The Conversion of Vitamin A₈ to Retinene₂ in a Fresh-water Fish*

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The stimulus for the present work arises from the discovery of Wald (1) that bullfrog tadpoles change their visual pigment from porphyropsin (retinene₂ chromophore) to rhodopsin (retinene₁ chromophore) during metamorphosis. Wilt (2) subsequently confirmed and extended this finding. The visual pigment conversion is stimulated by the thyroid hormone (3). Further analysis of this system demanded an extensive investigation of the comparative biochemistry of carotenoid and vitamin A metabolism. Since fresh-water fish have a pure porphyropsin system, it was decided that an investigation of chromophore synthesis in this instance would lead to an understanding of the mechanism of vitamin A₁ and retinene₂ synthesis (cf. Moore (4)).

The specific objectives of this report are (a) a description of radioisotopic tracer techniques used to investigate visual pigment chromophore synthesis; (b) the demonstration that the retinene of porphyropsin can be formed from vitamin A₁; (c) the localization of the site of vitamin interconversion in the eye; and (d) a preliminary analysis of the role of the liver in vitamin A interconversion.

EXPERIMENTAL PROCEDURE

Mature green gills, a hybrid of Lepomis species (sunfish), were collected in local streams and used for all experiments. The vitamin A was dissolved in 5 parts of Tween 80,¹ then mixed with 95 parts of 1% bovine serum albumin, and subsequently injected intraperitoneally or intraocularly. In one type of experiment (Table III, Experiment 1), the bovine serum albumin contained 1 × 10⁻⁴ μg triiodothyronine.

The tritiated vitamin A₁ used in these studies was generously donated by Hoffman-La Roche and Company, Basle. It was supplied as the acetate ester with a specific activity of 308 pc per mg. The site of labeling is indicated in Fig. 1, which also presents the structure of vitamin A₁. The retinenes are the aldehydes of the A vitamins.

Vitamin A and retinene analyses were carried out by the standard Carr-Price antimony trichloride colorimetric reaction (2) in a Beckman DK-2 recording spectrophotometer. This instrument was also used to record visual pigment spectra. The absorption maxima and values in the SbCl₃ reaction (Baker, reagent grade) in this laboratory are: vitamin A₁, 618 μμ, 4400; retinene₂, 661 μμ, 4000; vitamin A₂, 694 μμ, 4400;

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² Nutritional Biochemicals Corporation.

Vitamin A was extracted from liver by grinding the tissue to a dry powder with sand and sodium sulfate and extracting the powder with peroxide-free diethyl ether.

Visual pigments were prepared by standard procedures of Wald and his associates (8). Briefly, this consists of isolating rod outer segments in buffered 45% sucrose, washing the segments with neutral phosphate buffer, H₂O, 4% alum, and H₂O, lyophilizing and extracting the segments with petroleum ether, resuspending the segments in H₂O, washing with phosphate buffer, and extracting the segments overnight at 4° with 2% aqueous digitonin buffered with phosphate at pH 7.0. This procedure was carried out in dim red light. The ratio of absorption at the spectral minimum to absorption at the spectral maximum was usually 0.4 to 0.5, indicating a fair degree of purity of the pigments.

Usually one aliquot of unbleached visual pigment was extracted with petroleum ether and analyzed with SbCl₃ to assure that all of the free retinene and vitamin A were removed. The remainder of the pigment was routinely bleached in two steps by the method of Dartnall (9). One-third to one-half of the pigment was bleached with light composed of wave lengths from 610 μμ to 3000 μμ, which bleaches porphyropsin rapidly but leaves rhodopsin essentially intact. (The light source was a 300-watt tungsten bulb at 70 cm from the visual pigment. Cornning filter No. 2412 and a water heat-absorbing filter were used.) The products of the first bleaching were then extracted. A second bleaching was subsequently carried out by heating the extracted, partially bleached preparation to 70° and exposing the sample to white light.

