Biological activity of crystalline vitamin A_2 aldehyde

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The importance of retinene$_2$ in the visual cycle of fresh-water fish was recognized by Wald (1937), who described it as a 'deep yellow carotenoid' having an absorption maximum at 405 mμ (chloroform) in the ultraviolet and at 720–706 mμ in the antimony-trichloride colour reaction. Porphyropsin, which is formed by interaction of retinene$_2$ with scotopsin, has been characterized as a purple, light-sensitive pigment in the retina of the freshwater fish, with an absorption maximum in solution at 522 ± 2 mμ.

Morton (1944) showed that retinene$_1$ is the aldehyde of vitamin A_1, and later Ball, Goodwin & Morton (1948) obtained crystalline vitamin A_1 aldehyde by oxidation of vitamin A_1 alcohol with manganese dioxide. Similarly, retinene$_2$ was shown to be the aldehyde of vitamin A_2 (Morton, Salah & Stubbs, 1947), and it was also obtained in a crystalline form (Cama, Dalvi, Morton, Salah, Steinberg & Stubbs, 1952; Farrar, Hamlet, Henbest & Jones, 1952). It was further demonstrated (Cama, Dalvi, Morton & Salah, 1952) that vitamin A_2 aldehyde is converted into vitamin A_2 in vivo, but the biological activity of vitamin A_2 aldehyde was not quantitatively assessed.

Ames, Swanson & Harris (1955) in a study of the biological potencies of five isomers of synthetic vitamin A_1 aldehyde reported a biological activity on a molar basis of 91% for all-*trans* vitamin A_1 aldehyde in comparison to all-*trans* vitamin A_1 acetate, but Wendler, Rosenblum & Tishler (1950) reported that all-*trans* vitamin A_1 aldehyde was as active as vitamin A_1.

In this paper, the preparation of crystalline vitamin A_2 aldehyde by oxidation of vitamin A_2 alcohol, obtained from the liver oil of the freshwater fish *Wallago attu*, is described. The biological activity of crystalline vitamin A_2 aldehyde in comparison with the United States Pharmacopoeia vitamin A reference standard was determined quantitatively by a modified U.S.P. rat-growth assay.

EXPERIMENTAL

Materials

Solvents. Cyclohexane, for spectroscopic use, was obtained from British Drug Houses Ltd. Light petroleum (b.p. 40–60°), obtained from Burmah-Shell Company, was purified by letting it react with a solution of $KMnO_4$ for a few days, washing it free of $KMnO_4$ with water, drying over $CaCl_2$ and distilling twice before use. Diethyl ether was purified by leaving it over sodium wire; before use it was freshly distilled to remove peroxides. Benzene was of A.R. quality. Ethanol, for spectroscopic use, was

prepared by refluxing rectified spirit with potassium hydroxide and zinc dust for 6 h and distilling twice before use.

Reagents. Antimony trichloride, acetic anhydride, potassium hydroxide, anhydrous sodium sulphate and manganese dioxide were laboratory reagents obtained from British Drug Houses Ltd. Alumina, specially prepared for chromatography, was obtained from Merck and Co. It was de-activated with known amounts of water (usually 5–10%, v/w), stirred in slowly under light petroleum. The U.S.P. vitamin A reference standard was obtained from Hoffmann-La Roche through the courtesy of Voltas Ltd, Bombay.

Animals. Litter-mate male albino rats, bred in the animal house of this Institute, were used. To avoid vitamin A storage during weaning, the young rats, when they were 15 days old, were placed on a diet from which carrots, milk and shark-liver oil were omitted.

