Preparation and Properties of Retinal-oxidizing Enzyme from Rat Intestinal Mucosa*

(Received for publication, August 11, 1969)

DAVID J. MOFFA; F. J. LOTSPEICH, AND R. F. KRAUSE
From the West Virginia University School of Medicine Department of Biochemistry, Morgantown, West Virginia 26506

SUMMARY

An enzyme which converts retinal to retinoic acid was purified from rat intestinal mucosa approximately 160-fold via a combination of acetone precipitation, ammonium sulfate precipitation, and DEAE-cellulose ion exchange chromatography. The purified preparation appeared to be homogeneous in the ultracentrifuge, on ion exchange chromatography, and on polyacrylamide gel electrophoresis. The molecular weight of the enzyme was approximately 80,000 and its isoelectric point was in the neighborhood of 6.7. The enzyme preparation contained approximately 2 moles of iron per mole of enzyme. The absorption spectrum of the enzyme exhibited a maximum at 280 mμ and a much smaller band around 410 mμ.

Purified retinal-oxidizing enzyme from rat intestinal mucosa stoichiometrically and irreversibly converted retinal to retinoic acid. The latter was identified as the product of the reaction by ultraviolet absorption spectrum, thin layer chromatography, and gas chromatography. The product was approximately 96% pure as determined by its \( R_{f} \) value.

The enzymatic reaction exhibited Michaelis-Menten kinetics with a \( K_{m} \) of approximately \( 3.0 \times 10^{-4} \) M. The rate of the reaction was constant for 120 min, directly proportional to the enzyme concentration, and maximal when GSH, NAD, FAD, and Fe\(^{2+} \) were added. The pH optimum was approximately 7.7. The reaction proceeded well under both aerobic and anaerobic conditions. The enzyme acted upon both the all-trans and 13-cis forms of retinal.

Reduced nicotinamide adenine dinucleotide noncompetitively inhibited the reaction and was found to exhibit a control over the utilization of retinal in vitro. Thiols and metal ions stimulated the reaction, while thiol inhibitors and chelators inhibited the reaction. It is suggested that retinal-oxidizing enzyme is a metalloprotein which requires sulfhydryl groups for maximal enzymic activity.

In the presence of H\(_{2}\)O\(_{2}\), the oxidation of retinal by retinal-oxidizing enzyme appeared to resemble a dehydrogenase rather than an oxygenase or oxidase.

The discovery of Ares and van Duy (1), and others (2, 3), of the growth-promoting activity of retinoic acid in retinol-deficient rats has suggested the involvement of the acid in the pathway of retinol metabolism. Retinol, a product of the enzymatic oxidation of retinol, has been oxidized \( \textit{in vitro} \) to retinoic acid by liver aldehyde dehydrogenase, liver aldehyde oxidase, and milk xanthine oxidase (4). Recently, retinoic acid has been detected in the blood, liver, intestine, and other tissues of the rat after administration of retinal (5, 6). The formation of retinoic acid from retinol (7), retinal (5, 8), and \( \beta \)-carotene (8) has been shown in several laboratories. On the other hand, other investigators (9) have indicated that under normal conditions the major pathway of retinal absorption involves its reduction to retinol, while a small amount of retinal is oxidized to the acid. Thus, the role of retinoic acid as an obligatory metabolite of retinol has not yet been established.

An enzyme responsible for the oxidation of retinal to retinoic acid has been partially purified from rat liver via ammonium sulfate precipitation (10). Recently, Crain, Lotspeich, and Krause (8) have shown the enzymatic conversion of retinal to retinoic acid by an enzyme system from rat intestinal mucosa. This report presents a method for isolating the retinal-oxidizing enzyme from rat intestine and describes some of its properties.

EXPERIMENTAL PROCEDURE

Materials—All-trans retinal, 13-cis retinal, all-trans retinol, and \( \beta \)-carotene were purchased from Distillation Products Industries, Rochester, New York. Substrate dispersions consisted of 5.0 μmoles/0.1 ml of acetone containing 5% Triton X-100, purchased from Rohm and Haas, Philadelphia, Pennsylvania. The solutions were stored at 0–5°C for 2 to 3 weeks or less and were stable for this period of time. Xanthine, NAD\(^{+} \), NADH, GSH, and FAD were purchased from Sigma. These solutions were prepared immediately before use and chilled in ice. Diethylaminoethyl cellulose was purchased from Schleicher and Schuell Company, Keene, New Hampshire, and was prepared as described by Peterson and
Preparation of Reductase—A crude preparation from rat intestine, termed 45 to 70% (NH₄)₂SO₄ fraction, was used as a source of retinal reductase. This enzyme fraction was prepared by essentially the same procedure as described by Mahadevan, Murthy, and Ganguly (10) for a similar enzyme in liver. This fraction possessed an activity of 1.15 units/0.2 ml (0.9 mg of protein).

Preparation of Crude Enzyme Extract—The method of preparing the soluble fraction from rat intestinal mucosa was a modification of the procedure described by Crain et al. (8). Male albino rats of the Wistar strain, fasted overnight and killed by direct cardiac puncture. The proximal half of the small intestine was removed, flushed out with two 10-ml portions of 0.9% NaCl solution, and cut lengthwise. The mucosa was scraped off and homogenized in 0.1 mol sodium phosphate buffer, pH 7.7 (8 volumes per g of mucosa), in a Dounce homogenizer. The homogenate was centrifuged at 100,000 × g (40,000 rpm) for 30 min in a Spinco model L ultracentrifuge. The supernatant was filtered through cheesecloth, collected, and stored frozen until used. This fraction was termed the soluble fraction and constituted the crude enzyme preparation.