Retinene released by bleaching was extracted at 4° by adding 0.2 volume of cold glacial acetic acid and shaking with 2 volumes of cold petroleum ether. The petroleum ether extraction was routinely carried out three times. The retinene of bleached

The absorption maximum of retinene₂ in the Carr-Price reaction is somewhat variable. Pure retinene₂ has an absorption maximum at 755 μμ (5). Plack (6) has recently shown that, if acetic anhydride is omitted from the reaction mixture and the antimony trichloride is distilled, the maximum is 741 μμ. Previous studies showed that retinene₂ isolated from natural sources gave a maximum at 705 μμ (2, 7). In spite of the differences in reports from different laboratories, we find that the use of standard procedures and Baker antimony trichloride leads to a very reproducible spectrum with its maximum at 718 μμ.
pigment molecules is extracted by petroleum ether after addition of acid, and the cold acetic acid does not appreciably release bound retinene from unbleached visual pigment. The remaining unbleached visual pigment can then be re-bleached with white light, as described above, and re-extracted with petroleum ether. Finally, the pigment residue is extracted with cold acetone to remove any retinene that was not extracted by petroleum ether-acetic acid. Commonly, petroleum ether does not extract all of the remaining retinene of fully bleached preparations. The extracts of retinene are reduced to dryness under reduced pressure and transferred to CHCl need for the colorimetric analysis. Application of this procedure to mixtures of porphyropsin and rhodopsin from retinas of metamorphosing Rana catesbeiana demonstrated its suitability for separating retinene from retinenez.

After colorimetric analysis of retinene, radioactivity was determined by liquid scintillation counting procedures. The SbCl3, which interferes with liquid scintillation counting, is removed from the Carr-Price reaction mixture by shaking three times with 0.5 volume of 9% HCl. The chloroformic solution is transferred to a scintillation counting vial and reduced to dryness with a stream of N2. Acid is added and the residue is extracted with acetone (Curve E). The chloroformic solution is transferred to a scintillation counting vial and reduced to dryness under reduced pressure and transferred to CHCl need for the colorimetric analysis. Application of this procedure to mixtures of porphyropsin and rhodopsin from retinas of metamorphosing Rana catesbeiana demonstrated its suitability for separating retinene from retinenez.

Specifically labeled H3-vitamin A2 was prepared from H3-vitamin A1 by the method of Jones et al. (10) with N-bromosuccinimide after addition of carrier vitamin A1. The crude yield of vitamin A2 was 30%. The product was purified by chromatography (three times) on water-weakened alumina, with petroleum ether and benzene used as a developing solvent system. It was not possible to crystallize the vitamin. Antimony trichloride reaction curves of these two retinene fractions, respectively. They are both characteristic of pure retinene. The first extraction quantitatively extracted the released retinene, but the second, after white light bleaching, extracted only a portion of the retinene. The remainder of the retinene was extracted with acetone (Curve E).

Table I presents the data for the above and similar experiments. The retinene2 bleached from the pigment was radioactive. The incorporation of label reaches a maximum between 1 and 2 weeks at 20°, the temperature at which the fish were kept.

There are two possibilities of artifacts in this experiment. First, it is possible that vitamin A2 is only oxidized to retinene, and that the latter is then coupled to opsin. The radioactivity of the retinene would be due to the presence of small amounts of radioactive retinene1 admixed with the large population of retinene2 molecules. An inspection of Table I rules out this possibility. The specific activity of the retinene released by the bleaching with red light, which bleaches predominantly porphyropsin, is approximately the same as the specific activity of the retinene released by the second bleaching with white light, which would release both retinenes.

RESULTS

Conversion of Vitamin A1 to Retinene-The results of a typical experiment. Six fish were given injections of 340,000 c.p.m. of H3-vitamin A1 acetate. After 7 days the fish were dark-adapted and killed, and the visual pigment was prepared. The pigment (Curve A) has a broad absorption maximum at 515 to 520 μm, which is indicative of porphyropsin. (The maximum at pH 7 is 518 μm.) After partial bleaching with red light, 61% of the pigment remains. (Preparations examined during bleaching showed no shift of absorption maximum, indicating homogeneity of the pigment. In two cases, however, difference spectra revealed the presence of a second pigment, constituting only a few percent of the total, with a difference spectrum maximum of 538 μm.) Partial bleaching results (Curve B) in a new maximum at 400 to 405 μm, which is indicative of the release of retinene. The free retinene2 was extracted, the pigment was re-bleached with white light, and the retinene was re-extracted. Curves C and D are the antimony trichloride reaction curves of these two retinene fractions, respectively. They are both characteristic of pure retinene. The first extraction quantitatively extracted the released retinene, but the second, after white light bleaching, extracted only a portion of the retinene. The remainder of the retinene was extracted with acetone (Curve E).

