Anhydro vitamin A is the unsaturated hydrocarbon formed when vitamin A loses a molecule of water (1). This occurs with extreme ease when a solution of vitamin A alcohol in anhydrous solvent is treated with a trace of catalyst, such as a strong mineral acid. The resulting compound has six conjugated double bonds in place of the original five which, apparently, have shifted from their normal position. The formation of anhydro vitamin A, as postulated by Meunier et al. (2), involves an ionization of vitamin A in the presence of acid, followed by a shift of the positive charge to a carbon atom in the ionone ring where the molecule eventually regains its electrical neutrality by losing a proton from this carbon atom according to the accompanying scheme.

The early work of Embree (3) on crude concentrates of anhydro vitamin A indicated that this material had no detectable growth-promoting power. However, a bioassay on the first crystalline preparation (1) showed a biological potency of about 15,000 U. S. P. units per gm., approximately 0.4 per cent of the activity of crystalline vitamin A alcohol. It was thought that this slight activity might be caused by contamination with a small amount of unchanged vitamin A; therefore two more crystalline preparations were made. These were scrupulously purified by repeated chromatographic adsorption and recrystallization. Both were found to have the same degree of biological activity as the first preparation, and this activity could, therefore, be considered an inherent property of the compound.

The questions with which the present paper is concerned then arose. Does anhydro vitamin A per se exert physiological activity, or is a small amount of it reconstituted into vitamin A by the animal body, or is it changed into an entirely new compound possessing some degree of vitamin A activity? The experimental work described below shows that a new compound is formed in vivo which is intermediate in activity between vitamin A and the ingested anhydro vitamin A.

The general experimental procedure was as follows: Vitamin A-de-
ficient rats were fed crystalline anhydro vitamin A at a level high enough to produce some liver storage of the anhydro vitamin A derivative. At the end of approximately 3 weeks the animals were sacrificed and the livers removed and extracted with ethyl ether. No anhydro vitamin A or vitamin A could be detected in the liver extract. However, a com-

\[
\text{Vitamin A alcohol (+ acid catalyst)}
\]

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{H}_2\text{C} & \quad \text{C} = \text{CH} \quad \text{C} = \text{CH} \quad \text{C} = \text{CH} \quad \text{C} = \text{CH} \quad \text{C} = \text{CH} + \text{OH}^- \\
\text{H}_2\text{C} & \quad \text{C} \\
\text{CH}_3 & \quad \text{CH}_3 \\
\text{H}_2 & \\
\text{CH}_3 & \quad \text{CH}_3 \\
\end{align*}
\]

\[
\text{Anhydro vitamin A}
\]

ound possessing certain characteristics of both these substances was found. When this material was fed to vitamin A-deficient rats, it was found to have about 20 times the activity of the original anhydro vitamin A.

The new compound produces a blue color with antimony trichloride with an absorption maximum at about 612 mp, in the same region as the colors
given by anhydro vitamin A, vitamin A alcohol, and other derivatives of vitamin A such as its esters and ethers. Since these compounds all have the same blue color extinction on a molecular basis, it seems reasonable to assume that the new compound behaves in the same manner, thus affording a basis for the quantitative biological comparison of this substance with crystalline vitamin A and anhydro vitamin A.

Since only a tiny amount of this compound greatly diluted with extraneous fatty material was obtained, rather indirect observations were made in an attempt to elucidate its structure. The results of these studies indicate that the new substance may be a structural isomer of vitamin A, having the formula shown in the diagram.

\[
\begin{align*}
\text{CH}_2 & \quad \text{CH}_2 \\
\text{H}_2\text{C} & \quad \text{C} = \text{CH} \quad \text{C} = \text{CH} \quad \text{C} = \text{CH} \quad \text{C} = \text{CH} \quad \text{CH}_2\text{CH}_2\text{OH} \\
\text{H}_2\text{C} & \quad \text{C} \\
\text{H} & \\
\end{align*}
\]

Rehydro vitamin A

Because of the manner in which this compound is apparently formed, the name "rehydro vitamin A" is proposed. It is conjectured that the vitamin A-deficient animal, in its attempt to utilize anhydro vitamin A, is able to add the elements of water to a small portion of the anhydro vitamin A, but is incapable of shifting the double bonds to their normal position. Thus, the animal effects a partial return toward the normal vitamin A structure and in so doing effects only a partial return toward the high activity of vitamin A.

