Anhydrovitamin A₂ and Rehydrovitamin A₂. II

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In a previous publication (1), we reported that when 1 mg of a pure concentrate of anhydrovitamin A₂ (4'-ethoxy anhydrovitamin A₁, E₁₁₁₇₈ at 370 mμ = 2248 in light petroleum ether), and 0.5 mg of α-tocopherol are fed daily to vitamin A-deficient rats, growth is resumed, xerophthalmic lesions are cured, and a new compound named rehydrovitamin A₁ showing absorption maxima at 330, 348, and 365 mμ can be obtained from the livers. In analogy to rehydrovitamin A₁ (2), a tentative structure (4'-ethoxy rehydrovitamin A₁) was assigned to rehydrovitamin A₁ (1). Since anhydrovitamin A₂ has an ethoxy group in the β-ionone ring (3), the growth-promoting activity of this compound was surprising.

In this paper, the biological activity of crystalline anhydrovitamin A₃ assayed by the rat growth method of U.S.P. XIII (4), is reported. Further, the site of conversion of anhydrovitamin A₃ to rehydrovitamin A₄ in the rat is discussed.

EXPERIMENTAL PROCEDURE AND METHODS

Solvents and Reagents—These were obtained and purified as described earlier (1).

The U.S.P. vitamin A reference standard was used in the bio-assay.

Crystalline Anhydrovitamin A₃—Anhydrovitamin A₃ was prepared by the action of ethanolic hydrogen chloride on vitamin A₂ alcohol as described earlier (1). The material was purified by repeated chromatography and crystallized four times.

Estimation of Biological Potency of Crystalline Anhydrovitamin A₃—The biological potency of crystalline anhydrovitamin A₃ was assessed by the rat growth method of U.S.P. XIII (4). Both male and female rats were used in separate bio-assays. The daily supplements of anhydrovitamin A₃ and vitamin A acetate reference standard were fed in 0.1 to 0.15 ml of refined, deodorized groundnut oil containing 5 mg of α-tocopherol per ml.

On the last day of the bio-assay, 10 rats that had been fed anhydrovitamin A₃ were killed 3 hours after the last dose. The remaining 10 rats that had received this material were killed 24 hours after the last dose, and their livers, intestines, kidneys, spleens, lungs, and blood sera were analyzed for anhydrovitamin A₃ and rehydrovitamin A₃ by column and paper chromatography.

Intraintestinal Administration of Anhydrovitamin A₃—In one experiment, two vitamin A-deficient rats were mildly anesthetized, and the middle one-third of the intestine was ligated at two ends. An oily dispersion of 2 mg of anhydrovitamin A₃ and 1 mg of α-tocopherol was injected near the upper ligature, the incision sutured, and the animals returned to their cages. They were killed after 2 hours and the small intestines, between the two ligatures, and the livers were analyzed for anhydrovitamin A₃.

Intraperitoneal Administration of Anhydrovitamin A₃—An oily dispersion of 2 mg of anhydrovitamin A₃ and 1 mg of α-tocopherol was injected intraperitoneally daily for 10 days into a group of five vitamin A-deficient rats. On the 10th day, the animals were killed 3 hours after the last dose, and their tissues were analyzed.

Intramuscular Administration of Anhydrovitamin A₃—An oily dispersion of 2 mg of anhydrovitamin A₃ and 1 mg of α-tocopherol was injected into the leg muscle of five vitamin A-deficient rats. The intestinal tract, from stomach to caecum, had been removed from two of these rats. Three such injections were given at intervals of 1 hour. Two of the operated animals were killed 3 and 5 hours, respectively, after the first dose. The three unoperated animals were killed 3, 5, and 7 hours, respectively, after the first dose and the tissues of all the rats were individually analyzed for anhydro and rehydro derivatives.

When anhydrovitamin A₃ was given by a parenteral route, some unabsorbed material could be recovered near the site of injection.

Analysis of Lipid Extracts of Tissues by Column Chromatography—The lipid extracts of different tissues were saponified and the unsaponifiables were chromatographed over deactivated (water added to a level of 10%) alumina columns as described earlier (1). Unsaponifiables were always used, since anhydrovitamin A₃ and rehydrovitamin A₃ esters could not be separated on such columns. Anhydrovitamin A₃ could be eluted with light petroleum ether, whereas rehydrovitamin A₃ alcohol could be eluted with a mixture of 20% diethyl ether in light petroleum ether.

Analysis of Lipid Extracts of Tissues by Circular Paper Chromatography—Circular paper chromatograms on Whatman No. 1 filter paper disks that were impregnated with 1% Vaseline were run by the technique of Giri and Rao (5). Ethanol-water (75:25) was used as the developing solvent. The chromatograms were run in the dark for 6 to 8 hours and scanned in ultraviolet light, and the Rₚ values of the components were determined.

Anhydrovitamin A₃, rehydrovitamin A₃ ester, and rehydrovitamin A₃ alcohol could be readily separated from the tissue lipid extracts of the rats, even without saponification. Since these compounds had bright fluorescence in the ultraviolet light, they could be detected when present in trace.

