The Enzymatic Reduction of Retinal to Retinol in Rat Intestine*

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SUMMARY

The reduction of retinal to retinol was examined with enzyme preparations from homogenates of rat intestinal mucosa. Retinal reduction was catalyzed by a soluble mucosal enzyme which was purified 13-fold by ammonium sulfate precipitation, column chromatography, and heating (55° for 6 min). The enzyme was relatively heat-stable and had a molecular weight approximately in the range of 60,000 to 80,000. The partly purified reductase was unable to oxidize ethanol in the presence of NAD+. Retinal reduction required NADH or NADPH as cofactor. Both reduced nucleotides were effective; at low nucleotide concentration NADH was more effective, whereas at high concentrations the reaction rate was slightly greater with NADPH. The reaction was stimulated by the addition of glutathione and was inhibited by -SH inhibitors. No other cofactors were required. There was a sharp pH optimum near 6.3. Retinal reduction displayed typical Michaelis kinetics, with a $V_{\text{max}}$ of 2 x low6 moles of retinal formed per hour per mg of protein and with an apparent $K_m$ of 2 x low4 M. The enzyme appears to be a relatively nonspecific aldehyde reductase. Short and medium chain aliphatic aldehydes, of length C-2 to C-14, were actively reduced, with greatest activity being seen with aldehydes of length C-4 to C-8. Unsaturated C-18 fatty aldehydes were reduced at a lesser rate, but saturated aldehydes of length C-16 or greater were not reduced. The enzyme was stereospecific for 4R-NADH-4-3H1, and did not incorporate tritium from 4S-NADH-4-3H1 into the product retinol.

During the intestinal absorption of dietary β-carotene, the carotene is largely converted to vitamin A, and the newly synthesized vitamin A is then transported in the lymph mainly in the form of retinyl ester (1-4). The conversion of β-carotene into vitamin A involves, first, the central cleavage of β-carotene into 2 molecules of retinal (5, 7). This reaction is catalyzed by a soluble mucosal enzyme, and has recently been characterized in some detail (7, 8). The newly formed retinal is next mainly reduced to retinol, which in turn is esterified with fatty acids and transported via the lymph, largely in association with lymph chylomicrons (3, 4).

We have recently reported the results of studies designed to examine, in the rat, the anatomical and chemical pathways involved in the intestinal absorption of retinal (9). It was found that under normal conditions the major pathway of retinal absorption involved its reduction to retinol, which was then esterified and transported via the lymph in a manner similar to that of dietary retinol. A small proportion of retinal was found to be oxidized, and then transported via the portal vein and excreted in the bile in a manner similar to that of dietary retinoic acid.

Since the reduction of retinal to retinol is an important reaction in the pathway of vitamin A biosynthesis in intestinal mucosa, experiments were conducted in order to characterize the enzyme and the reaction involved in this reduction. The results of these studies are described in this paper.

EXPERIMENTAL PROCEDURE

Enzyme Preparation—The soluble fraction (designated Fraction S-104) of rat intestinal mucosa was prepared as previously described (7). Mucosal scrapings were homogenized in the same phosphate buffer-nicotinamide-MgCl2 solution described before (7), but at pH 7.4 instead of 7.7. The enzyme was purified further by precipitation with ammonium sulfate between 23 and 50% saturation. The precipitate was dissolved in a small volume of the buffer solution used for homogenization, and the ammonium sulfate (and other low molecular weight components) was removed by gel filtration on a column of Sephadex G-25 (Pharmacia). The protein was eluted from this column with 0.01 M potassium phosphate buffer, pH 7.4, and the enzyme was purified still further by fractionation on a column of DEAE-
Sephadex. The ratio of protein load (milligrams) to bed volume (milliliters) of DEAE-Sephadex A-50 (in 0.01 M potassium phosphate, pH 7.4) was 125:1. The enzymatic activity was recovered with the single protein peak eluted with 0.01 M potassium phosphate, pH 7.4. This enzymatically active solution was heated at 55° for 6 min and then centrifuged at 2000 rpm for 10 min at 4°. This resulted in the removal of some nonenzymatic protein with little or no loss in enzymatic activity. In some instances, additional purification (to a small extent) was achieved by gel filtration on a column of Sephadex G-200 (10).

