

The determination of vitamin A in animal tissues and its presence in the liver of the vitamin A-deficient rat

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Our attention was directed to this problem during a study of the effect of vitamin A deficiency on ubiquinone concentration in the rat, during which it was necessary to determine relatively small amounts of vitamin A in certain tissues (Edwin, Bunyan, Green & Diplock, 1962). The results of that work suggested that the amounts of vitamin A found by us were often considerably higher than expected. Accordingly, several methods for measuring vitamin A in animal tissues were investigated.

In the usual methods for determining vitamin A, the tissue is first digested with alkali and the lipid fraction (saponified or partially saponified) is then extracted with a solvent (nearly always diethyl ether) and finally dissolved in chloroform before measurement of the vitamin A by use of the antimony trichloride reagent. Most methods published are only simple modifications of this sequence. Liver, which usually contains many times more vitamin A than other tissues, is occasionally treated differently and assayed without preliminary alkali treatment. Thompson, Ganguly & Kon (1949) have already criticized the use of total tissue saponification and preferred to use a direct-extraction method for determining vitamin A in either intestine or liver. We have selected for study three exemplary methods that seem to cover the range used by most workers and compared them with the method used by us. The methods were compared for normal rat liver and also for rat kidney, which is a suitable tissue containing only small amounts of vitamin A.

Alkali-digestion method. This was the method of Davies (1933), who described it in some detail, although it had essentially been used for some years by Moore (1930) and was originally due to Rosenheim & Webster (1927). The tissue is heated on a steam-bath with 5% aqueous KOH for between 10 and 90 min, depending on the time taken to complete digestion. After addition of a measured amount of alcohol, the digest, which may not be totally saponified, is extracted with diethyl ether.

Total-saponification method. This was the method of Heaton, Lowe & Morton (1957), who used it to examine the vitamin A content of a range of rat tissues, including liver, kidney and intestine. The main difference from Davies's method is that much stronger alkali (60% w/w, KOH) is used and some ethanol is added during a second period of heating under reflux. The tissue and the fat are more or less completely saponified by this treatment.

Direct-extraction method. In this procedure, due to Ames, Risley & Harris (1954), liver is ground with anhydrous Na_2SO_4 , and the dry powder is then extracted in the

cold with diethyl ether. Ames *et al.* (1954) restricted the use of their method to liver, but we used it for kidney in some of the tests described below.

Extraction-saponification method. This was the method of Diplock, Green, Edwin & Bunyan (1960). The tissue is first pulverized with acetone and anhydrous Na_2SO_4 at -70° and then extracted in a Soxhlet with acetone. The lipid is saponified, in the presence of pyrogallol as an antioxidant, with strong alcoholic KOH for 5 min.

EXPERIMENTAL AND RESULTS

Measurements. Vitamin A was determined by measuring the colour it produced with SbCl_3 at $620 \text{ m}\mu$ on the Unicam S.P. 350 spectrophotometer.

Expt 1. The four methods were compared on samples of normal rat liver and kidney. Forty 6-month-old rats, which had been given adequate amounts of vitamin A during their lifetime, were killed. The livers were minced and mixed and the kidneys treated likewise; sixteen 5 g samples of each were then prepared and stored at -20° until required for analysis. Each tissue was analysed by the four methods in quadruplicate, each analysis being carried out on one 5 g sample. The four different methods were studied simultaneously. The results are given in Table 1. The alkali-digestion method

Table 1. *Expt 1. Vitamin A in rat liver and kidney, measured by four methods of analysis*

(Each method was applied to four 5 g samples, see this page)

Tissue	Vitamin A (i.u./g) (mean with standard deviation)					
	A. Alkali digestion	B. Total saponification	C. Direct extraction	D. Extraction- saponification		
Liver	368 ± 33	531 ± 21	621 ± 29	593 ± 38		
Kidney	3.7 ± 0.35	4.0 ± 0.49	6.6 ± 1.56	7.2 ± 0.63		
Level of statistical significance* of differences						
Difference	B - A	C - A	D - A	C - B	D - B	D - C
Liver	0.1 %	0.1 %	0.1 %	1 %	2 %	NS
Kidney	NS	0.1 %	0.1 %	1 %	0.1 %	NS

NS, not significant.