EXPERIMENTAL

Preparation of Anhydro Vitamin A—The method of preparing anhydro vitamin A originally used by Edisbury et al. (4) consists of dehydrating vitamin A alcohol in \( n/30 \) anhydrous alcoholic hydrogen chloride solution. We have shown (1) that the concentration of vitamin A must be kept below 1 per cent to obtain a good yield. The preparation of a sizable quantity of anhydro vitamin A by this method involves a cumbersome volume of acidic alcohol solution. A new method suitable for the preparation of large amounts of anhydro vitamin A was devised as follows: 100 gm. of a saponified vitamin A concentrate with \( E_{1\text{cm}}^{2\text{%}} = 1340 \) were dissolved in 600 ml. of benzene in a 1 liter round bottomed flask fitted with a condenser and water-collecting side arm. The solution was re-
fluxed for 15 minutes but no water could be seen in the side arm. 3 mg. of p-toluenesulfonic acid were added, and droplets of water immediately began to collect in the measuring tube. The theoretical amount of vitamin A present was approximately 75 gm. or 0.26 mole, which should yield 4.7 ml. of water. At the end of 2 hours, 4.6 ml. had been collected and the evolution of water had apparently ceased.

The benzene solution was cooled to room temperature and poured onto a column containing 900 gm. of aluminum oxide (Merck, according to Brockmann). The anhydro vitamin A was the most weakly adsorbed material and formed a bright yellow band which was washed through the column with Skellysolve F. The solvent was removed under nitrogen, leaving 62.5 gm. of orange oil, exhibiting absorption maxima in the ultraviolet at 351, 368, and 390 m\(\mu\) with \(E_{1\%}^{1\%}\) (368 m\(\mu\)) = 1800. This material was chromatographed again on a column of magnesia (Westvaco) containing 30 per cent Celite. The anhydro vitamin A fraction consisted of 44.0 gm. of viscous orange oil with \(E_{1\%}^{1\%}\) (369 m\(\mu\)) = 2290. This material was dissolved in 175 ml. of Skellysolve F and after storage for 3 days at \(-30^\circ\) yielded 11.2 gm. of crystals. Two recrystallizations from 30 per cent solutions in Skellysolve F gave a final yield of 5.5 gm. of orange prisms which melted at 76.5-77.5°. The ultraviolet absorption curve showed three maxima at 351, 371, and 392 m\(\mu\) with \(E_{1\%}^{1\%}\) = 2540, 3680, and 3200. The crystals were sealed in ampuls under a vacuum and stored at \(-30^\circ\) for future use.

Toxicity of Anhydro Vitamin A—The amount of rehydro vitamin A stored in the liver was very small compared to the amount of anhydro vitamin A fed, and it was therefore desirable to feed as large a quantity as possible without producing toxic symptoms.

5 mg. of crystalline anhydro vitamin A were fed daily in olive oil solution to each of eighteen male rats, 6 weeks old, which had received a vitamin A-free diet for a period of 3 weeks since weaning. At this level toxic effects were noted which did not appear on a daily dose of 2.5 mg.

One rat died after 9 days on the experiment. The bladder was distended with urine and both testes showed hemorrhage. Of the three animals which died on the 10th day, two showed hemorrhage in the epididymides. One rat died on the 12th day, showing a distended bladder and hemorrhage in one testis and both epididymides. The thirteen remaining rats were killed on the 13th day. In twelve of the animals there were hemorrhages in either the testes or epididymides. In one there was subcutaneous hemorrhage in the abdominal region, while in another a subcutaneous hemorrhage occurred near the right shoulder.