**Assay Procedure**—In most of these studies a spectrophotometric assay was used which depended on the fact that retinal, when dispersed in an aqueous solution, has a fairly broad absorption band with a peak at approximately 405 μm. Enzymatic activity was asayed by observing the decrease in absorbance at this wave length, thus measuring the rate and extent of disappearance of substrate from the system. A standard curve, prepared by plotting the concentration of retinal (dispersed in buffer) against the absorbance at 405 μm, was linear in the range of 10 to 50 μg of retinal per 2.5 ml. The procedure for the assay was as follows. To a 25-ml Erlenmeyer flask of amber glass was added 0.5 ml of 1 M potassium phosphate buffer, pH 6.1, 0.25 ml of 0.1 M GSH, enzyme solution, and water to a final volume of 5.0 ml. Freshly chromatographed (3) retinal (90 μg in 45 μl of acetone) was added via a Hamilton syringe, and the flask was then incubated at 37° for 2 min. The contents of the flask were divided equally between two cuvettes which were placed in a Beckman DB Spectrophotometer and maintained there at 37°. NADH (0.1 ml of a 10 mg per ml solution) was added to one cuvette to initiate the reaction, and the change in absorbance at 405 μm was recorded continuously. The activity was assayed as the initial rate of the reaction, in terms of micromoles of retinal reduced per min per mg of protein.

The spectrophotometric assay was validated by comparing the percentage conversion of retinal to retinol, measured spectrophotometrically as the percentage disappearance of retinal, with the percentage conversion of retinal to retinol measured directly with retinal-14C as substrate and isolating the product, retinol-14C, at the end of the incubation. To this end, retinal-14C was incubated with enzyme and NADH in a cuvette, and the reaction was monitored continuously at 405 μm. After the desired percentage of retinal had disappeared, the contents of the cuvette were extracted and the radioactive products were isolated by chromatography on alumina (3).

**Extraction, Chromatography, and Radioassay**—When the conversion of retinal to retinol was studied with the use of isotopes, the incubation mixtures were extracted with 20 volumes of CHCl3-CH3OH, 2:1 (v/v). A mixture of nonradioactive carriers, including 8 μg each of retinal, retinol, and retinoic acid and 20 μg of a mixture of retinyl esters, was added to each extraction mixture, followed by the addition of 5 volumes of 0.01 N H2SO4. The lower chloroform phase was collected and evaporated and the total lipid extract so obtained was chromatographed on a column of alumina (Woelm, activity grade III) as described previously (3). Radioassay of the separated fractions was carried out as reported previously (3, 7).

**Substrate Specificity**—The partially purified enzyme preparation was tested for its ability to reduce aldehydes other than retinal. Such activity was quantitatively assessed by measuring the aldehyde-dependent oxidation of NADH, as determined by monitoring the reaction at 340 μm in a cuvette. Experiments were carried out with the same incubation mixture described above (see "Assay Procedure"). NADH (0.4 μmole) was added to each cuvette, and the aldehyde was added in solution in acetone to one cuvette.

**Stereospecificity of Enzyme**—In order to examine the stereospecificity of the enzyme with respect to the pyridine nucleotide, the reaction was studied in the presence of two stereoisomeric forms of NADH, containing tritium instead of protium either at the 4A or the 4B position of NADH. It was then possible to measure the extent of tritium transfer from NADH to substrate, and therefore to determine the stereospecificity, with respect to NADH, of the enzyme.

Experiments were carried out in cuvettes in order to monitor spectrophotometrically (at 405 μm) the reduction of retinal. Retinal-15C (diluted with unlabeled retinal to a specific radioactivity of 0.009 μC per μmole) was used as substrate and the reaction was initiated by the addition of one of the H-labeled NADH preparations. The NADH-4-H preparations were diluted with unlabeled NADH before use to the following specific radioactivities: [A-4H]-NADH-4-H, 0.061 μC per μmole; [B-4H]-NADH-4-H, 0.090 μC per μmole. When the reaction rate had slowed considerably the contents of the cuvette were extracted and the product retinol was then isolated as described above. The retinol was simultaneously assayed for its content of 2H and 14C by radioassay in a Packard liquid scintillation counter.