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**Table I**

*Retinene formed from intraperitoneally injected vitamin A₁*

No retinene was detected in any preparation. Specific activity is expressed in counts above background per minute per microgram of retinene. The “after bleach” is retinene present in acetone extracts of visual pigment after bleaching and petroleum ether extraction.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of fish</th>
<th>Days until sacrifice</th>
<th>Radioactivity injected per fish</th>
<th>First bleach</th>
<th>Second bleach</th>
<th>After bleach</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Retinene</td>
<td>Specific activity</td>
<td>Retinene</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>100,000</td>
<td>1.315</td>
<td>208</td>
<td>0.258</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>2</td>
<td>343,000</td>
<td>1.42</td>
<td>241</td>
<td>0.351</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>7</td>
<td>345,000</td>
<td>1.31</td>
<td>1123</td>
<td>0.98</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>14</td>
<td>343,000</td>
<td>0.965</td>
<td>939</td>
<td>0.348</td>
</tr>
</tbody>
</table>

**Table II**

*Retinene formed from vitamin A₁ injected intracocularly*

No retinene was detected in any preparation. Specific activity is expressed in counts above background per minute per microgram of retinene.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of eyes</th>
<th>Duration of experiment</th>
<th>Radioactivity injected per eye</th>
<th>First bleach</th>
<th>Second bleach</th>
<th>Total counts in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Retinene</td>
<td>Specific activity</td>
<td>Retinene</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>6</td>
<td>26,000</td>
<td>0.59</td>
<td>346</td>
<td>0.27</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>24</td>
<td>26,000</td>
<td>0.43</td>
<td>414</td>
<td>0.65</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>2</td>
<td>11,500</td>
<td>0.885</td>
<td>125</td>
<td>0.70</td>
</tr>
</tbody>
</table>

The second possible source of confusion arises from the possibility that the radioactive vitamin A₁ is catabolized and that some of the tritium labels other compounds in the eye. This seems unlikely because the action of light is necessary to release radioactivity. Nevertheless, Krinsky (12) has shown that some phospholipid bound to visual pigment becomes susceptible to diethyl ether extraction after bleaching of visual pigments. It is not known whether this phospholipid can be extracted with petroleum ether. Krinsky's extractions were carried out with sucrose suspensions of rod outer segments. It is doubtful that petroleum ether could extract phospholipid from digitonin solutions of aluna-hardened, petroleum ether-extracted rod powders, as Krinsky pointed out. Furthermore, acetone extracts of bleached digitonin solutions of pigment contain radioactivity which can be quantitatively accounted for by retinene. The data support the conclusion that vitamin A₁ can be converted to retinene in *Lepomis*.

**Site of Conversion of Vitamin A₁ to Retinene**—Since it was demonstrated previously that thyroxin affects amphibian visual pigment conversion directly in the eye (3), it was considered of interest to see whether the fish eye can convert vitamin A₁ to retinene. Tritiated vitamin A₁ acetate was injected directly into the eye, and the visual pigments were isolated at short time intervals thereafter. These experiments are presented in Table II. The visual pigment chromophores are quickly labeled, but the participation of other organs cannot be ruled out because counts appear in the liver even after a few hours. Therefore, the experiment was carried out in organ culture in *vitro*. The eyes were aseptically removed, 5 μl of H₃-vitamin A₁ acetate were injected into the vitreous humor, and the eyes were placed in organ culture at 22°C. The eyes were kept under ordinary laboratory lighting conditions for 4 hours and then were dark-adapted before the visual pigments were extracted. Fig. 3 graphically presents a typical experiment of this type. Table III presents the results of these experiments. The visual pigment chromophore is rapidly labeled. Furthermore, the results of differential bleaching demonstrate that the retinene arises from vitamin A₁ in the eye in *vitro*. In Experiment 1 of Table III, the vitamin A₁ was suspended in 10⁻⁴ M triiodothyronine; this high concentration of hormone led to a reproducible lowering of the specific activity of the retinene by a factor of 2. Experiments 3 and 4 of Table III were designed to compare
the efficacy of vitamin A1 and A2. Vitamin A2 was approximately 3 times more effective in labeling the retinene molecule than was vitamin A1.