In a parallel experiment with a vitamin A concentrate, approximately 5.6 mg. of vitamin A were fed daily to each of six weanling rats for 5
weeks. No deaths occurred until the 33rd day of the experiment. By the 10th day, two of the rats were beginning to lose hair around the mouth, and, by the 21st day, four of the animals had spontaneously broken one or more legs. Similar toxic effects of high potency fish liver oils were observed by Vedder and Rosenberg (5). It appears that anhydro vitamin A has a toxic effect somewhat different from that of vitamin A.

**Feeding of Anhydro Vitamin A**—Vitamin A-deficient rats grew well when a daily supplement of approximately 0.1 mg. of anhydro vitamin A was added to the diet, but no detectable amount of material was stored in the liver at this level. In order to feed anhydro vitamin A at a level at which some liver storage would be obtained, but no symptoms of toxicity would appear, a daily dose of 1.0 mg. was used.

In the first feeding experiment, 55 male weanling rats were placed on a vitamin A-free and low vitamin E diet for a period of 14 days, at which time the absence of any stored vitamin A was assumed, since the liver extracts from five animals gave no blue color with antimony trichloride. The rats were then given a daily supplement of 1.0 mg. of crystalline anhydro vitamin A and 0.5 mg. of mixed tocopherols for a period of 18 days. (A preliminary experiment had shown that the simultaneous supplement of tocopherols increased the storage of vitamin A-active material in the liver by about 25 per cent.) During this 18 day period the animals gained an average of 60 gm. in weight. They were killed on the 19th day and the livers were removed, weighed, and stored at -30° until they were analyzed.

**Examination of Livers for Vitamin A-Active Material**

**Extraction**—The livers from the above animals (50 livers, 384 gm.) were minced in a Waring blender with 200 ml. of 0.05 N aqueous KOH and 50 ml. of ethanol. The suspension was extracted four times with 250 ml. portions of ethyl ether. The combined ether solutions were washed, dried over sodium sulfate, and filtered. Removal of the solvent under nitrogen left 11.0 gm. of a light yellow oil.

**Saponification**—A 7.0 gm. portion of this oil was saponified by warming under nitrogen to 80° for \( \frac{1}{2} \) hour in a solution of ethyl alcohol containing 2.0 gm. of KOH. The solution was diluted with water and extracted three times with ethyl ether. The combined ether extracts were washed, dried, and the solvent removed under nitrogen. The residue consisted of 0.435 gm. of viscous red oil.

**Ultraviolet Absorption Measurement**—Ultraviolet absorption curves were determined for both the whole oil from rat liver and for the unsaponifiable portion. The whole oil showed very strong extraneous absorption below 300 m\( \mu \), most of which disappeared upon saponification. Otherwise, the
two curves were similar in shape, with absorption maxima at 351 and 369 m\(\text{m}\), and an inflection at about 330 m\(\text{m}\). \(E_{1\%}^{\text{cm}}\) (351 m\(\text{m}\)) for the whole oil was 0.59 and for the unsaponifiable portion 10.2 (Fig. 1). The absorption curves of crystalline vitamin A alcohol and crystalline anhydro vitamin A are also reproduced in Fig. 1 for comparison. The ultraviolet curve of this new product is very similar in appearance to "isoanhydro vitamin A" (1) which is formed by prolonged treatment of vitamin A in dry alcoholic hydrogen chloride. However, its other physical properties described below are totally different, which precludes the possibility that the two substances are identical.