* Tested against pooled estimate of error (12 df).

of Davies gave considerably lower vitamin A values than any of the other three methods, namely about 60% of the highest value obtained by any of the other methods for both liver and kidney. The total-saponification method, when applied to liver, gave a mean vitamin A value that was about 10-15% lower than that obtained by the direct-extraction or the extraction-saponification method. When kidney, with much less vitamin A, was analysed by the total-saponification method of Heaton *et al.* (1957), again only about 60% was found. Our results confirm the superiority of the method of Ames *et al.* (1954) to methods A and B for liver, although it did not give significantly higher values than the extraction-saponification method. These two procedures gave essentially similar results with kidney also, although the extraction-saponification method gave rather higher values with this tissue. There appeared to

be a somewhat bigger variation in the analysis of kidney by the method of Ames *et al.* (1954) than occurs with the other three methods.

Expt 2. Edwin *et al.* (1962) have already commented on their finding that the livers of rats in the early stages of clinical vitamin A deficiency often contain small amounts of vitamin A. This observation appeared to be contrary to the results of most earlier workers; however, in view of the evidence described here indicating that the commonly used methods of analysis destroy some vitamin A (especially in a tissue of low potency), the matter was investigated more thoroughly. Four male and four female weanling rats of the Norwegian hooded strain were fed on the vitamin A-deficient diet, AO2, described by Edwin *et al.* (1962). They were weighed daily, and the onset of clinical vitamin A deficiency was recorded by the appearance of xerophthalmia and cessation of growth. Each rat was killed after it had entered the 'weight plateau' period, that is when its weight remained constant (to within about 3 g) for at least 3 consecutive days. Control rats given supplements of vitamin A (3-10 i.u./g diet) continued to grow during this period. Four of the eight rats had xerophthalmia at death, and one male rat did not show a clearly defined weight plateau but lost about 20% of its maximum weight rapidly over 4 days. The first rat was killed 42 days after the beginning of the experiment and the last one 10 days later.

Table 2. *Expt 2. Vitamin A in the livers of vitamin A-deficient rats*

Sex	Rat	Liver		
	Weight on 4 consecutive days before death (g)	Weight (g)	Vitamin A content (i.u./g)	Xerophthalmia
♂	143, 145, 147, 145	5.7	1.1	+
♂	133, 136, 138, 136	5.5	1.1	-
♂	102, 99, 88, 82	3.5	3.5	-
♂	167, 168, 172, 170	6.7	0.5	+
♀	92, 93, 95, 94	4.3	0.6	-
♀	137, 135, 138, 136	6.0	0.6	+
♀	126, 131, 132, 130	5.2	0.6	-
♀	116, 122, 122, 120	3.8	1.4	+

Statistical analysis of results

Sex	Vitamin A content of liver (i.u.) (mean with standard deviation)
♂	6.8 ± 3.9*
♀	3.7 ± 1.2**

* Significantly greater than zero, $P < 0.05$.

** Significantly greater than zero, $P < 0.01$.

Each liver was analysed for vitamin A. The tissue was extracted and saponified as described by Diplock *et al.* (1960). The non-saponifiable fraction was dissolved in 5 ml of 90% (v/v) aqueous ethanol (with warming, if necessary), and 5 ml of 1% (w/v) digitonin in the same solvent were added. After the mixture had been left to stand overnight, the digitonide was removed by centrifugation and the supernatant liquid diluted with water and then extracted twice with 10 ml of light petroleum (b.p. 40-60°). The extract, after evaporation to small bulk, was divided into two equal parts,

to one of which were added 10 μg vitamin A alcohol to act as a chromatographic marker. Both parts were then chromatographed on Whatman no. 1 paper, impregnated first with zinc carbonate and sodium fluorescein, as described by Green (1958), and then with liquid paraffin, as described by Eggitt & Ward (1953). The mobile phase was 50% (v/v) aqueous ethanol. After 17 h, during which time the solvent front migrated about 30 cm, the papers were dried in air and observed under u.v. light (Hanovia Chromatolite). The vitamin A in the extract appeared as a brownish quenching spot surrounded by a halo of yellow fluorescence (R_F 0.46) and was similar in colour to and identical in R_F with the spot containing authentic vitamin A. Both spots, however, ran slightly more slowly than a marker of pure vitamin A alcohol chromatographed on an adjacent lane, because of considerable amounts of hydrocarbon impurity in the test extract. (*N.B.* Occasionally, 50% aqueous ethanol partially elutes the absorbed sodium fluorescein, making observation of the spots difficult. If this occurs, it may be due to deterioration in the adsorptive power of the zinc carbonate on storage. Fresh papers should be used.)