RO-Enzyme Assay—A modification of Futterman’s method (12) was used for the determination of RO-enzyme activity. A 4.0-ml reaction mixture, consisting of 0.05 ml of retinal dispersion (2.5 μmoles), 2.45 ml of 0.1 mol sodium phosphate buffer (pH 7.7), 0.05 ml of the enzyme preparation, and either 1.00 ml of a solution containing 2 μmoles each of NAD⁺, FAD, and FeCl₂ or 1.00 ml of distilled water was incubated at 37°C in a Dubnoff shaker for 2 hours under O₂. Aliquots of 0.5 ml were withdrawn at 0 and 2 hours and extracted five times with 2 ml of an ether-ethanol mixture (9:1, v/v). The extracts were then pooled, and the ether was removed under a stream of nitrogen. Retinal and retinoic acid were separated by chromatography on DEAE-cellulose in the following manner. A column (1 × 4 cm) was prepared from a slurry of DEAE-cellulose in ethanol, converted to the hydroxy form by treatment with 10 ml of ammoniacal ethanol (ethanol, 240 ml; concentrated ammonia, 10 ml), and washed with 10 ml of ethanol. The pooled extracts of retinal and retinoic acid were added to the column and eluted with ethanol. Retinal was recovered in the first 10 ml of the effluent and quantitatively determined by comparison of its absorbance at 380 μm, in a Beckman DU spectrophotometer, to a standard sample of retinol. Retinoic acid was recovered in the 0.68 mg of protein).

The assay procedure was standardized by extracting and chromatographing known amounts of commercial retinal and retinoic acid. Results indicated that the method was reproducible, and that approximately 97% of retinal and 93% of retinoic acid were recoverable. The procedure was validated by isolating and identifying the product and by comparing the amount of product formed to the amount of substrate utilized. (The data are presented under “Results.”)

β-Carotene Oxygenase Assay—Reaction mixtures consisted of 0.025 ml of β-carotene dispersion (1.25 μmoles), 3.08 ml of 0.1 mol sodium phosphate buffer (pH 7.7), and 0.30 ml of enzyme preparation. Determination of β-carotene oxygenase activity was similar to the spectrophotometric method of Bracken, Myklestad, and Njaa (13). Enzymatic activity was expressed as micromoles of retinal produced per 2 hours per ml of enzyme preparation.

Xanthine Oxidase Assay—Reaction mixtures consisted of 0.01 ml of 4.5 × 10⁻³ M xanthine solution, 2.70 ml of 0.1 mol sodium phosphate buffer (pH 7.7), and 0.30 ml of enzyme preparation. The phosphate buffer and enzyme were pipetted into two 1-cm quartz cells. To the first cell (blank) was added 0.01 ml of 0.05 M NaOH. The reaction was initiated by placing 0.01 ml of xanthine solution into the second cell. Absorbance readings were taken in a Beckman DU spectrophotometer at 290 μm at 1-min intervals. Activity was expressed as the increase in absorbance at 290 μm per min per ml of enzyme preparation. Commercial milk xanthine oxidase (xanthine; oxygen oxidoreductase EC 1.2.3.2), purchased from Nutritional Biochemicals, possessed an activity of 3.80 units per ml.

Reducase Assay—The enzymatic reduction of retinal to retinol was followed spectrophotometrically in a similar manner as described for the RO-enzyme assay, except that the amount of retinol in the isolated products was measured by comparing the increase in optical density at 325 μm to a standard sample of retinol.

Other Procedures—Protein concentration of enzyme samples was determined by the method of Lowry et al. (14), non-heme iron content according to Ramsay (15), and carbohydrate by the phenol-sulfuric acid method (16).

Enzyme samples were dialyzed, with agitation, in Cellophane tubings twice against 90 volumes of distilled water at 0–4°C for periods of 1.5 hours.

Polyacrylamide disc electrophoresis was performed on 7.5% gels according to Davis (17).

Electrophoresis was carried out at pH 9.5 for 60 min at 2 to 5 ma per tube. Tracking dye was 0.005% bromophenol blue. Proteins were stained with 0.5% Bufalo blue black. Dyeing was carried out for 45 min in 7% acetic acid at 12.5 ma per tube.

Analytical ultracentrifugation was carried out in a Spinco model E ultracentrifuge with a 12-mm sector cell and an A-ND head. Centrifugations were performed at 40,000 rpm and 20°C. Thin layer chromatography was performed on glass plates (20 × 20 cm) previously coated with Silica Gel G (E. Merck AG, Darmstadt, Germany). Standards of retinoic acid, retinal, retinol, and methyl retinoate, along with unknown reaction products, were spotted and developed via ascending chromatography in a solvent system consisting of chloroform-methanol-water (65:20:2). Lipid spots were detected with I₂ vapor.

Methylated retinoic acid was subjected to gas chromatography with a Research Specialties Company model 600 series gas chromatograph, equipped with an H₂ flame detector, Gas. Chrom P support, and a 20% diethylenglycolsuccinate column measuring 5 feet × 0.25 inch. The temperature of the column was 130°C, and the flow rate was 32 ml per min. Retinoic acid was...
methyland by diazomethane. The latter compound was prepared according to Fieser and Fieser (18).

Anaerobic studies on the enzymatic reaction were conducted by incubating the reaction mixture in a Thunberg vessel with oxygen removed under vacuum.

Oxygen content of reaction vessels was measured polarographically with the use of a vibrating Teflon-covered platinum electrode patterned after Kahn (19). The electrode response characteristics were evaluated with known concentrations of ferricyanide ion.