Procedure

Preparation of crystalline vitamin A_2 aldehyde from W. attu *liver oil.* The oil was extracted from 250 g of *W. attu* livers with light petroleum as described in a previous publication (Balasundaram, Bamji, Cama, Sundaresan & Varma, 1958). The yield of the oil was 10 g. The method for the preparation of crystalline vitamin A_2 aldehyde was essentially that reported earlier (Cama, Dalvi, Morton, Salah, Steinberg & Stubbs, 1952). The crystalline material obtained, after four crystallizations from 5% (w/v) light petroleum solutions, had a m.p. of 76° with $E^{1\%}_{1\,cm} = 1438$ at 385 mμ (light petroleum).

One sample of crystalline vitamin A_2 aldehyde from a different batch of livers of *W. attu* showed a m.p. of 61°. Repeated recrystallization did not raise the melting point, which was sharp.

Analysis of crystalline vitamin A_2 aldehyde. Crystalline vitamin A_2 aldehyde with m.p. 76° was analysed for carbon and hydrogen.

Reaction of maleic anhydride with vitamin A_2 alcohol obtained by reduction of vitamin A_2 aldehyde with lithium aluminium hydride. The method for reduction of vitamin A_2 aldehyde with lithium aluminium hydride was that described by Cama & Morton (1953). From 15 mg of vitamin A_2 aldehyde, about 9 mg of vitamin A_2 alcohol with $E^{1\%}_{1\,cm} = 800$ at 350 mμ (light petroleum) was obtained. About 8 mg vitamin A_2 alcohol were then taken up in 10 ml benzene and treated with an equal volume of a 10% solution (w/v) of maleic anhydride in benzene at 27°. A 1 ml sample was taken out immediately, diluted to a suitable concentration, and the vitamin A_2 present in the solution was determined by the antimony-trichloride reaction at 693 mμ. Samples were again taken out every hour for 8 h and $E^{1\%}_{1\,cm}$ values at 693 mμ were determined.

Biological assay of crystalline vitamin A_2 aldehyde. The biological assay of crystalline vitamin A_2 aldehyde was carried out essentially as described in United States Pharmacopoeia XIV (1950).

When the young rats were 4 weeks old and weighed approximately 25–40 g, they were placed on the vitamin A-free diet made up of: casein (ether-extracted) 18,

starch 53·8, sucrose 15, refined groundnut oil 9, salt mixture (Hawk & Oser, 1931) 4, and cystine 0·2%. The vitamin A-free diet also included per kg diet: α-tocopheryl acetate 100 mg, 2 methyl-1,4-naphthaquinone 5 mg, ergocalciferol 100 μg, thiamine 5 mg, pyridoxine 5 mg, riboflavin 5 mg, biotin 0·5 mg, folic acid 0·5 mg, inositol 100 mg, and *p*-aminobenzoic acid 100 mg.

The rats were weighed at the same time each day at weekly intervals for the first 3 weeks and then every 3 or 4 days until their weights tended to become stationary. The ration was given *ad lib.* with an adequate supply of fresh tap water. A rat was considered depleted when its net gain in weight on four successive days did not exceed 1 g, provided that on two of these days the animal did not gain weight (Bliss & György, 1951). Eight litters of four male rats were used in a design consisting of two 4×4 latin squares, so that the factors in each square were litters, doses and order of depletion within each litter. When two or more litter-mates were depleted on the same day, they were allocated to doses on a body-weight basis.

The U.S.P. vitamin A reference standard was given to rats in quantities of 0·708 and 1·416 μg daily, equivalent to 2·06 and 4·12 i.u., and vitamin A_2 aldehyde in quantities of 1·79 and 3·58 μg, both in refined deodorized groundnut oil. The handling of rats during the test period was simplified by the device known as 'record days' (Bliss & György, 1951). The growth response for each rat in g/week was computed from the weekly weights. The weights of the rat on its initial record day and on the 7th, 21st, and 28th days thereafter were multiplied in turn by the coefficients -2 -1, 1 and 2, and the products added. The sum of these products was divided by 10 to obtain the growth in g/week.