Absorption Spectra and Antimony Trichloride Color Reaction—The ultraviolet absorption spectra and antimony trichloride color tests were performed with a Beckman model DU spectro-
photometer. For infrared analysis, a Perkin-Elmer Infra Cord was used.

RESULTS

Anhydrovitamin A<sub>2</sub>—The melting point of the crystals of anhydrovitamin A<sub>2</sub> was 80-81°C and the E<sub>1%cm</sub> at 369 µm was 3194. The E<sub>1%cm</sub> at 693 µm in the ShC<sub>2</sub> color test was 4500. Neither the melting point nor the extinction could be raised by further crystallizations. The yield of the crystalline material from a chromatographically pure concentrate (E<sub>1%cm</sub> = 2800) was about 30%.

Anhydrovitamin A<sub>2</sub> was used. 80-81°C was found.

<table>
<thead>
<tr>
<th>Sex of rats</th>
<th>Dose</th>
<th>Average gain in weight (g/week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>Anhydrovitamin A&lt;sub&gt;2&lt;/sub&gt;, 1 mg (U1)</td>
<td>3.0</td>
</tr>
<tr>
<td>Males</td>
<td>Vitamin A acetate, 0.600 µg (SI)</td>
<td>8.6</td>
</tr>
<tr>
<td>Males</td>
<td>Vitamin A acetate, 0.900 µg (SI)</td>
<td>11.11</td>
</tr>
<tr>
<td>Females</td>
<td>Anhydrovitamin A&lt;sub&gt;2&lt;/sub&gt;, 1 mg (U1)</td>
<td>4.9</td>
</tr>
<tr>
<td>Females</td>
<td>Anhydrovitamin A&lt;sub&gt;2&lt;/sub&gt;, 1.5 mg (U2)</td>
<td>6.2</td>
</tr>
<tr>
<td>Females</td>
<td>Vitamin A acetate, 0.600 µg (SI)</td>
<td>6.8</td>
</tr>
<tr>
<td>Females</td>
<td>Vitamin A acetate, 0.900 µg (SI)</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* In each group were six rats.

Biological Potency of Crystalline Anhydrovitamin A<sub>2</sub>—Dosage response of the rats used for the assay of crystalline anhydrovitamin A<sub>2</sub> in terms of mean gain in weight per week is given in Table I. The biological potency was found to be 330.9 µg and 326.4 µg per g of anhydrovitamin A<sub>2</sub> on the two doses, U1 and U2, respectively. The two levels obtained with female rats agree well and the graph of log dose against gain in weight for the test and standard substances are parallel, thus ensuring the validity of the bio-assay. In case of the male rats to which only one level of the unknown, but two levels of the reference standard were fed, the biological potency was obtained by ascertaining the micrograms of vitamin A acetate required to produce the same growth response as that obtained with 1 mg of the unknown substance. The results of the bio-assay with male rats gave a potency of 275.2 µg per g of anhydrovitamin A<sub>2</sub>.

A more accurate method for the bio-assay with larger numbers of animals as described in U.S.P. XIV (6) could not be used due to paucity of crystalline material as well as its low biological potency.

Qualitative Distribution of Anhydrovitamin A<sub>2</sub> and Rehydrovitamin A<sub>2</sub> in Different Tissues of Rat—Table II summarizes the results of tissue analyses when anhydrovitamin A<sub>2</sub> was administered by various routes. Due to the wide disparity in the ultraviolet extinctions of anhydrovitamin A<sub>2</sub> (E<sub>1%cm</sub> at 370 µm = 3100) and rehydrovitamin A<sub>2</sub> (E<sub>1%cm</sub> at 350 µm = 9) and different ShC<sub>2</sub> color test maxima (693 µm and 645 to 650 µm, respectively), quantitative estimations of the two derivatives in the various tissues could not be made. The generalizations made in Table II are based on the nature of absorption curves of the lipid extracts, the extinction values at 370 and 350 µm of the various fractions after column chromatography, and on the brightness of the fluorescent bands due to the two derivatives on paper chromatograms when scanned in ultraviolet light.

The mean RF values of compounds tested by circular paper chromatography were as follows. Anhydrovitamin A<sub>2</sub>, 0.35; rehydrovitamin A<sub>2</sub> acetate, 0; and rehydrovitamin A<sub>2</sub> alcohol, 1.0. Thus, the compounds under investigation, which had very different RF values, could be distinctly resolved, and the sequence of bands was always the same.

Rehydrovitamin A<sub>2</sub>—Rehydrovitamin A<sub>2</sub> alcohol obtained from the unsaponifiables of the livers of rats fed with anhydrovitamin A<sub>2</sub> after removal of the sterols and repeated chromatography, showed E<sub>1%cm</sub> at 348 µm = 9 and E<sub>1%cm</sub> at 645 µm = 15 in the ShC<sub>2</sub> color test.