Isotope experiments in H$_2$O were carried out under aerobic conditions. Twenty micromoles of retinal dispersion (0.4 ml) were incubated with 10.00 ml of 0.1 M sodium phosphate buffer (pH 7.7), 4.00 ml of H$_2$O (2.0 atom %), and 2.00 ml of purified RO-enzyme. The oxidation, which was allowed to proceed for 3 hours, resulted in approximately 70% conversion to retinoic acid. The product was extracted into diethyl ether, evaporated to dryness under N$_2$, and oxidized to CO$_2$ with Hg(CN)$_2$ (20). The latter procedure minimizes oxygen exchanges which might occur during the oxidation. Carbon dioxide was collected in an anaerobic vessel and determined mass 46:44 ratio with a mass spectrometer.

The supernatant was discarded and the precipitate was immediately centrifuged at -3°C for 10 min at 7500 rpm (6800 x g). Stirring in a -4°C alcohol bath. Acetone, chilled to -5°C, was added slowly below the liquid surface of the soluble fraction to bring the suspension to an acetone concentration of 50%. The suspension remained at -4°C for an additional 10 min, and then was centrifuged at -4°C for 10 min at 7500 rpm (8500 × g). The supernatant was discarded and the precipitate was immediately dried over a stream of N$_2$ to remove traces of acetone. The sample was then dissolved in 0.1 M sodium phosphate buffer, pH 7.7. This preparation is termed the 50% acetone fraction.

Ammonium Sulfate Fractionation—Samples of the 50% acetone fraction (100 ml each) were treated with solid ammonium sulfate at 30, 40, 50, and 60% saturation levels according to a nomogram presented by Dixon and Webb (21). The 30, 40, 50, and 60% saturated fractions were dissolved in 25, 20, 15, and 20 ml of distilled water, respectively.

Dialysis—The 50% (NH$_4$)$_2$SO$_4$ fraction was dialyzed twice against 0.001 M phosphate buffer, pH 7.7, for a total of 3 hours in order to prepare the enzyme fraction for a DEAE-cellulose ion exchange separation. Longer periods of dialysis against buffer resulted in a considerable loss of enzymatic activity.

First DEAE-cellulose Chromatography—The dialyzed 50% ammonium sulfate fraction was chromatographed on DEAE-cellulose. The effluent was collected in 5-ml fractions with a Gilson fraction collector, scanned for protein via absorbance at 280 nm in a Beckman DU spectrophotometer, and assayed for RO-enzyme activity. The results of a typical separation of the 50% saturated fraction on DEAE-cellulose are shown in Fig. 1A. The conditions used for the column separation are described in the legend accompanying the figure. The RO-enzyme peak area (tubes 20 to 28) was labeled DEAE$_1$ fraction and stored in a frozen state. This enzyme fraction was quite unstable during extensive freezing and thawings; however, very little activity was lost when samples were frozen and thawed only once or twice. Thus, whenever the enzyme preparation was used for assaying purposes, the samples were frozen and thawed only twice.

Ammonium Sulfate Concentration and Dialysis—The DEAE$_1$ fraction was concentrated by the addition of ammonium sulfate (90 to 95% saturation) and centrifuged for 30 min at 7500 rpm (9000 × g), and the resulting precipitate was dissolved in a small volume of 0.001 M sodium phosphate buffer, pH 7.7. The sample was then prepared for a second DEAE-cellulose separation by dialyzing against 0.001 M phosphate buffer, pH 7.7.

Second DEAE-cellulose Chromatography—The dialyzed, concentrated DEAE$_1$ fraction was chromatographed on DEAE-cellulose under the same conditions as the first DEAE-cellulose column except that elution was carried out with a linear pH gradient (50 ml of 0.01 M sodium phosphate buffer, pH 7.7, and 50 ml of 0.01 M sodium phosphate buffer, pH 4.0). The results of a typical separation are shown in Fig. 1B. The fraction containing RO-enzyme activity (tubes 9 to 16) was termed DEAE$_{II}$.

**Purity of Enzyme**—The relative homogeneity of the DEAE$_{II}$ fraction was tested by ultracentrifugation, ion exchange chromatography, and polyacrylamide gel electrophoresis. Table I illustrates a summary of the specific activities, degree of purification, and yields of the various enzyme fractions. The final

![Figure 1. Chromatographies on DEAE-cellulose.](https://www.jbc.org/)

**RESULTS**

**Purification of Retinal-oxidizing Enzyme**

*Acetone Fractionation*—The soluble fraction was cooled with stirring in a -4°C alcohol bath. Acetone, chilled to -90°C, was added slowly below the liquid surface of the soluble fraction to bring the suspension to an acetone concentration of 50%. The suspension remained at -4°C for an additional 10 min, and then was centrifuged at -3°C for 10 min at 7500 rpm (8500 × g). The supernatant was discarded and the precipitate was immediately dried over a stream of N$_2$ to remove traces of acetone. The sample was then dissolved in 0.1 M sodium phosphate buffer, pH 7.7. This preparation is termed the 50% acetone fraction.

*Ammonium Sulfate Fractionation*—Samples of the 50% acetone fraction (100 ml each) were treated with solid ammonium sulfate at 30, 40, 50, and 60% saturation levels according to a nomogram presented by Dixon and Webb (21). The 30, 40, 50, and 60% saturated fractions were dissolved in 25, 20, 15, and 20 ml of distilled water, respectively.

*Dialysis*—The 50% (NH$_4$)$_2$SO$_4$ fraction was dialyzed twice against 0.001 M phosphate buffer, pH 7.7, for a total of 3 hours in order to prepare the enzyme fraction for a DEAE-cellulose ion exchange separation. Longer periods of dialysis against buffer resulted in a considerable loss of enzymatic activity.