RESULTS AND DISCUSSION

In this study, vitamin A_2 aldehyde could be obtained by manganese-dioxide oxidation of vitamin A_2 alcohol within 16 h, whereas in the previous studies (Cama, Dalvi, Morton, Salah, Steinberg & Stubbs, 1952), reaction with manganese dioxide for 4–7 days was necessary. The purity of vitamin A_2 obtained in the present studies from the liver oil of *W. attu* (vitamin A_2/vitamin A_1 = 10/1; Balasundaram, Cama, Sundaresan & Varma, 1956*a*) compared to the oils which were mixtures of vitamins A_1 and A_2, used in the previous studies, should partly explain this behaviour. It is also probable that because of the greater activity of the manganese dioxide used in the present study, the formation of vitamin A_2 aldehyde was achieved in 16 h.

The spectroscopic properties of two samples of vitamin A_2 aldehyde with m.p. 76° and 61° now obtained, were:

	Vitamin A_2 aldehyde, m.p. 76°	Vitamin A_2 aldehyde, m.p. 61°
$E^{1\%}_{1\,cm}$ (light petroleum) at 385 mμ	1438	1567
$E^{1\%}_{1\,cm}$ at 735 mμ in the $SbCl_3$ colour test	4065	4174

The discrepancy in the melting points of two samples of crystalline vitamin A_2 aldehyde was previously suggested to be due to the phenomenon of *cis–trans* isomerism in vitamin A_2 aldehyde preparations (Cama, Dalvi, Morton, Salah, Steinberg &

Stubbs, 1952; Farrar *et al.* 1952). However, the results now obtained do not support such a view, because the sample with m.p. 61° had a higher extinction coefficient than the one with m.p. 76°. It may be that the difference in melting point without any difference in absorption spectrum is due to the change in crystal structure. It is probable that the crystalline samples with m.p. 76° and 61° are the dimorphic forms of the all-*trans* isomer. This phenomenon has also been observed in previous studies on geometrical isomers of vitamin A_1 aldehyde (Robeson, Blum, Dieterle, Cawley & Baxter, 1955).

Table 1. *Growth rate (g/week) over 4 weeks of individual litter-mate rats receiving crystalline vitamin A_2 aldehyde or vitamin A_1 acetate*

Source and daily dose of vitamin A	Litter no. 1	2	3	4	5	6	7	8
Vitamin A_1 acetate								
2·06 i.u.	8·2	9·3	12·8	12·8	12·7	12·6	13·0	18·9
4·12 i.u.	13·7*	11·3	13·8	17·5	18·9	13·5	14·3	18·3
Vitamin A_2 aldehyde								
1·79 μg	10·2	11·6	10·4	15·6	8·8	12·9	12·6	16·1
3·58 μg	13·4	9·5	15·2	17·6	18·1	16·9	16·2	13·6

* Missing value, estimated by conventional least squares technique.

Crystalline vitamin A_2 aldehyde with m.p. 76° was shown to be all-*trans* by the study of the maleic-anhydride reaction of vitamin A_2 alcohol obtained by reduction of vitamin A_2 aldehyde with lithium aluminium hydride. The percentage recovery of vitamin A_2 after reaction with maleic anhydride for 7 h was 7·4; after 18 h it was 5·4, the low recovery values thus indicating that vitamin A_2 was all-*trans*.

The results of microanalysis confirmed that vitamin A_2 aldehyde (m.p. 76°) has the formula $C_{20}H_{26}O$. Required: C, 85·04; H, 9·29; O, 5·66. Found: C, 84·65; H, 9·75; O, 5·60.

Biological assay of vitamin A_2 aldehyde. From the rates of gain in weight of the rats, given in Table 1, the potency of the vitamin A_2 aldehyde was estimated statistically to be equivalent to 95% of that of the corresponding dose of vitamin A_1 acetate, with fiducial limits of 61% and 143% for the relative potency. Thus, by this assay, crystalline vitamin A_2 aldehyde has a biological activity of 1 098 000 i.u./g, which is about 33% of the activity of crystalline vitamin A_1 alcohol (the defined potency of crystalline vitamin A_1 alcohol is $3·33 \times 10^6$ i.u./g).