**Labelled Retinal**—Retinol-15C (8.45 μC per μmole) was a generous gift from Hoffmann-La Roche, Basel, Switzerland. Retinol-15C was prepared by oxidation of retinol-4C with MnO2 (11) and the retinal was purified by chromatography on an alumina column (3).

**Labelled Nucleotides**—NAD+4-H (specific radioactivity, 250 μC per μmole) and ethanol-1-3H (specific radioactivity, 100 μC per μmole) were purchased from New England Nuclear.

[A-4H]-NADH-4-H was prepared by reduction of unlabeled NAD+ with ethanol-1-H, with an enzyme (alcohol dehydrogenase) stereospecific for the 4A-hydrogen of NADH. Ethanol-1-3H, 50 μmoles (5 mC), was added to a 25-ml Erlenmeyer flask containing 5 μmoles of NAD+ in 3 ml of 0.01 M Tris-HCl buffer, pH 8.6. The reaction was started by the addition of 50 μg of crystalline yeast alcohol dehydrogenase (Sigma). The reduction of the NAD+ at 37° was observed by measuring the absorbance at 340 μm of diluted portions of the incubation mixture. At frequent intervals, after the reaction had slowed markedly, additional alcohol dehydrogenase and unlabeled ethanol were added. When about 50% of the NAD+ had been reduced, the reaction was stopped by heating at 80° for 3 min. The contents were quantitatively transferred to a round bottom flask (A).

[B-4H]-NADH-4-H was prepared in a cuvette by the addition of 50 μl of unlabeled ethanol to an incubation mixture containing 0.20 μmole of NAD+4-H (50 μC) and 50 μg of yeast alcohol dehydrogenase in 2.7 ml of 0.01 M Tris-HCl buffer, pH 8.6. After incubation at 37°, the reaction quickly approached equilibrium as determined by the absorbance at 340 μm, and additional alcohol dehydrogenase (50 μg) and ethanol (50 μl) were added to the cuvette. When approximately 70% of the NAD+ had been reduced, the contents of the cuvette were transferred to a round bottomed flask (B) and...
TABLE I
Partial purification of retinal reductase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Specific enzyme activity ( \times )</th>
<th>Retinal recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction S-104</td>
<td>1162</td>
<td>1.07 ( \times ) 1.0</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, 23 to 50%</td>
<td>604</td>
<td>2.05 ( \times ) 1.9</td>
<td>99</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>149</td>
<td>7.30 ( \times ) 6.9</td>
<td>88</td>
</tr>
<tr>
<td>Sepharose G-200</td>
<td>84</td>
<td>0.90 ( \times ) 8.5</td>
<td>61</td>
</tr>
<tr>
<td>Heating</td>
<td>47</td>
<td>14.00 ( \times ) 13.1</td>
<td>53</td>
</tr>
</tbody>
</table>

* This preparation was derived from 21 rats.
* Millimicromoles of retinal reduced per min per mg of protein.
* Cf. Fraction S-104.
* 55° for 6 min.

TABLE II
Validation of spectrophotometric assay of retinal reduction to retinol

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Retinal reduced</th>
<th>Retinol-¹⁴C formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(spectrophotometric assay)</td>
<td>%</td>
</tr>
<tr>
<td>3</td>
<td>13.6</td>
<td>13.2</td>
</tr>
<tr>
<td>6</td>
<td>27.7</td>
<td>24.9</td>
</tr>
<tr>
<td>0.5</td>
<td>44.6</td>
<td>35.8</td>
</tr>
<tr>
<td>16.0</td>
<td>49.0</td>
<td>46.3</td>
</tr>
</tbody>
</table>

the cuvette was rinsed with 0.5 ml of a solution of unlabeled NADH (5.1 \( \mu \) moles added) and then with 0.5 ml of H₂O. The flask was then heated as described above.