**First DEAE-cellulose Chromatography**—The dialyzed 50% ammonium sulfate fraction was chromatographed on DEAE-cellulose. The effluent was collected in 5-ml fractions with a Gilson fraction collector, scanned for protein via absorbance at 280 nm in a Beckman DU spectrophotometer, and assayed for RO-enzyme activity. The results of a typical separation of the 50% saturated fraction on DEAE-cellulose are shown in Fig. 1A. The conditions used for the column separation are described in the legend accompanying the figure. The RO-enzyme peak area (tubes 20 to 28) was labeled DEAE$_1$ fraction and stored in a frozen state. This enzyme fraction was quite unstable during extensive freezing and thawings; however, very little activity was lost when samples were frozen and thawed only once or twice. Thus, whenever the enzyme preparation was used for assaying purposes, the samples were frozen and thawed only twice.

**Ammonium Sulfate Concentration and Dialysis**—The DEAE$_1$ fraction was concentrated by the addition of ammonium sulfate (90 to 95% saturation) and centrifuged for 30 min at 7500 rpm (9000 × g), and the resulting precipitate was dissolved in a small volume of 0.001 M sodium phosphate buffer, pH 7.7. The sample was then prepared for a second DEAE-cellulose separation by dialyzing against 0.001 M phosphate buffer, pH 7.7.

**Second DEAE-cellulose Chromatography**—The dialyzed, concentrated DEAE$_1$ fraction was chromatographed on DEAE-cellulose under the same conditions as the first DEAE-cellulose column except that elution was carried out with a linear pH gradient (50 ml of 0.01 M sodium phosphate buffer, pH 7.7, and 50 ml of 0.01 M sodium phosphate buffer, pH 4.0). The results of a typical separation are shown in Fig. 1B. The fraction containing RO-enzyme activity (tubes 9 to 16) was termed DEAE$_{II}$.
TABLE I

Purification of retinal-oxidizing enzyme

| Fraction   | Total protein | Total RO-enzyme activity | Specific activity | Fold purification | Yield% |
|------------|---------------|--------------------------|-------------------|-------------------|--------|
| Soluble    | 850.0         | 53.60                    | 0.06              | 1.00              | 100    |
| 50% acetone| 55.6          | 46.41                    | 1.00              | 2.07              | 89     |
| 50% (NH₄)₂SO₄| 7.5       | 11.42                    | 0.91              | 25.33             | 21     |
| DEAE      | 2.8           | 10.67                    | 3.41              | 30.50             | 20     |
| DEAEII    | 0.9           | 9.64                     | 3.30              | 19.00             | 16     |

a Specific activity is defined as total RO enzyme activity per mg of protein.
b Expressed as the percentage of RO-enzyme activity recovered from the soluble fraction.

preparation was purified 160 fold with a yield of 16%. The purified fraction exhibited a single protein band when subjected to polyacrylamide gel electrophoresis. Elution patterns on DEAE-cellulose chromatography and Sephadex G-200 chromatography are shown in Figs. 1B and 2A, respectively. In each case, a single protein peak was observed which was closely superimposable on a single activity peak. The enzyme exhibited a homogeneous sedimentation pattern in the ultracentrifuge and possessed an $s_{20, w}$ of 3.6.

**Molecular Weight**—The molecular weight of the retinal-oxidizing enzyme was estimated by thin layer and column chromatography on Sephadex G-200 according to Andrews (22). Fig. 2B shows a plot of the log molecular weight against $V_o$, the elution volume, and Fig. 2C is a plot of the log molecular weight against the distance of migration. The molecular weight was approximately 75,000 to 80,000.

**Absorption Spectrum**—The absorption spectrum of the purified enzyme preparation, measured in a Beckman DU spectrophotometer, is presented in Fig. 3. The preparation exhibited a $\lambda_{max}$ at 280 nm and an $A_{280}/A_{260}$ ratio of 1.35.

**Lipid, Carbohydrate, and Iron Analyses**—Lipid material contained in the various enzyme fractions was quantitatively estimated by extracting various enzyme fractions with ether-ethanol (9:1) and determining the amount of ether-extractable solid remaining after evaporation of the extracts under N₂. The results indicated that ether-extractable material (<0.025% w/v) was present only in the soluble and 50% acetone fractions, while the 50% (NH₄)₂SO₄, DEAE₁, and DEAEII fractions were devoid of any ether-extractable material.

Carbohydrate analysis via the phenol-sulfuric acid method (16) indicated that the soluble, 50% acetone, and 50% (NH₄)₂SO₄ fractions contained approximately 154, 73, and 19 μg per ml of sugars, respectively. The DEAE₁ and DEAEII fractions did not contain any detectable carbohydrates.

Non-heme iron, estimated by the dipyridyl method (15), was present in all enzyme fractions. The purified fraction (DEAEII) contained about 0.153% iron by weight. This value corresponds to a $M_{min}$ of approximately 36,500, and, assuming that the enzyme possesses a molecular weight of 80,000 (Fig. 2, B and C), then approximately 2 moles of iron per mole of enzyme are present in the purified fraction.

**Stoichiometry and Requirements**—The enzymatic conversion of retinal to retinoic acid exhibited a stoichiometric relationship. In 2 hours, approximately 1.25 μmoles of retinal were utilized in the formation of 1.10 μmoles of retinoic acid. The requirements for maximal RO-enzyme activity are presented in Table II. Maximum rate of retinal oxidation occurred when GSH, FAD, NAD⁺, and Fe²⁺ were added to the reaction mixture.

**Identification of Reaction Product**—Several criteria were used to identify retinoic acid as the oxidation product of retinal. The isolated product and synthetic retinoic acid had similar absorption spectra with a $\lambda_{max}$ at 350 nm. The $E_{1%}^{1\text{cm}}$ value at 350 μm in ethanol for the isolated product was 1409. This represents approximately 96% pure retinoic acid. Table III summarizes the results of thin layer chromatography of the...
TABLE III
Migration of reaction product on thin layer chromatography

| Sample                        | RF |
|-------------------------------|----|
| Retinal standard              | 0.70 |
| Retinoic acid standard        | 0.60 |
| Retinol standard              | 0.72 |
| Methyl retinate standard      | 0.79 |
| Reaction productb             | 0.60 |
| Reaction substrateb           | 0.78 |
| Methylated productc           | 0.79 |

* Both standard retinoic acid and the reaction product were methylated with diazomethane.

b Refers to the substrate (retinal) and product (retinoic acid) fractions from the enzyme reaction mixture described under Experimental Procedure."