The biological activity of vitamin A_2 has long been engaging the attention of various workers. Karrer, Geiger & Bretscher (1941) first claimed that vitamin A_2 did not possess any biological activity. However, Karrer & Bretscher (1943) modified their view by showing that vitamin A_2 had 10% of the activity of vitamin A_1 and they attributed it to the capacity of the rats to transform a small portion of vitamin A_2 into vitamin A_1. In contrast, Gillam, Heilbron, Jones & Lederer (1938) observed that vitamin A_2 did possess biological activity, and later Gillam (1938) confirmed this observation by showing that vitamin A_2 is stored in the livers of animals which consume

diets containing vitamin A_2. The relative distribution of vitamins A_1 and A_2 in fish and sea birds was studied by Lovern, Morton & Ireland (1939), who concluded that vitamin A_2 does not replace vitamin A_1 in all functions.

Shantz, Embree, Hodge & Wills (1946) proved that vitamin A_2 is not converted into vitamin A_1 in vivo, but showed that in rats given vitamin A_2, porphyropsin replaces rhodopsin in the retinas. Later, Shantz & Brinkman (1950) observed, with pure non-crystalline natural vitamin A_2, a biological activity equal to 40% of that of crystalline vitamin A_1, and Farrar *et al.* (1952) showed that synthetic all-*trans* vitamin A_2 had 30% of the activity of vitamin A_1. Our study provided an opportunity for checking the biological activity of vitamin A_2 by biological assay of crystalline vitamin A_2 aldehyde. It has been well known that crystalline all-*trans* vitamin A_1 aldehyde has a biological potency almost equal to that of crystalline all-*trans* vitamin A_1 (Wendler *et al.* 1950). By analogy, the biological activity of vitamin A_2 aldehyde would probably be equal to that of vitamin A_2 if vitamin A_2 were obtained in a crystalline form. Thus it may well be that vitamin A_2 possesses a biological activity equal to 33% of that of crystalline vitamin A_1 alcohol. This figure is in agreement with that reported by Farrar *et al.* (1952) and Shantz & Brinkman (1950).

The spectroscopic properties of vitamin A_2 were studied in detail by Shantz (1948), Farrar *et al.* (1952) and Cama & Morton (1953). Taking the biological activity of vitamin A_2 as 40% of that of vitamin A_1, Cama & Morton (1953) showed that the conversion factor for $E^{1\%}_{1\,cm}$ at 352 mμ (cyclohexane) is 1000, and for the $SbCl_3$ colour test at 693 mμ it is 345. We will use these values until such time as vitamin A_2 is obtained in a crystalline form and its biological potency determined. As the biological potency of vitamin A_2 is quite appreciable, we consider that in the analysis of marine oils, in which vitamin A_2 is usually present to an extent of 10% (Balasundaram, Cama, Sundaresan & Varma, 1956*b*), the determination of vitamin A_2 may well be undertaken as a routine estimation, and its biological activity allowed for by analysts.

SUMMARY

1. Samples of crystalline vitamin A_2 aldehyde with melting points 76° and 61° were prepared by manganese-dioxide oxidation of vitamin A_2 alcohol from the liver oil of the freshwater fish *Wallago attu*.

2. Vitamin A_2 aldehyde (m.p. 76°) was reduced to vitamin A_2 alcohol and tested with maleic anhydride. The low recovery of vitamin A_2 obtained after 18 h showed that the alcohol and its precursor, vitamin A_2 aldehyde, were all-*trans*.

3. Vitamin A_2 aldehyde (m.p. 76°) was assayed biologically against U.S.P. vitamin A reference standard and was shown to possess a potency of 1098000 i.u./g, which is approximately 33% of the activity of crystalline vitamin A_1 alcohol.

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