Henbest et al. (3) have reported that anhydrovitamin A<sub>2</sub> shows a strong absorption band at 1100 cm<sup>-1</sup> in the infrared spectrum, which is due to the ethoxy group in the β-ionone ring. Similar observation was made by us on the preparation of crystalline anhydrovitamin A<sub>2</sub> used in the present study. To ascertain whether rehydrovitamin A<sub>2</sub> also has an absorption band at 1100 cm<sup>-1</sup>, the infrared spectrum of the above preparation of rehydrovitamin A<sub>2</sub> (although probably not very pure,
as judged by its low extinction), was taken in carbon tetrachloride (20 mg/0.1 ml). No absorption band was recorded at 1100 cm⁻¹ in the infrared spectrum of rehydrovitamin A₂.

**DISCUSSION**

**Crystalline Anhydrovitamin A₂**—The spectroscopic properties of crystalline anhydrovitamin A₂ prepared in the present study were in good agreement with those reported by the earlier workers (7, 8). The results of elemental analysis also suggest that the preparation was pure. The melting point 80—81° is, however, lower than that reported by Shantz (7) (89.5°) and Farrer et al. (8) (87—88°). The low melting point in the present instance was not due to lack of purity, since the melting point was sharp and could not be raised by recrystallizations. The disparity in the melting point may be due to the phenomenon of dimorphism as was shown to occur in case of the aldehyde of vitamin A₁ (9). The low yield of the crystals (30%) suggests the presence of other isomeric forms in the chromatographically pure concentrate of anhydrovitamin A₂ which resist crystallization.

**Growth-promoting Activity of Crystalline Anhydrovitamin A₂**—It is clear from the bio-assay that anhydrovitamin A₂, in spite of the presence of a substituent ethanolic group in the β-ionone ring (3), has biological activity. It is, however, difficult to judge if anhydrovitamin A₂ is biologically active per se or whether the active form is the rehydro derivative. Rehydrovitamin A₁ has been shown to be more active than anhydrovitamin A₁ by Shantz (2), who has suggested that probably rehydrovitamin A₁ is active. A similar possibility may well be true for anhydrovitamin A₂ and rehydrovitamin A₂.

The infrared absorption band at 1100 cm⁻¹ (corresponding to an ethoxy group in the β-ionone ring) is strong in the infrared spectrum of anhydrovitamin A₂, but is completely absent from the spectrum of rehydrovitamin A₂. Although the preparation of rehydrovitamin A₂ used for the infrared spectrum was probably not very pure, this striking difference in the infrared spectrum of anhydrovitamin A₂ and rehydrovitamin A₂ cannot be neglected; it is likely that the 4'-ethoxy group is completely absent in the molecule of rehydrovitamin A₂. The available data are inadequate at the moment to speculate about a new structure for rehydrovitamin A₂. Higher E₁%ım values for rehydrovitamin A₂ could not be obtained though the material was chromatographically and spectroscopically homogeneous. This is in line with the observation of Shantz (2) on rehydrovitamin A₁. Since compounds with a substituent group in the β-ionone ring are known to be biologically inactive, it may be that the biological activity of anhydrovitamin A₂ is due to its conversion to rehydrovitamin A₂ in vivo.

**Site of Conversion of Anhydrovitamin A₂ to Rehydrovitamin A₂**—When anhydrovitamin A₂ was administered by oral or intraintestinal routes, a mixture of varying amounts of anhydrovitamin A₂ and rehydrovitamin A₂ was detected in all the tissues (Table II). However, the results of tissue analysis of the rats that were killed 2 hours after receiving the first intraintestinal dose of anhydrovitamin A₂ suggest that rehydrovitamin A₂ appears in the intestine before it appears in the liver and hence that the intestine is an important site for the conversion of anhydrovitamin A₁ to rehydrovitamin A₂.

When anhydrovitamin A₂ was given parenterally, significant amounts of rehydrovitamin A₂ could not be detected in any of the tissues although the injected material reached the intestine via the blood (Table II). Thus, it seems that the conversion can occur in the intestine only during the process of absorption and that the other tissues are probably not effective. The trace amount of rehydrovitamin A₂ which was detected in the liver extract of rats which had received intraperitoneal injection of anhydrovitamin A₂ for 10 days, may have been formed during the resorption of a small amount of anhydrovitamin A₂ which might have passed into the intestinal lumen with bile.

The intestine has often been found to be an active site for biochemical reactions involving carotenoids and vitamin A and hence the above observation is not surprising. However, the extent of conversion of the anhydro derivative is rather small and even the unconverted material passes through the intestinal wall. Here it is interesting to recall that many species that convert β-carotene to vitamin A also pass unchanged β-carotene through the intestinal wall.

**SUMMARY**

Crystalline anhydrovitamin A₂ has a biological activity of 328 μg per g of anhydrovitamin A₂ in female rats and 275 μg per g of anhydrovitamin A₂ in case of male rats when fed with 0.5 mg of α-tocopherol.

Anhydrovitamin A₂, which contains the 4'-ethoxy group, when fed to rats is deposited in the liver as rehydrovitamin A₂. The intestine has been indicated as the site for this conversion. The 4'-ethoxy group is probably absent in rehydrovitamin A₂ as shown by the complete absence of a 1100 cm⁻¹ band in its infrared spectrum.

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