### TABLE II
Requirements for RO-enzyme activity

| Treatment                        | RO-Enzyme activitya |
|----------------------------------|---------------------|
| None                             | 100                 |
| Enzyme omitted                   | 3                   |
| NAD+ added                       | 136                 |
| NADH added                       | 32                  |
| GSH added                        | 125                 |
| FAD added                        | 122                 |
| FeCl2 added                      | 152                 |
| Several cofactors addedc         | 155                 |
| Anaerobic                        | 101                 |

a The percentage of RO-enzyme activity of the treated sample compared to the activity of the nontreated sample.
b The control reaction contained 1.0 ml of distilled water instead of the 1.0 ml of cofactor solution.
c A solution (1.0 ml) containing 2 μmoles each of NAD+, GSH, FAD, and FeCl2.
d See “Experimental Procedure” under “Other Procedures.”

diamethyl product, its methylated derivative, and standards of retinal, retinol, retinoic acid, and methyl retinolate.

The methylated product and methyl retinonate were analyzed via gas chromatography as described under “Experimental Procedure.” The results indicated that both samples possessed similar patterns and retention times (9.5 min).

**Effects of Time and Enzyme Concentrations**—Fig. 4 illustrates the effect of time and enzyme concentration on the conversion of retinal to retinoic acid. In these experiments, the reaction mixture (8.00 ml) consisted of 0.1 ml of retinal dispersion (2.5 μmoles), 6.90 ml of 0.1 M sodium phosphate buffer (pH 7.7), and 1.00 ml of the enzyme preparation. The rate of the reaction was constant for 120 min and proportional to the concentration of enzyme.

**Effect of Substrate**—Enzyme activity was determined at various concentrations of retinal. The results are presented in Fig. 5, as determined by the Lineweaver-Burk double reciprocal plot. The enzymatic reaction exhibited Michaelis-Menten kinetics with a $K_m$ of $3.0 \times 10^{-4}$ M retinal and a $V_{max}$ of 2.7 μmoles/2 hours per reaction mixture (4.68 $\times 10^{-8}$ M per sec).

**Effect of pH**—The effect of pH on enzymic activity was studied in varying pH values of sodium phosphate buffer (0.1 M). The pH curve was rather broad with an optimum at approximately pH 7.7.

**Effect of Dialysis**—Dialysis of enzyme preparations against distilled water for several hours resulted in approximately 29% loss of enzymic activity. This loss of activity was restored by the addition of 0.5 μmole of each of the following to the reaction vessels: GSH, FeCl2, FAD, and NAD+.

---

**FIG. 3.** Absorption spectrum of purified RO-enzyme. Blank consisted of 0.1 M sodium phosphate buffer, pH 7.7.

**FIG. 4.** Effects of time and enzyme concentration on RO-enzyme (ROE) activity. A, the effect of time; B, the effect of RO-enzyme concentration.
Fig. 5. Effect of substrate concentration on the rate of retinal oxidation. The data are plotted according to the Lineweaver-Burk double reciprocal plot.

TABLE IV
Effect of various reagents on RO-enzyme activity

| Addition                        | Amount | Activation or inhibition |
|---------------------------------|--------|-------------------------|
| None                            | 0      | 0                       |
| GSH                             | 20     | +58                     |
| GSH                             | 2      | +25                     |
| Cysteine                        | 2      | +6                      |
| Ascorbic acid                   | 2      | +13                     |
| FeCl$_2$                        | 2      | -34                     |
| 2-Iodoacetamide                 | 5      | -5                      |
| p-chloromercuribenzoate          | 1      | -85                     |
| $\alpha$,$\alpha'$-Dipryridyl    | 2      | -18                     |
| $\alpha$,$\alpha'$-Dipryridyl + FeCl$_2$ | 2, 4$^b$ | -9                     |
| Sodium cyanide                  | 2      | -18                     |
| Atabrine                        | 1      | -9                      |
| Atabrine + FAD                   | 1, 0.5$^c$ | +4                     |

$^a$ A positive sign indicates activation, while a negative sign indicates inhibition.

$^b$ Two micromoles of $\alpha$,$\alpha'$-dipryridyl plus 4 $\mu$mole of FeCl$_2$ dissolved in distilled water.

$^c$ One micromole of atabrine plus 0.5 $\mu$mole of FAD dissolved in distilled water.

Effect of Other Reagents—Several reagents were used to affect the rate of enzymatic oxidation of retinal. The results are presented in Table IV. Reagents were added in 1.0 ml volume to the reaction mixture and the RO-enzyme activity was determined immediately after addition. Reduced glutathione stimulated and p-chloromercuribenzoate inhibited the enzymatic reaction. This suggests that the enzyme requires sulfhydryl groups for activity. Likewise, metal ions, especially ferrous ions, appear to be required by the enzyme since the rate of the reaction was inhibited by the chelator, $\alpha$,$\alpha'$-dipryridyl, and stimulated by the addition of ferrous ions. Atabrine slightly inhibited the enzymic reaction and FAD reversed the inhibition.

Substrate Specificity of RO-Enzyme—The ability of RO-enzyme to oxidize various compounds was tested and the results are presented in Table V. All-trans retinal was more actively oxidized than the 13-cis form. Purified RO-enzyme did not attack $\beta$-carotene, all-trans retinol, all-trans retinoic acid, and xanthine. On the other hand, an impure sample of RO-enzyme, e.g. the 50% (NH$_4$SO$_4$) fraction, did oxidize $\beta$-carotene and xanthine.