**Antimony Trichloride Reaction Product**—The rat liver oil was found to give a blue color with antimony trichloride solution, and the absorption curve of this reaction product was determined on a General Electric recording visual spectrophotometer. The absorption spectra of the various fractions all showed only a single band with a sharp maximum at 612 m\(\text{m}\). \(E_{1\%}^{\text{cm}}\) (612 m\(\text{m}\)) for a fraction of chromatographically purified whole rat liver oil was 3.33. In the ultraviolet region, this fraction had

---

**Fig. 1.** Ultraviolet absorption spectra in ethanol. Curve 1, 0.152 per cent solution of saponified liver oil extract from rats fed anhydro vitamin A; Curve 2, 0.0008 per cent solution of crystalline vitamin A alcohol; Curve 3, 0.00044 per cent solution of crystalline anhydro vitamin A.
a value of 2.30 for $E_{1\%}^{1\%}$ (351 m$\mu$). Thus, the ratio $E(612 \text{ m}$\mu$)$ to $E(351 \text{ m}$\mu$)$ for this new substance is 1.45, about the same as the ratio $E(620 \text{ m}$\mu$)$ to $E(372 \text{ m}$\mu$)$ for anhydro vitamin A.

**Chromatographic Adsorption**—1 gm. of the whole rat liver oil was adsorbed on a column containing 20 gm. of magnesium oxide mixed with 25 per cent Celite. When developed with 200 ml. of Skellysolve F, 85 per cent of the material having an absorption maximum at 351 m$\mu$ was washed through the column. When 0.2 gm. of the unsaponifiable portion (representing about 3.0 gm. of the original oil) was diluted to 1 gm. with Wesson oil and treated in an identical manner, the material with an absorption maximum at 351 m$\mu$ was found to be very strongly adsorbed near the top of the column.

**Solvent Partition**—The adsorption experiments indicated that the rehydro vitamin A was stored in the rat liver as an ester, and was strongly adsorbed after saponification because of the free hydroxyl group. This was confirmed by solvent partition experiments, which were carried out in the following manner: Skellysolve F and 83 per cent aqueous ethanol were shaken together until they were mutually saturated at room temperature. 1.0 gm. of the whole oil and 0.2 gm. of the unsaponifiable portion were dissolved in separate 25 ml. portions of the Skellysolve. Each solution was then extracted seven times with 25 ml. portions of the 83 per cent ethanol. The extinction at 351 m$\mu$ for each extract and for each of the residual petroleum ether solutions was determined on a Beckman spectrophotometer. It was found that the petroleum ether-83 per cent ethanol distribution ratio for the unsaponified material was 98:2, while that for the saponified portion was 45:55. These are approximately the ratios found for vitamin A fatty acid esters and free vitamin A alcohol, respectively.

**Attempted Dehydration of Rehydro Vitamin A**—Some of the saponified material was dissolved in 0.1 N anhydrous alcoholic hydrogen chloride and allowed to stand for 30 minutes. No change in the ultraviolet absorption spectrum was observed, nor was there any change in the solvent distribution ratio.

**Biological Potency of Rehydro Vitamin A**—Previous work has shown that vitamin A alcohol, vitamin A esters, and anhydro vitamin A produce the same intensity of blue color with antimony trichloride on a molecular basis. It was assumed that the antimony trichloride blue product of rehydro vitamin A has about the same molecular extinction coefficient as have these above derivatives of vitamin A.

A preliminary biological assay on three vitamin A-depleted rats over a 3 week feeding period indicated that, on the basis of blue color, the new compound possessed approximately one-tenth the activity of vitamin A.
and 25 times the activity of anhydro vitamin A. A confirmatory assay on six additional animals was carried out after preparation of a second batch of the conversion product as follows:

93 vitamin A-deficient rats were fed 1.25 mg. daily of crystalline anhydro vitamin A for a period of 32 days. At the end of this time the animals had grown an average of 115 gm. and were sacrificed. The livers were removed, minced, and extracted repeatedly with ethyl ether and alcohol. Most of the phospholipides were precipitated with acetone and the remaining oil (8.2 gm.) was saponified. After removal of sterols, the unsaponifiable portion was dissolved in 3 gm. of Wesson oil for bioassay. The ultraviolet absorption maximum of this preparation was at 351 μm with $E_{1%}^{1%} = 2.3$. The apparent vitamin A potency by blue color was 2500 units per gm.

