CCXXXVIII. THE COLORIMETRIC DETERMINATION OF VITAMIN A BY THE ALKALI DIGESTION METHOD.

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THE alkali digestion method for the colorimetric estimation of vitamin A which was first adopted by Rosenheim and Webster [1927], is used in this laboratory both in work upon tissues from experimental animals and upon samples of human liver obtained at autopsy [Moore, 1932]. Since the latter work has involved large numbers of determinations on specimens available at varying times after death it appeared desirable (1) to study the technique of the method with a view to simplifying the procedure as far as possible so as to permit rapid working, (2) to examine the effect of ageing on the stability of the vitamin in case unsuspected deterioration should be taking place. The present paper is concerned with the collection of information on these two points.

EXPERIMENTAL.

Standard procedure for determination of vitamin A in liver samples.

In early experiments Moore [1930] extracted the vitamin A from alkaline digests of rat liver by four extractions with ether and washed the combined ether extracts with three changes of water. This procedure presumably ensured almost complete extraction of the vitamin and was therefore suitable for application in a limited number of experiments when the reliability of the results was of primary importance. In routine work however the large number of extractions and washings with indefinite volumes of water involves loss of time, both directly and through the increased danger of the formation of emulsions. Steps were therefore taken to simplify the technique so as to obtain results of reasonable accuracy in rapid routine work. It was found that if the digest was given a preliminary shaking with a small proportion of alcohol a single ether extraction was sufficient to extract almost all (usually 90 % or more) of the vitamin A. Moreover, two washings of the ether extract were sufficient to secure freedom from alkali, while by adherence to the correct proportion of alcohol, ether and water the formation of emulsions could be avoided except in rare instances. By the use of the following method it is possible to average 3-4 assays per hour.

Extraction of vitamin. About 5 g. liver are weighed to the nearest 0.1 g. in a 50 cc. beaker, and minced with scissors into 10 cc. of 5 % aqueous KOH. (In the case of specimens of human liver collected at hospitals a convenient plan is to place the specimen in a small bottle containing the potash. Mincing may be postponed until the specimen is received in the laboratory.) The material is then transferred to a 50 cc. conical flask, which is corked and placed

in cold storage if the determination is not to be carried out at once. To digest the tissues the mixture is heated, preferably in a steam-oven, until complete solution has taken place. The digest is then transferred to a 100 cc. graduated funnel and is shaken strongly (for about 10 secs. each time) first with 5 cc. of ethyl alcohol and then with the addition of 50 cc. of ether. (B.D.H. "A.R." ether and ether of sp. gr. 0.730 give identical results in ordinary work.) The layers are allowed to settle out, and the aqueous layer is discarded. 5 cc. of water are now added and vigorously shaken together with the ethereal layer. After settling, the aqueous layer is discarded and the washing completed by gentle agitation with 50 cc. of water. The ether fraction is next filtered by suction through a layer of anhydrous sodium sulphate¹, contained in a sintered glass funnel, into a 100 cc. wide-necked flat-bottomed flask (as used in Soxhlet extractions) the sulphate being washed with a little ether. The ether is evaporated off rapidly on a water-bath, preferably under suction from a filter-pump.

Colorimetric assay. The residue after evaporation, which should be quite clean and dry in appearance², is dissolved in 5 cc. of chloroform (a further dilution is sometimes necessary in the case of specimens giving high values). Portions of this solution, varying from 0.02 to 0.5 cc., according to the amount of vitamin present, are delivered by an appropriate blood pipette into a 1 cm. cell, or a test-tube of 1 cm. bore which has been checked against a cell. The volume is made up to 0.5 cc. with chloroform, and the pipette is washed out by sucking up the chloroform and expelling it again. 2 cc. of the SbCl_3 reagent are then added and the blue colour, which should give a reading of 3 to 5, is matched quickly in a Lovibond tintometer. Results are calculated in Moore's units as follows.

Example. 4.9 g. of liver were digested and extracted. Residue from extract was dissolved in 5 cc. CHCl_3 . 0.02 cc. of this solution made up to 0.5 cc. with CHCl_3 gave a reading of 4 B 1 Y (neglected).