Tritium label not present in nucleotide molecules was removed by freeze-drying the two preparations. Each residue was dissolved in 2 ml of H₂O and again lyophilized. This procedure was repeated for Flask A to ensure complete removal of unreacted tritiated ethanol. The \( ^{3} \)H-labeled reduced pyridine nucleotides were then purified by chromatography on small (1 x 10 cm) columns of DEAE-cellulose, as described by Pastore and Friedkin (12). The purified nucleotides (after lyophilization) were isolated by preparative chromatography on carboxymethyl Sephardex or DEAE-cellulose.

Enzyme Purification—Preliminary experiments, with \(^{14}C\)-labeled retinal as substrate, showed that the whole mucosal homogenate actively reduced retinal-\(^{14}C\) to retinol-\(^{14}C\). All of the enzymatic activity was found in the soluble supernatant fraction (Fraction S-104) of the mucosal homogenate.

The partial purification of retinal reductase from Fraction S-104 is summarized in Table I. Almost all of the reductase activity of Fraction S-104 was recovered in the protein fraction precipitated with ammonium sulfate between 23 and 50%. The protein fractions precipitated with ammonium sulfate between 0 and 23% and between 50 and 90% were inactive. Significant further purification was obtained by fractionation of the active (NH₄)₂SO₄ precipitate on DEAE-Sepharose. A critical ratio of protein load to bed volume of DEAE-Sepharose was required in order to avoid considerable loss of activity. Thus, with a load of 12.5 mg of protein per ml of bed volume of DEAE-Sepharose, 3.6 fold purification was obtained with very little loss of activity. If the column bed volume was increased so as to lower this ratio, up to 75 to 80% of the activity was lost.

Subsequent chromatography on Sepharose G-200 resulted in only a slight additional purification, as shown in Table I. Gel filtration of the enzyme on a Sepharose G-200 column standardized by chromatography on it of proteins of known molecular weight (13) showed that the enzyme had a molecular weight in the range of approximately 60,000 to 80,000. By heating the enzyme-containing fraction at 55° for 5 min, another 1.5-fold purification was obtained, with minimal loss of activity. The final purification after these steps was approximately 13-fold as compared to the S-104 preparation. No further significant purification, without considerable loss of activity, could be obtained by fractionation on carboxymethyl Sephadex or DEAE-cellulose.

In most of the experiments reported below, the enzyme was purified as described in Table I, except for the omission of gel filtration on Sephadex G-200. The procedures generally resulted in a purification of about 10- to 12-fold. Because the enzyme was quite labile to further attempts at purification, the properties of the enzyme and of the reaction were studied with enzyme purified to this extent.

Cofactor Requirements—There was no change in the absorbance, at 405 mp, of retinal dispersed in the incubation system described, unless NADH or NADPH was added to the system. There was thus an absolute requirement for a reduced pyridine nucleotide in order for this reaction to take place. No other cofactors were required for enzymatic activity, although the addition of GSH or cysteine stimulated activity (see below).

Validation of Spectrophotometric Assay—Table II shows that the percentage of retinol \(^{14}C\) formed from retinal \(^{14}C\) correlated well with the amount of retinal reduced as assayed spectrophotometrically, with 0.6 mm thick layers of Silica Gel G, with \( n \) -hexane-diethyl ether-acetic acid, (70:30:1, v/v/v), as solvent. Bile salts, Tween 80, thios, and inhibitors were obtained as described previously (7).

Effect of pH—As shown in Fig. 1, the reductase reaction
had a narrow optimal pH range. The maximal rate was observed at about pH 6.3, and the rate rapidly decreased as the pH was raised above 6.3 or lowered below 6.0.

**Rate of Reaction**—The reaction velocity was constant for 4 to 5 min and then decreased progressively until about 10 to 15 min, after which time very little further reduction occurred.

**Effect of Enzyme Concentration**—The initial reaction rate was directly proportional to the enzyme concentration in the range from 0 to 0.6 mg of protein (per 2.5 ml). The rate could be increased further, but not linearly, by increasing the enzyme concentration beyond 0.6 mg in 2.5 ml. In most of the experiments reported here an enzyme concentration of 0.4 to 0.6 mg in 2.5 ml was used in order to work in the linear range of enzyme concentration.