The vitamin A in the extract was eluted with ethanol (3 ml) and then determined (in chloroform) with the SbCl_3 reagent. The colour obtained was always clear blue, and faded, as does the colour with pure vitamin A, to give a colourless or faintly pink solution. The results are given in Table 2. Analysis of variance showed that the mean liver vitamin A contents of these deficient rats were significantly different from zero.

DISCUSSION

The determination of vitamin A in animal tissues (especially those of low potency) is a problem that, unlike those arising during the analysis of high-potency oils, has received little attention. Though there appears to be ample evidence that high-potency oils containing vitamin A acetate can be saponified under proper conditions without appreciable loss (Brunius, 1958), several workers have expressed doubts about the quantitative recovery of vitamin A from natural oils after saponification (Cama, Collins & Morton, 1951; Moore, 1957, p. 46). Jones & Haines (1943) demonstrated that, with such oils, small losses do occur. However, in relation to the other analytical problems that arise with high-potency oils, these small saponification losses are not often considered to be a major cause for concern. Our results show that, with animal tissues, particularly with those of low potency, the loss of vitamin A can be (and with the classical methods of analysis probably is) of a more significant order. Thompson *et al.* (1949) studied Davies's method with several tissues and also concluded that it gave low results compared with a direct-extraction method. Ames *et al.* (1954) found that, with calf liver, their direct-extraction method gave results about 10% higher than a typical saponification method. The superiority of the method of Ames *et al.* (1954) with liver, as our results show, suggests that if saponification must be used the time should be kept as short as possible. Unfortunately, direct-extraction methods are not practical with all types of tissue, and, as Thompson *et al.* (1949) have shown, residues after extraction usually still contain some vitamin A. With low-potency tissues, such as kidney, the quantities remaining may be significant; even if they were

not, the lipid from low-potency tissues must generally be saponified before vitamin A can be measured accurately. The superiority of the extraction-saponification procedure of Diplock *et al.* (1960) for low-potency tissues is partly due to the short saponification time used (the method is derived from that originally used for vitamin E by Tošić & Moore, 1945), but is also in part due to the use of pyrogallol as an antioxidant during the saponification. In several preliminary experiments, not reported here, we have found that the presence of pyrogallol increases the recovery of vitamin A from low-potency tissues, whichever saponification procedure is adopted. The use of an antioxidant during the saponification stage of vitamin A determinations does not seem to have been previously considered, although, as Thompson *et al.* (1949) showed, vitamin A is subject to oxidative loss. Maintenance of an inert atmosphere during saponification of vitamin A esters has occasionally been advocated with low-potency material; however, it may be no more effective than it is in preventing oxidation of vitamin E.

By a combination of paper-chromatographic separation and the extraction-saponification procedure, significant and sometimes appreciable amounts of vitamin A can be found in livers of rats in the early stages of clinical vitamin A deficiency. In several experiments, not recorded, it has been shown that vitamin A cannot be detected in such livers by other procedures: the small quantity present may be destroyed, or, if paper chromatography is not used, its reaction with antimony trichloride may be obscured by impurities present. Several workers have, in the past, concluded that the young rat grows at almost the optimal rate in the absence of a detectable concentration of vitamin A in its liver (cf. Lewis, Bodansky, Falk & McGuire, 1942). Moore (1957, p. 298) summarized the evidence of several workers as well as his own experiments and concluded that 'rats may often continue to grow for several weeks after examination of the livers of their litter mates has shown their reserves to be exhausted'. Similar results were obtained by Dann (1932) and Baumann, Riising & Steenbock (1934). Sherman & Campbell (1945) and Sherman & Trupp (1949), however, found that growth and survival of rats were both diminished, even when they received enough vitamin A to produce detectable storage in the liver, and Guilbert & Hart (1934) and Guilbert & Hinshaw (1934) found some vitamin A in the livers of calves and poultry showing clinical and pathological signs of avitaminosis A. Our experiments indicate that clinical signs of vitamin A deficiency in the rat begin when there is still some vitamin A in the liver, but special methods of analysis have to be used to determine the small amounts likely to be present.

SUMMARY

1. A comparative study of four methods for determining vitamin A has been made, with normal rat liver and kidney as test tissues.
2. It has been shown that substantial losses of vitamin A occur when such tissues are digested or saponified with alkali in the absence of an antioxidant. These losses can be avoided if the tissue is first extracted with acetone and the extract then saponified for a short time in the presence of pyrogallol.

3. With this method of purification and subsequent paper chromatography of the extracts, it has been found that the livers of rats showing early clinical signs of vitamin A deficiency still contain some vitamin A. The amounts present are likely to be destroyed if total digestion or saponification of the tissue is used as a method of analysis.

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