Role of Liver—Exploratory experiments have been carried out to determine whether the liver can also carry out the conversion of vitamin A1 to A2. After injection of large amounts of nonradioactive vitamin A into the body cavity of fish, vitamin A1 is present in appreciable amounts in liver tissue after 24 hours. The liver of these fish normally contains only vitamin A2. On subsequent days, the vitamin A1 disappeared from the liver tissue, being absent 4 to 5 days after injection. In order to distinguish conversion of A1 to A2 from other mechanisms of loss of A1 from the liver, livers of fish injected with vitamin A1 were placed in organ culture. The ratio of A1 to A2 was followed daily for 3 days. There was no disappearance of vitamin A1 or build-up of vitamin A2 under these conditions.

DISCUSSION

The results support the conclusion that intraperitoneally injected tritiated vitamin A1 leads to labeling of the retinene molecule. It that retinene2 that is labeled is supported by the following facts. (a) Radioactivity is only released in substantial amounts by bleaching the visual pigments. The small amounts of radioactivity extractable with petroleum ether from unbleached pigments are accompanied by retinene extraction. This small amount of retinene is extractable only after addition of acetic acid. (b) Incompletely bleached and petroleum ether-extracted visual pigments remain extractable with petroleum ether, and the acetone-extractable counts are accompanied by extraction of retinene. (c) There is a quantitative correspondence of counts released and retinene extracted in the different extracts. The specific activity is relatively constant. (d) The acetone-extractable counts in bleached extracted pigments probably cannot be explained by phospholipid extraction.

The results also indicate that this conversion can take place in the eye, in vitro. Apparently the eye can convert vitamin A1 to retinene. Whether this conversion takes place between vitamin A2 and vitamin A1 or between the corresponding retinenes is not elucidated by the data. Whether the eye is the major site of conversion of vitamin A1 is also not clear. It is difficult to determine whether there are important extracollateral sites of conversion of vitamin A1 because isotopic tracer methods are not feasible until satisfactory physical means of separating small amounts of vitamin A1 and A2 become available.

At any rate, in the present experiments, the liver is unable to convert substantial amounts of vitamin A1 to vitamin A2 under conditions of organ culture. This may be due to the insufficiency of culture methods to the tissue. In the living animal, vitamin A1 is stored by the liver and quickly disappears (cf. (13)). The organ culture results suggest a utilization, catabolism, or excretion of vitamin A1 rather than conversion to vitamin A2.

Vitamin A2 is also an effective precursor of retinene2. The experiment presented here indicates it is approximately 3 times more efficient in labeling retinene2 in the eye in vitro than is vitamin A1. The more effective utilization of vitamin A2 is also apparent after intraperitoneal injection of vitamin A2 into the living animal; it is probably fair to state the general conclusion that vitamin A2 does lead to a quantitatively more efficient labeling of the retinene molecule.

This study raises many more questions than it solves and points up the need of further intensive study on comparative biochemistry of vitamin A metabolism. Are there different provitamins for the two vitamins A? Are there two metabolic systems, extracellular and ocular, for metabolizing vitamins A, and what is the relative quantitative importance of the two systems? What is the evolutionary and physiological importance of the shift from a vitamin A2 system in fresh-water fish to the vitamin A1 system of marine fish and land vertebrates? What is the mechanism of this shift of systems in some amphibian tadpoles? Experiments 3 and 4 of Table III bear on this latter point. The introduction of relatively large quantities of thyroid hormone into the eye reduces the conversion of vitamin A1 to retinene2. This would suggest that the bullfrog tadpole can convert vitamin A1 to retinene2 in the eye, leading to the formation of porphyropsin. The thyroid hormone would reduce or eliminate this ability and favor the utilization of retinene2 to form rhodopsin. This hypothesis is under active investigation, and it is already clear that the bullfrog tadpole eye can convert vitamin A1 to retinene2.

SUMMARY

The formation of the retinene2 chromophore of fish porphyropsin has been investigated by isolation of the chromophore after injection of tritiated vitamin A1. Vitamin A1 is an effe
tive precursor of retinene₂. This conversion takes place in the isolated eye in organ culture. The liver is able to store vitamin A₁ but cannot convert substantial amounts of vitamin A₁ to vitamin A₂ in organ culture. Vitamin A₂ is a more effective precursor of retinene₂ than is vitamin A₁. Triiodothyronine (1 x 10⁻⁴ M) reduces the conversion of vitamin A₁ to retinene₂.

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