**Effect of Substrate Concentration**—This is shown in Fig. 2. The reaction rate increased rapidly in the range from 0 to 100 μmoles of added retinal (added to 2.5 ml of incubation mixture); addition of larger amounts of substrate produced only small further increases in rate. When 1/v was plotted against 1/s (Fig. 2, inset) a straight line resulted, from which the following figures were calculated: $V_{\text{max}} = 2.0 \times 10^{-5}$ mole of retinol formed per hour per mg of protein; $K_m = 2.0 \times 10^{-5}$ M. It should be noted that $K_m$ represents only an apparent value for the Michaelis constant, since retinal is an insoluble substrate and was dispersed in the incubation medium from an acetone solution.

**Pyridine Nucleotide Specificity**—Before studying the pyridine nucleotide specificity of the enzyme, the stability of NADH and NADPH in the incubation mixture lacking retinal was examined. This was done by monitoring the absorbance at 340 mλ after the addition of one or the other reduced pyridine nucleotide to one of the cuvettes. It was found that the concentration of added NADH decreased only very slightly during the first 3 min, whereas NADPH was oxidized fairly rapidly in the absence of added aldehyde. The identity of the endogenous substrate responsible for NADPH oxidation is not known, although oxidized GSSG (probably added in small amounts along with the GSH) may have been responsible. In order to stabilize the level of NADPH, a NADPH-regenerating system was therefore added to incubations with NADPH. This was achieved by means of the additions indicated in the legend to Fig. 3. From Fig. 3, it can be seen that the enzyme had a much higher affinity for NADH as a hydrogen donor, because only small additions of this cofactor were required for maximal reaction rates. In the presence of similar amounts of NADPH, the reaction rate was much slower although, at high concentrations of the pyridine nucleotides, the velocity reached a slightly higher value in the presence of NADPH than when NADH was present. The nucleotide concentrations which provided approximately half of the maximal velocity were: NADH, $4 \times 10^{-5}$ M, and NADPH, $4 \times 10^{-5}$ M.

**Effects of Addition of Detergents**—Addition of sodium glycocholate or of Tween 80 had an inhibitory effect on the reduction of retinal to retinol (see Table III).

**Effects of Thiols and Thiol Inhibitors**—As shown in Table IV, both GSH and cysteine stimulated the reaction to a small extent; in contrast, mercaptoethanol was slightly inhibitory. The thiol inhibitors $N$-ethylmaleimide and $p$-hydroxymercuribenzoate were strongly inhibitory.

**Substrate Specificity**—The specificity of the enzyme preparation was investigated by measuring the rate of oxidation of NADH in the presence of several aldehydes other than retinal. The aldehydes which were tested ranged from C-2 to C-22 in carbon chain length and included saturated and unsaturated
Fig. 3. Pyridine nucleotide specificity for retinal reduction. The figure shows the initial rate of the reaction plotted against the number of millimicromoles of NADH or NADPH added per 2.5 ml of incubation mixture. In the experiments with NADPH, each incubation mixture contained 2 μmoles of glucose-6-P and 1 mg of glucose-6-P dehydrogenase (Sigma) as well as the usual additions.

**Table III**

Effects of detergents on retinal reduction

<table>
<thead>
<tr>
<th>Detergent added</th>
<th>Amount</th>
<th>Initial rate of retinal reduction (μmoles/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>17.8</td>
</tr>
<tr>
<td>Sodium glycocholate</td>
<td>11 μmoles</td>
<td>13.7</td>
</tr>
<tr>
<td>Sodium glycocholate</td>
<td>30 μmoles</td>
<td>7.5</td>
</tr>
<tr>
<td>Tween 80</td>
<td>2.5 mg</td>
<td>10.3</td>
</tr>
<tr>
<td>Tween 80</td>
<td>7.5 mg</td>
<td>5.3</td>
</tr>
</tbody>
</table>

compounds purchased or prepared as described under “Experimental Procedure.” Because the assay measured the aldehyde-dependent oxidation of NADH, it was assumed that the rate of NADH oxidation was the same as the rate of aldehyde reduction.