Blue units (B.U.) per g. of liver = $\frac{4 \times 2.5 \times 5}{0.02 \times 4.9} = 500$ to nearest significant figure.

The 2.5 in the top line of the calculation refers to the total volume of the reaction mixture.

*The effect of various modifications in the technique of extraction
on the apparent vitamin A content.*

The following experiments were carried out on various specimens of ox-, pig- and sheep-liver with a view to testing the efficiency of the above technique and examining the effect of variations in procedure.

Technique of digestion. Portions of ox-liver (specimen A) were digested for varying periods (10–90 minutes) with 5 %, or in one case 10 %, aqueous KOH. The digests were then extracted and the residues assayed in the usual way. Although the technique of digestion affected the yield of fat (*i.e.* the degree of saponification) and consequently the blue value per unit of fat, there was no variation in the blue value per g. of liver.

¹ The anhydrous sodium sulphate used in this work was supplied by Messrs B.D.H. and had given good results over a number of years. Just after sending this paper to press, however, the quality of sulphate supplied was changed. The new sulphate, which was a much finer powder, was quite valueless when used in the way described in the text. Dr F. H. Carr kindly submitted alternative types of sulphate for trial. The quality described as "dense" gave satisfactory results.

² The presence of moisture will cause clouding on adding the SbCl_3 reagent. This clouding may be prevented by adding a drop of acetic anhydride before the reagent, but resort to this procedure should be unnecessary if the extraction is carried out correctly.

The action of alcohol in facilitating the extraction of the vitamin. The purpose of using alcohol is both to prevent the formation of emulsions and to facilitate the extraction of the vitamin. The ability of ether, unaided by alcohol, to extract the vitamin from the alkaline liver digests varied from specimen to specimen. The examples given below will serve to illustrate this point.

Example 1. Good extraction of vitamin A by ether alone. 5 g. of ox-liver (B) were digested by heating. Extraction with 50 cc. of ether alone yielded 1000 B.U. per g. of liver. A second extraction with 50 cc. of ether and 5 cc. of alcohol yielded 62.5 B.U. per g. Most of the vitamin A in this case, therefore, could be obtained by one extraction with ether in the absence of alcohol.

Example 2. Poor extraction of vitamin A by ether alone. 5 g. of pig-liver (C) were digested by heating and were extracted with 50 cc. of ether, yielding 5 B.U. of vitamin A per g. A second extraction with 50 cc. of ether and 5 cc. of alcohol yielded 20 B.U. per g. Conversely when 5 g. of liver were first extracted with 50 cc. of ether and 5 cc. of alcohol 20 B.U. per g. were obtained, while a second extraction with 50 cc. of ether and 5 cc. of alcohol gave only 7.5 B.U. per g. In this case therefore ether by itself extracted only a small proportion of the vitamin, but by using ether and alcohol most of the vitamin could be obtained in the first extraction.

Similar results were obtained in the case of a sample of pig-liver (D) richer in the vitamin, which gave a result of 500 B.U. per g. when the extraction was carried out by gentle shaking (5 secs.) with ether in the presence of alcohol, but only 100 (gentle shaking for 4 secs.) to 200 B.U. per g. (vigorous shaking for 30 secs.) in the absence of alcohol. These results afford a good illustration of the action of alcohol in facilitating the extraction of the vitamin.

The technique of digestion also appeared sometimes to affect the ease of extraction by unaided ether. Thus in one series of experiments on pig-liver (E) the surprising result was obtained that ether could extract the vitamin after digestion of the tissues at room temperature, but not if the digestion had been carried out by the usual procedure of heating in a steam-oven. The disadvantages of using unheated digests in routine work lie in the danger of forming emulsions, and loss of time through blockages in the separating funnel.

Efficiency of the standard process of extraction. The following examples will serve to indicate the proportion of the vitamin extracted.

	1st extraction (alcohol and ether)	2nd extraction B.U.	3rd extraction
Sheep-liver (F)	1250	25	—
Ox (G)	350	30	0
Human (H)	25	0	—
Pig (C)	20	7.5	—

In all cases the bulk of the vitamin was obtained in the first extraction.

