589	1 064	0.575	1 042	0.578	0.609
310	1 533	0.838	1 562	0.844	1 497	0.832	0.844
320	1 734	0.949	1 765	0.955	1 708	0.948	0.950
325	1 828	1.000	1 851	1.000	1 800	1.000	1.000
328	1 787	0.977	1 788	0.967	1 764	0.980	
330	1 732	0.947	1 728	0.934	1 704	0.964	0.957
340	1 325	0.725	1 319	0.713	1 335	0.742	0.772
350	819	0.448	782	0.423	824	0.457	0.509
360	441	0.241	409	0.221	458	0.255	0.278

The measurements indicate that the difference between the absorption curve of vitamin A in different solvents is only small in the range 310—350 mμ, which is the range in which the requirements of the new international rules for the determination of vitamin A must be fulfilled. Therefore it seems superfluous to take into consideration the deviation which appears when calculating the concentration of vitamin A on the basis of the absorption curve measured in the mixture of petroleum ether and ethyl ether. It should be mentioned that petroleum ether without any addition is used as a blank since it was found that a difference in the light absorption of ethyl ether and the petroleum ether used in the range above 280 mμ cannot be detected. At 270 mμ, however, the absorption of the petroleum ether was significantly higher than that of ethyl ether.

Furthermore, pure vitamin A was applied to the determination of losses during chromatography. A solution of vitamin A in petroleum ether was measured on the Beckmann spectrophotometer prior to and after chromato-

graphy. A column of dicalcium phosphate as described above was used, and a quantity of vitamin A of 150—200 μg which after chromatography was taken up in 50 ml. Four experiments of this type were performed. The loss was found to be ½—2 %. In Table 2 are given the measurements at different wave lengths for one of these experiments.

Experiments on solutions with other light absorbing substances

The applicability of chromatography to the purification of impure extracts was investigated on a series of vitamin A solutions from extracts of widely different materials. Some of these experiments are shown in Table 3 and the effect of chromatography is illustrated by the values of $\frac{E_{310}}{E_{325}}$ and $\frac{E_{350}}{E_{325}}$. It becomes clear from Table 3 that these ratios in the solution after chromatography are very much like those found for pure vitamin A dissolved in petroleum ether (Table 1).

Table 2. Loss during chromatography of 140 µg pure vitamin A.

Wave length mµ	$E_{1\text{ cm}}$	
	Directly, without chromatography	After chromatography
280	0.103	0.088
290	0.189	0.180
300	0.306	0.302
310	0.436	0.430
320	0.489	0.478
325	0.513	0.502
328	0.501	0.492
330	0.485	0.473
340	0.373	0.363
350	0.227	0.220
360	0.122	0.117

For the majority of the materials Table 3 shows a considerable decrease in $E_{1\text{ cm 325 m}\mu}^{1\%}$ due to chromatography. Of course, it cannot be decided on the basis of these experiments whether this decrease actually corresponds to unspecific absorption in the original solution. A comparison of these investigations with the experiments on pure vitamin A discussed above and showing

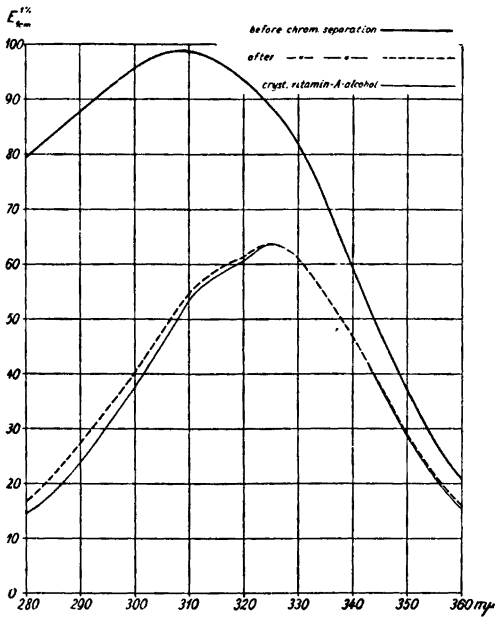

Fig. 1. Absorption-curves of extract from whale-liver-conc. before and after chromatography.

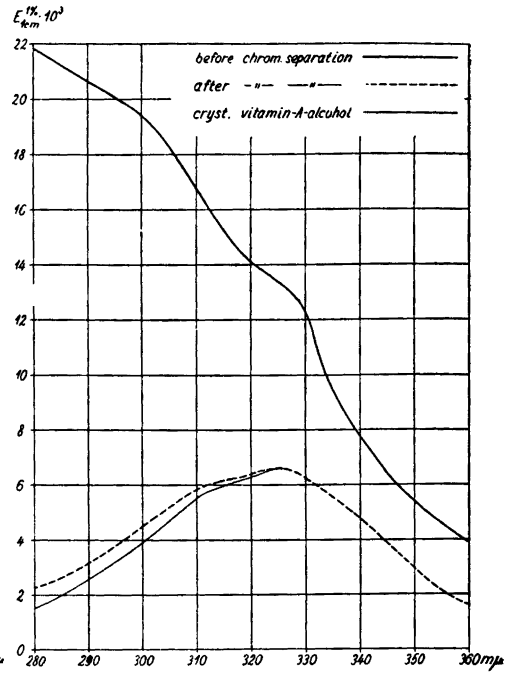

Fig. 2. Absorption-curves of extract from vitaminized oats before and after chromatography.

very small losses after chromatography leave no doubt that this is actually the case.