The results of these experiments are summarized in Figs. 4 and 5. Fig. 4 shows the rate of oxidation of NADH in the presence of straight chain, saturated aliphatic aldehydes. Aldehydes of 2 to 14 carbons in length were actively reduced, whereas longer straight chain saturated aldehydes (including cetyl, stearyl, and benzenyl aldehydes) were not reduced in this system. Maximal activity was seen with aldehydes of intermediate chain length (C-4 to C-8), whereas both shorter and longer chain aldehydes were reduced at a lesser rate. The rate of retinal reduction with the same enzyme preparation used for the experiment shown in Fig. 4 was similar to the rate of reduction of tetradecyl aldehyde.

Fig. 5 shows the rate of NADH oxidation in the presence of unsaturated, branched chain, and aromatic aldehydes. All of the compounds shown were actively reduced. In contrast to their saturated counterparts, the unsaturated long chain aldehydes (oleyl, linoleyl, and linolenyl) were actively reduced, the rate of reduction increasing with increasing unsaturation.

The effect of the addition of detergents on the reduction of long chain saturated aldehydes was studied, in order to determine whether better dispersion of these aldehydes would increase their availability to the enzyme for reduction. However, no such stimulation was observed, since addition of either

**Table IV**

Effects of -SH compounds and inhibitors

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Amount added</th>
<th>Initial rate of retinal reduction (μmoles/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td></td>
<td>12.3</td>
</tr>
<tr>
<td>GSH</td>
<td>5</td>
<td>15.0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>25</td>
<td>18.3</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>10</td>
<td>18.0</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>5</td>
<td>17.3</td>
</tr>
<tr>
<td>N-Hydroxymercuribenzoate</td>
<td>25</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.7</td>
</tr>
</tbody>
</table>

* GSH was omitted from the usual incubation mixture during this experiment.
12 μmoles of sodium glycocholate or of 5 mg of Tween 80 (per 2.5 ml) was without effect. Since the enzyme preparation being investigated catalyzed the reduction of aldehydes to alcohols, experiments were conducted to determine whether the enzyme would also be able to oxidize an alcohol to an aldehyde. Under all conditions explored, no oxidation of ethanol, in the presence of NAD+, was observed, even under those conditions (e.g. pH 8 to 9) in which liver and yeast alcohol dehydrogenases were highly active.

**Stereospecificity of Retinal Reductase**—As described under “Experimental Procedure,” triplicate incubations were carried out with each tritium-labeled diastereoisomer of NADH, and the product of the reaction (retinol) was isolated and assayed for 3H and 14C. In each experiment, the yield of retinol, as measured by the 14C content of the retinol fraction obtained by chromatography on alumina columns, was between 28 and 34%. These values closely agreed with the amount of retinal reduced as calculated from the change in absorbance at 405 nm during the incubation. Table V shows the content of 3H and 14C in the product retinol after each incubation. Very similar results were obtained within each set of triplicate incubations. An average of 0.60 μg atom of 3H was incorporated from [A-3H]-NADH-4-3H1 into each micromole of retinol formed. In contrast, an average of only 0.031 μg atom of 3H was incorporated per μmole of retinol from [B-3H]-NADH-4-3H1. These results clearly indicate that the reductase responsible for retinal reduction is stereospecific for the hydrogen atom at the 4A position of NADH.

**Discussion**

The experiments reported here have defined many of the properties of the enzyme in rat intestinal mucosa which is responsible for the reduction of retinal to retinol. The enzyme is a soluble protein with an approximate molecular weight in the range of 60,000 to 80,000. The enzyme is relatively heat-stable (at 55° for 6 min) and requires a reduced pyridine nucleotide, either NADH or NADPH, as hydrogen donor for aldehyde reduction. Retinal reduction, as studied in vitro, has a rather sharp optimum pH near 6.3, and displays typical Michaelis kinetics, with a Vmax of 2 × 10⁻⁵ moles of retinol formed per hour per mg of protein (of partially purified enzyme) and with an apparent Kₘ of 2 × 10⁻⁸ M. Since retinal is a water-insoluble substrate, which was presented to the enzyme in the form of a fine dispersion, the value of the apparent Kₘ should be interpreted with caution.