This solution was fed to six vitamin A-deficient weanling rats at three levels, two rats per level. The three levels were 77, 44, and 22 apparent blue color units of vitamin A per day.

The two animals on the highest level had gained over 50 gm. apiece at the end of 12 days and feeding was discontinued. At the end of 28 days, the two rats on the middle level had gained an average of 54 gm. apiece and the pair on the lowest level had gained an average of 20 gm. each. By comparison with other vitamin A assays in progress in these laboratories at this time, it was calculated that the lower level was equivalent to approximately 1.5 units and the middle level to approximately 3 units of vitamin A per day. Thus, despite the small number of animals used, these results essentially confirm those of the preliminary assay, indicating that the biological potency of the in vivo conversion product, rehydro vitamin A, is about one-fifteenth that of vitamin A itself, but is 15 to 20 times greater than that of the original anhydro vitamin A.

**DISCUSSION**

The behavior of this new compound with regard to its relative adsorption affinity and solvent distribution before and after saponification indicates that it contains a hydroxyl group and is stored in the liver as a fatty acid ester. The ultraviolet absorption spectrum exhibits a triple peak similar to anhydro vitamin A but the position of these maxima shows that one double bond of the molecule of anhydro vitamin A has been lost. As further confirmation of the double bond structure of rehydro vitamin A, it can be compared to the synthetic hydrocarbons containing five conjugated double bonds, previously synthesized by the author (6). The ultraviolet spectra are almost identical. Since a double bond system in conjugation with a hydroxyl group, as in vitamin A, usually exhibits only a single absorption maximum, the hydroxyl group of rehydro vitamin A is probably isolated from the unsaturated portion of the molecule.
The failure of rehydro vitamin A to be dehydrated in 0.1 N anhydrous alcoholic hydrogen chloride is further evidence of its structure, since vitamin A and many similar compounds readily lose a molecule of water under these conditions. The stability of this substance to a mild anhydrous acid solution would indicate that the ionization postulated by Meunier (2) as a part of the vitamin A dehydration mechanism does not occur in this case, probably because the hydroxyl group is not activated by conjugation with an unsaturated system. Another possible requirement is that the hydroxyl group should be able to undergo an allylic shift to a tertiary carbon atom before dehydration can take place. In either case, these conditions do not appear to be present in the molecule of rehydro vitamin A, indicating that it is probably a primary alcohol whose hydroxyl group is isolated from the double bond system.

On the other hand, antimony trichloride appears to be a strong enough reagent to bring the double bond system and the hydroxy group into conjugation, thus producing a blue color in the same region as given by vitamin A and anhydro vitamin A. Antimony trichloride has been shown to have this effect on other compounds containing two isolated chromophoric groups,1 and a similar shift of double bonds may account for the blue-green color produced by the action of antimony trichloride on vitamin A, and anhydro vitamin A (7). Thus the structure of rehydro vitamin A given earlier in this paper is proposed as an interpretation of the above experimental findings.

SUMMARY

The growth-promoting activity in the rat of the hydrocarbon anhydro vitamin A has been shown to be due to a new compound formed in vivo. A small amount of this substance is stored in the liver as an ester. When extracted from the liver and fed to other vitamin A-deficient rats, this compound has been found to be about 20 times as active as the original anhydro vitamin A, although only about one-fifteenth as active as vitamin A. From a study of its properties, a tentative formula and the name "rehydro vitamin A" have been proposed.

Anhydro vitamin A is toxic to rats at about the same level as vitamin A. The symptoms of toxicity, however, are somewhat different. Doses of 5 mg. per day induce hemorrhage, especially in the testes and epididymides of male rats.

An easy method of preparing anhydro vitamin A in large amounts has been described.

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