In order to demonstrate more clearly the applicability of chromatography to the purification of vitamin A solutions the absorption curve in the range 280—360 mμ before and after chromatography is given for three of the experiments shown in the table (Figs. 1—3).

Figs. 1—3 show that the absorption curves after chromatography fall very closely to the absorption curve of vitamin A, which seems to be an excellent criterion in favour of the specificity of the chromatographic-spectrophotometric method.

With regard to the applicability of chromatography to the purification of different extracts it should be mentioned that margarine extract is the only one among the materials investigated which could not be purified so effectively that a spectrophotometric determination could be performed, the reason being the sesame oil content of margarine which, according to Danish law, must be added to margarine as marking ingredient.

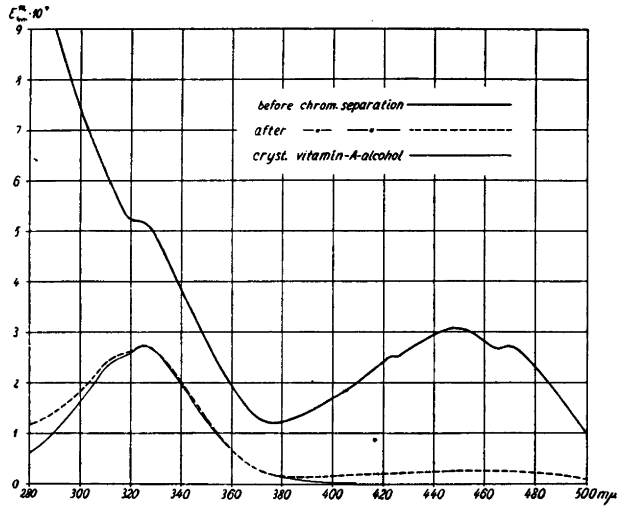

Fig. 3. Absorption-curves of extract from milk before and after chromatography.

DISCUSSION

Before the new international regulations for the determination of vitamin A had been accepted in 1949, it was possible to determine the content of vitamin A in fish liver oil without purification of the solution obtained after saponification and ether extraction prior to the measurement of the absorption spectrum. In this procedure the conversion factor 1600 compensated to some extent for the impurities present in the solution. For whale liver oil and its concentrates application of this conversion factor was more problematic because these products always show a considerably higher unspecific light absorption than does fish liver oil, which means that the maximum of the absorption curve may be found at a much lower wave length than that of pure vitamin A. Until recently, spectrophotometry has scarcely been applied to the determination of vitamin A in any other vitamin A containing materials.

In the new international regulations prescribing the conversion factor 1900 it was found necessary to formulate certain requirements with respect to the absorption curve forming the basis for the determination and the calculation by means of the given factor. It is required that the ratio between the extinction and the maximum extinction measured at any wave length in the range 310 to 350 mμ does not deviate by more than 0.02 from the same ratio for pure vitamin A. Here we are confronted with the question concerning the proper course of the absorption curve for pure vitamin A. In the communication published by the Medical Research Council it is stated that either

Table 3. Comparison between the absorption curves of different extracts before and after chromatography.

Materials	Before chromatography			Chromatography material	After chromatography			Calculated international units per gram
	$E_1^{1\%}{}_{\text{cm}}$ 328 mμ	$\frac{E_{310\ m\mu}}{E_{325\ m\mu}}$	$\frac{E_{350\ m\mu}}{E_{325\ m\mu}}$		$E_1^{1\%}{}_{\text{cm}}$ 325 mμ	$\frac{E_{310\ m\mu}}{E_{325\ m\mu}}$	$\frac{E_{350\ m\mu}}{E_{325\ m\mu}}$	
Whale liver oil	4.23	1.10	0.43	$CaHPO_4$	3.49	0.86	0.47	6 400
» » »	7.47	1.09	0.49	»	4.75	0.86	0.45	9 000
» » conc.	25.20	1.26	0.47	»	16.44	0.86	0.47	31 200
» » »	89.0	1.11	0.41	»	6.38	0.86	0.45	121 000
Cod liver oil	0.349	0.89	0.52	»	0.312	0.85	0.51	590
» » »	1.122	0.85	0.54	»	1.039	0.84	0.50	1 970
» » »	0.590	0.88	0.54	»	0.540	0.84	0.53	1 025
Butter	0.02075	1.02	0.50	»	0.01352	0.84	0.45	25.7
»	0.01715	1.00	0.51	»	0.01086	0.85	0.46	20.6
Liver paste	0.0703	0.89	0.57	»	0.0452	0.87	0.47	85.9
» »	0.0974	0.90	0.68	»	0.0468	0.87	0.45	88.9
Vitaminized oats	0.01480	1.23	0.50	»	0.00661	0.88	0.44	12.6
» »	0.01126	1.33	0.49	»	0.00446	0.91	0.46	8.5
Vitamine fodder	0.0492	1.45	0.55	Al_2O_3	0.0140	0.90	0.50	26.6
Milk	0.000517	1.19	0.54	$CaHPO_4$	0.000274	0.89	0.47	0.52
Liver oil paste	0.96	1.53	0.35	Al_2O_3	0.088	0.87	0.49	170