Anaerobic Oxidation of Retinal—RO-Enzyme activity was determined under anaerobic conditions. Samples were incubated at 37°, with agitation, in Thunberg tubes from which the oxygen was previously evacuated. Oxygen content was monitored polarographically. The RO-enzyme activity of a typical anaerobic sample is included in Table II. Polarographic analyses of the anaerobic mixture showed that the oxygen content was negligible. (Aerobic incubations yielded a 2.0-$\mu$A response while anaerobic incubations showed a response of less than 0.3-$\mu$A.)

Enzymatic Incorporation of $^{18}$O from H$_2^{18}$O—The enzymatic oxidation of retinal was performed in a H$_2^{18}$O medium as described under "Experimental Procedure." Incorporation of $^{18}$O into retinoic acid from H$_2^{18}$O was confirmed by extracting the product into diethyl ether, oxidizing to CO$_2$, and determining the mass 46:44 ratio with a mass spectrometer. The main peaks observed upon mass analyses were CO$_2$ and HCN, the latter resulting from the Hg(CN)$_2$ treatment. A 46:44 ratio of 2.49% was obtained. This value corresponds to 1.21 atom % $^{18}$O. Since the natural abundance of $^{18}$O in CO$_2$ is approximately 0.2%, then the corrected value for the $^{18}$O content is 1.0 atom % excess.

Mass spectrometer analysis was performed by Morgan-Schaffer Corporation, Montreal, Quebec, Canada.
Comparison of effects of NAD$^+$ and NADH on RO-enzyme activity

| Previous incubations | Dialysisa | RO-Enzyme activity | Additional treatmentb | RO-Enzyme activity |
|----------------------|-----------|---------------------|-----------------------|---------------------|
| None                 | None      | 1.54                | NAD$^+$               | 1.80                |
| None                 | None      | 1.54                | NADH                  | 1.22                |
| None                 | None      | 1.54                | NAD$^+$ + NADHII      | 1.24                |
| NAD$^+$              | None      | 1.80                | NADH                  | 1.20                |
| NADH                 | None      | 1.22                | NAD$^+$               | 1.28                |
| None                 | Twice against H$_2$O | 1.01 | None | None |
| NAD$^+$              | Twice against H$_2$O | 1.21 | NADH | 0.84 |
| NADH                 | Twice against H$_2$O | 0.95 | NAD$^+$ | 1.10 |

Enzyme and 0.5 μmole of cofactor were previously incubated for 10 min before treatment.

Inhibition by NADH—Since it was observed that NADH inhibited the enzymatic oxidation of retinal (Table II), the inhibition was studied at various NADH concentrations and at two substrate concentrations. Data are plotted in Fig. 6 in a manner described by Johnson, Eyering, and Williams (24), i.e. a plot of $V_0/V_I$ against NADH concentration. The results indicate that alterations in substrate concentration did not significantly affect the inhibition by NADH. This finding suggests that the inhibition is noncompetitive.

8-Diphosphopyridine nucleotide (reduced form), purchased from Sigma, is known to form "inhibitors" when stock solutions are kept for long periods of time, frozen or otherwise. Inhibitor formation can occur without a decrease in optical density at 340 mμ (23). In order to minimize this problem, all solutions were prepared in phosphate buffer immediately before use.

Effect of NAD$^+$ and NADH on Enzymatic Oxidation and Reduction of Retinal—The results of direct additions of NAD$^+$ and NADH to the reaction mixture and preliminary incubations with the enzyme preparation are presented in Table VI. The effect of dialysis on the previously incubated samples is also shown. The data suggest that the inhibition by NADH was not reversed by the direct addition of NAD$^+$, instead, the removal of the former via dialysis was necessary to effect a reversal by NAD$^+$. On the other hand, the stimulation by NAD$^+$ was reversed by the direct addition of NADH. In addition, an equal molar mixture of NAD$^+$ and NADH resulted in depression of the enzymatic activity. When a source of retinal reductase activity is added to the RO-enzyme preparation without adding either NAD or NADH, the major product formed is retinoic acid (Table VII). Maximum production of retinol occurred with the addition of NADH or an equal molar combination of NAD$^+$ and NADH, while minimum production of retinol occurred in the presence of NAD$^+$.
Retinal-oxidizing enzyme preparations contain 2 moles of iron per mole of enzyme and thawings. The absorption spectrum of the purified enzyme indicated that the \( \lambda_{\text{max}} \) was approximately 280 nm and that an additional absorbance occurred in the visible region (410 nm). This latter finding suggests that other light-absorbing materials are present in the purified preparation. In addition, the preparation contains a very slight trace of yellowish color. The \( A_{446}:A_{280} \) ratio was relatively low (1.35) and could indicate the presence of some molecule such as TPN or DPN in the enzyme preparation.

Investigations into the kinetics of the enzyme reaction indicate that the conversion of retinal to retinoic acid is stoichiometric and exhibits Michaelis-Menten kinetics. The enzyme possesses a fairly strong affinity for retinal as indicated by its \( K_m \) value.\(^\text{1}\) The reaction rate is constant over a relatively long period of time (120 min) and is proportional to the enzyme concentration. The optimum pH for the enzyme reaction is approximately 7.7.

Retinoic acid was identified as the reaction product by several criteria. First, it exhibited an absorption spectrum identical with the spectrum of commercial retinoic acid. The \( E_{1%}^1 \) value at 350 nm for the product indicated that it was 96% pure retinoic acid. Second, on thin layer chromatography the product and its methylated derivative had the same \( R_f \) values as their corresponding standards. Finally, the methylated derivative and methyl retinoate exhibited the same pattern and retention time on gas chromatography.