This study of retinal reduction was undertaken as part of an investigation of the pathways involved in the biosynthesis of vitamin A from its natural precursor, β-carotene. During its intestinal absorption, β-carotene is first cleaved into 2 molecules of retinal. The retinal is then mainly reduced to retinol, which in turn is esterified and transported from the gut via the intestinal lymphatics (3, 4).

Previous communications from this laboratory have characterized in some detail the reaction involved in the cleavage of β-carotene into 2 molecules of retinal (7, 8). The reaction requires the presence of molecular oxygen; its mechanism appears to be that of a dioxygenase reaction (6, 8). Although the mucosal enzymes responsible for β-carotene cleavage and for retinal reduction are both soluble proteins, the properties of the two enzymes are quite different. As studied in vitro, the cleavage reaction has an optimum pH near 7.7, and requires the addition of an appropriate detergent or detergent-lipid combination for enzyme activity. In contrast, retinal reduction has a lower pH optimum (6.3) and is inhibited by the addition of detergents. In addition, retinal reduction requires the addition of a reduced pyridine nucleotide, whereas the cleavage reaction requires molecular oxygen. Both reactions are stimulated by the addition of GSH and inhibited by thiol inhibitors.

Previous studies of the reduction of retinal to retinol have focused mainly on the retina and on the liver. Almost two decades ago Wald reported the reduction of retinal to retinol with homogenates of frog and cattle retinas (14, 15), and observed a requirement for reduced NADH for this reaction. More recently, Futterman examined retinal reduction with calf retina preparations, and found that reductase activity was due to a particulate enzyme localized in the visual cell outer segments (16). Both NADH and NADPH were found to be effective hydrogen donors for retinal reduction with washed visual cell outer segment preparations. Futterman also reported that NADPH was more effective than NADH in this regard, and

![Fig. 5. The rate of reduction of unsaturated, branched chain and aromatic aldehydes.](image)

<table>
<thead>
<tr>
<th>Table V</th>
<th>Stereospecificity of retinal reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled nucleotide added</td>
<td>Yield of retinol from retinal</td>
</tr>
<tr>
<td>[A-3H]-NADH-4-3H1</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>28</td>
</tr>
<tr>
<td>[B-3H]-NADH-4-3H1</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
</tbody>
</table>
that NADPH appeared to be the principal physiological reducing agent for retinal in the visual cycle.

The reduction of retinal to retinol in rat liver was studied in detail by Zachman and Olson (17). The enzyme catalyzing this reduction was found to be a soluble protein, and was purified 2- to 3-fold. With NADH as cofactor, the pH optimum of the reaction was found to be 5.9, and the $K_m$ for retinal was 2 to $3 \times 10^{-4} \text{M}$. The enzyme was inhibited by the addition of detergent (TWEEN 80 or sodium deoxycholate). On the basis of stability and fractionation studies, and of a comparison of properties, it was concluded that rat liver retinal reductase and alcohol dehydrogenase are the same enzyme. This conclusion was consistent with the previous observations by Bliss (18) that crystalline horse liver alcohol dehydrogenase was able to catalyze the reversible dehydrogenation of retinol to retinal in the presence of NADH. The equilibrium constant for the oxidation of retinol to retinal with this enzyme was reported to be $3.3 \times 10^{-9} \text{M}$ (18).

Considerable information is available about the properties of horse liver alcohol dehydrogenase (19). The enzyme has a very broad substrate specificity, and can catalyze the oxidation of a variety of aliphatic and aromatic alcohols. The molecular weight of the enzyme is about 84,000. NADH is the effective cofactor, although the enzyme can also utilize NADPH, albeit slowly, for ethanol oxidation (19, 20). In contrast to the liver enzyme, yeast alcohol dehydrogenase is unable to oxidize retinol to retinal (18).

Very little information has previously been available about the reduction of retinal to retinol in the intestine. Previous studies in vivo (21) and in vitro (22) showed the occurrence of this reaction in intestinal tissue and homogenates, but provided no detailed information about the reaction or the enzyme involved.