can the value given by Morton and Stubbs [5] be used or the curve can be redetermined by measuring a solution of the new biological standard against a solution of the oil from which the standard is made.

However, as is shown in Table 1 of the present paper, it becomes clear that there is no complete agreement between the author's measurements on pure synthetic vitamin A and the values given by Morton and Stubbs. Therefore, the question arises whether it is correct to use Morton and Stubbs' measurements; in which case the requirements as to the purity of the measuring solution are lower, the ratio $\frac{E_{350}}{E_{max}}$ (ca. 0.51) being higher than the corresponding ratio (0.46) measured on pure vitamin A as used in the present experiments.

If Morton and Stubbs' requirements are considered sufficient, many fish liver oils will in fact pass as being satisfactory, while none of the fish liver oils are satisfactory if the requirements correspond to what has been described in the present work (*cf.* the results on cod liver oil given in Table 3). The

requirement as to the course of the vitamin A curve given in the communications of the Medical Research Council, *viz.* measurement on the new standard solution, does not correspond to the curve for vitamin A alcohol, but for vitamin A ester, and is therefore applicable only when the determination is performed on the non-saponified oil solution. Without saponification, however, no material will lead to an absorption curve which is in satisfactory agreement with the curve for vitamin A acetate; consequently, as mentioned in the communication, it will always be necessary either to correct according to Morton and Stubbs [1] or to perform purification which, in practice, means chromatography.

Chromatography involves a separation of vitamin A ester from vitamin A alcohol and therefore the question arises whether the two components should be determined separately, which seems, however, very impractical. Saponification appears to be preferable and in this way the total vitamin A can be determined, as is the case in the present work.

Hence, if the curve for vitamin A acetate, measured as the international standard, is to be the basis for the evaluation of the specificity of the absorption curve, the determination must be performed applying Morton and Stubbs' correction to the directly measured curve. According to the communication of the Medical Research Council the correction is permissible only if the measured curve has a maximum at the proper wave length, *i. e.* at 325—328 mμ. If this does not hold, as in the case of most whale liver oils, a correct determination is possible only in connection with chromatography, but previous saponification is definitely to be preferred.

Since the above described technique of chromatography leads to absorption curves which are in good agreement with those measured on pure synthetic vitamin A, it appears reasonable to take this curve as the basis of the evaluation. If this curve is given preference, not one of the absorption curves for fish liver oil will show satisfactory agreement and, consequently, either the correction according to Morton and Stubbs or a purification through chromatography must be applied.

As chromatography is simple and not very time-consuming, and since there is little chance for the introduction of significant errors, it appears most convenient always to use chromatography, even in those cases where application of the correction method is permissible according to international rules. The very narrow wave length range applied in the correction method, which causes a multiplication of the spectrophotometric measuring uncertainty, no doubt results in an uncertainty of the calculated result that is much higher than the uncertainty of the chromatographic determination.

SUMMARY

A method for the chromatographic separation of vitamin A from petrol-ether-extracts of different materials is described. The method gives in connection with spectrophotometric measurement of the absorption curve a specific determination of vitamin A in a majority of materials containing this vitamin.

In experiments with pure vitamin A the losses during chromatography were found to be ½—2 %.

Values for the absorption at 310, 325 and 350 mμ, with and without chromatographic separation, of extracts from whale liver oils, cod liver oils, liver paste, butter, milk, vitaminized oats and fodder mixtures are given.

The determination of vitamin A in fish liver oils and whale liver oils is discussed in relation to the new international regulations concerning this problem and it is suggested that hereby the chromatographic separation should be preferred to the correction according to Morton and Stubbs.

REFERENCES

1. Medical Research Council, London, Department of biological standards. *The second international standard for vitamin A.* (1949).
2. Morton, R. A., and Stubbs, A. L. *Analyst* **71** (1946) 348.
3. Gridgemann, N. T., Savage, J. P., and Gibson, G. P. *Analyst* **73** (1948) 662.
4. Barua, R. K., and Morton, R. A. *Biochem. J.* **45** (1949) 308.
5. Morton, R. A., and Stubbs, A. L. *Biochem. J.* **42** (1948) 195.

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