Although undialyzed preparations of RO-enzyme, without external additives, can catalyze the oxidation of retinal, significant increases in the reactions occur when such cofactors as NAD, GSH, FAD, and \( \text{Fe}^{2+} \) are added to the preparation (Table II). Maximum activity occurs when all cofactors are present. In addition, the same cofactors can restore the RO-enzyme activity lost during dialysis. Therefore, it appears that the enzyme requires several cofactors that act in concert to bring about the oxidation of retinal. This statement is also supported by our observation that RO-enzyme preparation exhibit a low \( A_{420}:A_{340} \) ratio and a small absorption peak at 410 nm, thereby suggesting that other nonprotein, light-absorbing substances are associated with the purified enzyme preparation. It is probable that these substances may be cofactors which are strongly bound to the enzyme since they were not removed during purification. Although we have not been successful in showing the oxidant of the reaction, oxygen does not appear to be a likely candidate since the conversion of retinal to retinoic acid takes place under anaerobic conditions (Table II). Apparently, various electron carriers are involved in the oxidation process; however, the terminal acceptor for these electrons has not yet been identified.

Liver retinal oxidase (10) and several other enzymes (26) involved in oxidation reactions have been shown to be flavoproteins which require metal ions and sulfhydryl groups for activity. Evidence reported in this paper suggests that RO-enzyme may require certain metal ions, particularly ferrous ions, inasmuch as the ferrous ion stimulates RO-enzyme activity and the metal chelator \( \alpha, \alpha'- \text{dipyridyl} \) inhibits the reaction (Table IV). In addition, RO-enzyme preparations contain 2 moles of iron per mole of enzyme. Cyanide, which inhibits several metalloflavoenzymes (27), also inhibits RO-enzyme activity (Table IV). Ferric ions, on the other hand, inhibit RO-enzyme activity. The precise relationship between ferrous, ferric ions, FAD, and RO-enzyme activity is unknown.

Retinal-oxidizing enzyme may also require intact sulfhydryl groups for activity. Sulfhydryl reagents, such as GSH and ascorbate, stimulate RO-enzyme activity while sulfhydryl inhibitors, such as 2-iodoacetamide and \( \text{p}-\)chloromercuribenzoate, inhibit the reaction.

Data from anaerobic incubations, \( \text{H}_2\text{O} \) incubations, and polarographic analysis of the reaction mixture suggest that the enzymatic reaction does not use atmospheric oxygen; instead, it utilizes the oxygen from water. The amount of dissolved oxygen in 4.0 ml of water at standard pressure is approximately 0.97 \( \mu \)moles (28). Under anaerobic conditions, the 4.0-ml RO-enzyme reaction mixture would contain considerably less oxygen. Polargraphic analysis of the reaction mixture under aerobic and anaerobic conditions showed a decrease in microamperes from 2.0 to 0.3 \( \mu \)A, respectively. This decrease would correspond to a significant decline in oxygen concentration. Consequently, one would not expect to find an adequate amount of oxygen available to effect the conversion of 1 to 2 \( \mu \)moles of retinal to retinoic acid. Furthermore, enzymatic oxidation of retinal in the presence of \( \text{H}_2\text{O} \), under aerobic conditions, resulted in a significant incorporation of \( \text{H}^4 \)O into retinoic acid. Hence, the reaction utilizes the oxygen of water, thereby resembling a dehydrogenase rather than an oxidase or oxygenase reaction.

Retinal-oxidizing enzyme preparations oxidize both the all-trans and \( 13\text{-cis} \) forms of retinal; however, the former compound was the better substrate (Table V). The enzyme would not convert retinoic acid into retinal in the presence or absence of NADH, thus indicating that the RO-enzyme reaction is irreversible. In addition, retinal reductase, \( \beta \)-carotene oxidase, and xanthine oxidase activities were absent in the RO-enzyme preparation.

Previous reports differ on the effect of NAD\(^+\) and NADH on RO-enzyme activity. For example, Mahadevan et al. (10), using rat liver retinal oxidase, showed stimulations with NADH, while Crain et al. (8), using the rat intestinal oxidase, reported acceleration of the rate by both NAD\(^+\) and NADH. In this present study, we find that NAD\(^+\) increases RO-enzyme activity while NADH depresses the rate of this reaction. Our data indicate that NAD\(^+\) acts as a noncompetitive inhibitor of this enzyme (Fig. 6). We found no indication that NADH interfered with the assay method or that NADH had been oxidized to NAD\(^+\) by the enzyme preparation. (The latter statement stems from the observation that the absorbance of a mixture of enzyme and NADH at 340 nm did not change during 2 hours of incubation.)

In experiments in which one cofactor was previously incubated with enzyme (Table VI), and the other cofactor was added later, the results indicate that NADH can reverse the effect of NAD\(^+\), while the opposite relationship does not hold true. Both effects can be partially reversed by dialysis.

Since it has been shown by others (25) that retinal reductase from rat intestine requires NADH for the conversion of retinal to retinol, an experiment was carried out to study the effect of NAD\(^+\) and NADH on a solution containing both the reductase and the retinal-oxidizing enzyme. The results presented in

\(^{\text{1}}\) The authors must emphasize that the \( K_m \) value for ROE may merely represent an apparent value since retinal is water-insoluble and dispersed in the incubation mixture from an acetone solution (25).

\(^{\text{2}}\) The effect of NADH on the substrate (retinal) and product (retinoic acid) was tested. Incubations of known amounts of either substrate or product or both with NADH did not alter the efficiency of the assay technique.
Table VII show that without the addition of either cofactor the
oxidation reaction predominates and very little reduction occurs.
(The small amount of reduction that does take place is probably the result of endogenous NADH present in the crude retinal reductase preparation.) When NADH alone is added to the system, the RO-enzyme activity increases (24%) with no significant reductase activity. Conversely, the addition of NADH results in a depression of RO enzyme activity (34%) and a substantial increase (8 times) in the reductase activity. Thus, with the addition of equal molar amounts of NADH and NADPH, the above results suggest that NADH exerts a stronger control over the oxidation and reduction of retinal than NADH.