In the present experiments, the intestinal enzyme responsible for retinol reduction was found in the soluble supernatant fraction of the mucosal homogenate, from which it was purified about 13-fold. The partially purified enzyme preparation appeared to be relatively nonspecific with regard to its aldehydic substrate. Thus, short and medium chain aliphatic aldehydes, of length C-2 to C-14, were actively reduced, with the greatest activity being seen with aldehydes of intermediate (C-4 to C-8) chain length. Branching, chain, aromatic, and unsaturated aldehydes were also actively reduced. Unsaturated C-18 fatty aldehydes were reduced at a lesser rate, but saturated aldehydes of length C-16 or greater were not reduced. It is, however, possible that differences in the physical properties of the dispersions of the long chain aldehydes, rather than degree of unsaturation, were responsible for the differences observed with the various fatty aldehydes. In view of the broad range of substrates reduced by the mucosal preparation, the enzyme responsible for retinal reduction appears to be a relatively non-specific aldehyde reductase, rather than a specific retinal reductase. The apparent nonspecificity of the enzyme could, of course, be due to the presence of several aldehyde reductases, with different specificities, in the enzyme preparation used in these experiments. It should, however, be noted that other, highly purified, hydrogen-transferring enzymes, including horse liver alcohol dehydrogenase (19) and the recently characterized aromatic aldehyde-ketone reductase from rabbit kidney (23), have been observed to possess a broad range of substrate specificity. The mucosal enzyme was not able to catalyze the dehydrogenation of ethanol, even under conditions favorable for this reaction with yeast and liver alcohol dehydrogenase. It appears, therefore, that the mucosal enzyme is primarily a reductase, and not an alcohol dehydrogenase working in reverse. In this regard the mucosal enzyme resembles the aromatic aldehyde-ketone reductase from rabbit kidney, since the latter enzyme could not be shown to catalyze the reverse reaction, the dehydrogenation of carbinols, under favorable experimental conditions (23).

The mucosal enzyme is relatively nonspecific not only with regard to its aldehyde substrate, but also with regard to the pyridine nucleotides. Both NADH and NADPH were effectively used in the reaction, although at very low nucleotide concentrations (below about $2 \times 10^{-4} \text{M}$) NADH was the much more effective cofactor. The mucosal enzyme thus resembles the enzyme from calf retina (16) in its ability to utilize either NADH or NADPH for retinal reduction. The ability to use either NADH or NADPH for reduction of an aldehyde has been reported for mevaldate reductase from pig liver and from rat liver (24). In contrast, aromatic aldehyde-ketone reductase from rabbit kidney is specific for NADPH (23), and alcohol dehydrogenases from yeast and liver are absolutely or relatively specific for NADH (19).

An examination of the stereospecificity of the mucosal reductase, with regard to the hydrogen transferred to retinol from the reduced pyridine nucleotide, clearly indicated that the enzyme was stereospecific for the hydrogen atom at the 4A position of NADH. The mucosal enzyme thus resembles alcohol dehydrogenase in being specific for the hydrogen at the 4A position of NADH (25). In contrast, aromatic aldehyde-ketone reductase was shown to be stereospecific for the 4B hydrogen of NADPH (23). It is highly likely that the mucosal reductase would show the same stereospecificity with NADPH as with NADH, since identical stereospecificities for the 4A hydrogen of both NADH and NADPH have been observed with enzymes which use both pyridine nucleotides (e.g. mevaldate reductase (24)). In the study reported here, an average of 0.60 µg atom of $^3$H was incorporated from $[^A-^3H]-\text{NADH}-4\text{H}$ into each micromole of retinol formed. This value probably differs from the theoretical value of one because of an isotope effect, in which the labeled nucleotide molecules were used less rapidly than the unlabeled ones.

The absolute configuration at C-4 of the dihydronicotinamide ring in 'A'-tritio-NADH has been shown to be $R$ by Cornforth et al. (26); in the 'B'-tritio-NADH the configuration is $S$. The mucosal enzyme responsible for retinal reduction is therefore an $R$-specific reductase.

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