Comparison of the alkali digestion method with the Soxhlet extraction method using various solvents. A specimen of fresh ox-liver (I) giving 55 B.U. per g. by the alkali digestion method was weighed and minced finely. Portions of 5 g. each were taken, mixed with an equal volume of anhydrous sodium sulphate and extracted for 1 hour in a Soxhlet extraction apparatus with the following solvents—ether, light petroleum, chloroform, acetone, benzene. Although the amount of material extracted seemed to depend largely on the solvent used, the yield of vitamin A remained constant. Thus, in confirmation of the work of Simmonet *et al.* [1931] and of Wolff [1932] good agreement was observed between results obtained by the Soxhlet and alkali digestion methods.

Rate of deterioration of vitamin A in untreated liver and liver digests on standing.

A large specimen of ox-liver (J), rich in vitamin A, was minced, stirred up and divided into 5 g. portions, which were divided into 3 groups and treated as follows: Group (1) immediate digestion by heating with 5 % KOH: Group (2) slow digestion by KOH at room temperature. Group (3) storage at room temperature without any preservative treatment. Portions from each group were taken at intervals and the vitamin A content assayed by the standard procedure. The results of this experiment, and also those of a similar experiment using a pig-liver (K) of low vitamin A content, are shown in Table I.

Table I. *Rate of deterioration.*

Time in days	Group 1 Digested by heating in 5 % KOH	Group 2 Digested by standing at room temperature	Group 3 Standing without KOH at room temperature
Ox-liver (high vitamin A reserves).			
0	1000	1000	1000
3	1000	1000	1000
7	1000	1000	600
14	1000	1000	400
42	600	600	400
Pig-liver (low vitamin A reserves).			
0	20	20	20
7	20	20	10
14	20	17.5	8
21	15	10	4
28	10	8	1
35	3	10	0
42	3	12.5	0

It will be seen that some deterioration took place in all groups after prolonged standing. Deterioration, however, was less rapid in the liver treated with alkali than in the untreated liver. Thus in the untreated liver definite signs of deterioration were evident in 7 days, while no deterioration (except for a very small fall in the case of the pig-liver, group 2) took place in 14 days in the liver treated with alkali. In the ox-liver treated with alkali deterioration to the extent of 40 % was observed after 6 weeks. In the pig-liver deterioration was more rapid.

DISCUSSION.

The procedure for the estimation of vitamin A in tissues described above is designed to permit rapid routine work upon specimens showing very wide variations in vitamin A content, as found in human livers, and is not intended for use when the highest attainable accuracy in individual experiments is required. For work of this type it is recommended that the alkaline digest should be extracted twice or three times with ether, and that the fat obtained should be saponified before carrying out the colorimetric assay. The effect of these refinements on the final result would vary slightly from specimen to specimen. Additional extractions might be expected to increase the final result by as much as 10 %. Saponification should have little effect in the case of normal mammalian specimens, but might cause increased results in the case of livers of abnormally high fat content.

Similarly the experiments on the determination of the vitamin have not been carried out with a view of ascertaining the ideal conditions for ensuring

its stability, but rather to obtain information as to its behaviour under routine conditions involving periods of storage at ordinary temperatures, *e.g.* during transmission by post. Under these conditions the vitamin is more stable in alkali than in untreated specimens, and the tissues should therefore be placed in potash immediately on dissection for this reason as well as for the further consideration of the removal of danger to the worker in the case of pathological specimens. The results indicate that there is little danger of deterioration in digested specimens provided the assay is carried out within 14 days after death.

SUMMARY.

A simplified form of the alkali digestion process for the assay of vitamin A in tissues by the colorimetric method has been devised. The process permits rapid working and gives results substantially in agreement with those obtained by the Soxhlet extraction method.

At room temperature vitamin A deteriorates less rapidly in liver specimens treated with potash than in untreated tissues kept without preservative treatment. In the case of specimens of liver transferred immediately *post mortem* to potash solution and then stored at room temperature no serious decrease in vitamin A content is to be anticipated if the assay is carried out within 14 days after death.

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