At present, the metabolic significance of these results cannot be defined, but it is tempting to speculate that the tissue level of NADH may play a significant role in controlling the metabolic fate of retinal. For example, at high tissue concentrations of NADH, the retinal reductase reaction would be stimulated while the retinal-oxidizing reaction would be depressed, thereby resulting in an increased yield of retinol from retinal, whereas, at low levels of NADH, the retinal reductase reaction would be depressed while the oxidizing reaction would be increased, thereby resulting in a higher yield of retinoic acid from retinal. Since in most animals β-carotene is the common precursor of retinal (4, 29), this type of control could possibly serve as a means of regulating the animal’s supply of retinol and retinoic acid. Recently, Roberts and DeLuca (30) suggested that a control mechanism may be functioning at a step between retinol and retinoic acid. Since retinoic acid is detected in very small amounts in animal tissues, and since it is more toxic than retinol or retinol (31), the above mechanism might assist the animal in controlling the production of retinoic acid.

Acknowledgments—We wish to thank Dr. Harold Resnick and Mr. Edward Plow for carrying out the ultracentrifugal analysis, Mr. William Passwaters, Department of Chemistry, West Virginia University, for the polarographic analysis, and Mrs. Sue Badiei for her technical assistance.

REFERENCES

1. ARENS, J. F., AND VAN DORP, D. A., Nature, 157, 190 (1946).
2. DOWLING, J. E., AND WALD, G., Proc. Nat. Acad. Sci. U. S. A., 46, 557 (1960).
3. THOMPSON, S. Y., GANGULY, J., AND KON, S. K., Brit. J. Nutr., 3, 50 (1949).
4. OLSON, J. A., Pharmacol. Rev., 19, 550 (1967).
5. DESHMUKH, D. C., MALATHI, P., AND GANGULY, J., Biochim. Biophys. Acta, 107, 120 (1965).
6. DUNAGIN, P. E., ZACHMAN, R. D., AND OLSON, J. A., Biochim. Biophys. Acta, 90, 432 (1964).
7. EMERICK, R. J., ZELE, M., AND DELUCA, H. F., Biochem. J., 102, 600 (1967).
8. CHAIN, F. D., LOTTSPEICH, F. J., AND KRAUSE, R. F., J. Lipid Res., 8, 249 (1967).
9. FIDGE, N. H., SHIRATORI, T., GANGULY, J., AND GOODMAN, JEW. S., J. Lipid Res., 9, 103 (1968).
10. MARADEYAN, S., MURTHY, S. K., AND GANGULY, J., Biochem. J., 85, 320 (1962).
11. PETESEN, E., AND SOEREN, H., IN S. P. COLLOWICK AND N. O. KAPLAN (EDITORS), IN METHODS OF ENZYMOL., VOL. V, ACADEMIC PRESS, NEW YORK, 1961, P. 3.
12. BUTTERMAN, S., J. BIOL. CHEM., 237, 677 (1962).
13. BRACKKAN, O. R., MYKLESTAD, H., AND NIAA, L. R., ACIA CHEM. SCAND., 14, 779 (1960).
14. LOWRY, O. H., ROBERGOUTH, N. J., FARR, A. L., AND RANDALL, R. J., J. BIOL. CHEM., 193, 255 (1951).
15. RAMSAY, W. N. M., Biochem. J., 49, 494 (1954).
16. DUBOIS, M., GILES, K. A., HAMILTON, J. K., REBERS, P. A., AND SMITH, F., ANAL. CHEM., 28, 350 (1956).
17. DAVIS, R. J., ANN. N. Y. Aead. Sci., 121, 404 (1944).
18. FIEBER, L., AND FIEBER, M., REAGENTS FOR ORGANIC SYNTHESIS, John Wiley and Sons, New York, 1967, P. 1116.
19. KAHN, J. S., ANAL. Biochem., 9, 380 (1964).
20. LEE, J. S., ANAL. CHEM., 34, 835 (1962).
21. DIXON, M., AND WEBB, E., ENZYMES, ED. 2, ACADEMIC PRESS, NEW YORK, 1964, P. 40.
22. ANDREWS, P., Biochem. J., 91, 222 (1964).
23. FAWCETT, C. P., CIOTTI, M. M., AND KAPLAN, N. O., Biochem. Biophys. Acta, 54, 210 (1960).
24. JOHNSON, F. H., EYING, H., AND WILLIAMS, R. W., J. Cell. Comp. Physiol., 20, 247 (1942).
25. FIDGE, N. H., AND GOODMAN, J. D., J. BIOL. CHEM., 243, 4372 (1968).
26. WHITE, A., HANDLER, P., AND SMITH, E., PRINCIPLES OF BIOCHEMISTRY, ED. 4, McGRAW-HILL BOOK COMPANY, INC., NEW YORK, 1960, P. 364.
27. MAHLER, H. R., AND COOKE, E. H., BIOLOGICAL CHEMISTRY, HARPER AND ROW, PUBLISHERS, INC., NEW YORK, 1966, P. 206.
28. MOORE, W. J., PHYSICAL CHEMISTRY, PRENTICE-HALL, INC., ENSLEWOOD CLIFFS, NEW JERSEY, 1967.
29. GOODMAN, D. S., HUANG, H. S., KANAI, M., AND SHIRATORI, T., J. BIOL. CHEM., 242, 5543 (1967).
30. ROBERTS, A. B., AND DELUCA, H. F., Biochem. J., 102, 600 (1967).
31. GANGULY, J., J. SCI. IND. RES., 26, 110 (1967).
