Purification and characterization of enzymes metabolizing retinaldehyde, propionaldehyde, and octanaldehyde from four human livers and three kidneys were done to identify enzymes metabolizing retinaldehyde and their relationship to enzymes metabolizing other aldehydes. The tissue fractionation patterns from human liver and kidney were the same, indicating presence of the same enzymes in human liver and kidney. Moreover, in both organs the major NAD-dependent retinaldehyde activity copurified with the propionaldehyde and octanaldehyde activities; in both organs the major NAD-dependent retinaldehyde activity was associated with the E1 isozyme (coded for by aldhl gene) of human aldehyde dehydrogenase. A small amount of NAD-dependent retinaldehyde activity was associated with the E2 isozyme (product of aldhl gene) of aldehyde dehydrogenase. Some NAD-independent retinaldehyde activity in both organs was associated with aldehyde oxidase, which could be easily separated from dehydrogenases. Employing cellular retinoid-binding protein (CRBP), purified from human liver, demonstrated that E1 isozyme (but not E2 isozyme) could utilize CRBP-bound retinaldehyde as substrate, a feature thought to be specific to retinaldehyde dehydrogenases. This is the first report of CRBP-bound retinaldehyde functioning as substrate for aldehyde dehydrogenase of broad substrate specificity. Thus, it is concluded that in the human organism, retinaldehyde dehydrogenase (coded for by raldh1 gene) and broad substrate specificity E1 (a member of EC 1.2.1.3 aldehyde dehydrogenase family) are the same enzyme. These results suggest that the E1 isozyme may be more important to alcoholism than the acetaldehyde-metabolizing enzyme, E2, because competition between acetaldehyde and retinaldehyde could result in abnormalities associated with vitamin A metabolism and alcoholism.

In mammalian organisms retinoids and their derivatives are important in regulation of diverse physiological functions. Retinoic acid has only recently been recognized as a major hormone in cell differentiation and development (1, 2). It is also thought to be a causative agent in diseases such as cancer (3) and more recently, schizophrenia (4). In mammals, biosynthesis of retinoids proceeds via central or excentric cleavage of carotene to retinaldehyde followed by its reduction to retinol or oxidation to retinoic acid (5–7).

Enzymes with broad substrate specificity such as alcohol and aldehyde dehydrogenases have been known for a long time to include retinaldehyde among their many substrates (8–10). Efforts have been made to identify aldehyde dehydrogenase isozyme active with retinaldehyde (11–13). The mouse enzymes were found to have activity (11) as well as the human E1 isozyme (12, 13). However, more recently, cytosolic NAD-linked retinaldehyde dehydrogenases, more specific toward all-trans-retinaldehyde and assumed to be distinct from aldehyde dehydrogenase of broad substrate specificity, have been purified from rat liver (Ref. 14, retinaldehyde dehydrogenase 1) and kidney (15). In addition, rat retinaldehyde dehydrogenase was shown to utilize cellular retinol-binding protein (CRBP)1-bound retinaldehyde as substrate (14). The gene for retinaldehyde dehydrogenase 2 (RALDH2) was cloned from developing mouse eye (16) and rat testis (17) and in both cases the enzyme was characterized by expressing its cloned gene. The primary structures of mouse and rat enzymes and substrate specificity of the enzyme from rat testis exhibited all characteristic features of aldehyde dehydrogenase. There are differences in the distribution of aldehyde dehydrogenase isozymes between human and rat livers. While in the human liver the mitochondrial enzyme, E2 (coded for by aldhl gene), is expressed at approximately the same level as the cytoplasmic enzyme, E1 (coded for by aldhl gene), in the rat liver the product of the aldhl gene is the major enzyme (18). Rat cytoplasm contains very little aldehyde dehydrogenase activity and a large number of aldehyde dehydrogenases (19), of which some have not yet been identified. Moreover, gene duplication of aldhl must have occurred in the rat (20–22) and other animals (23) while only one aldhl gene is known in humans.

Human aldehyde dehydrogenases have been well characterized. We purified and characterized two human liver aldehyde dehydrogenases (E1 and E2) (24); human liver glutamic semialdehyde dehydrogenase (25) and human betaine aldehyde/y-aminobutyraldehyde dehydrogenase (E3, GenBank ALDH9) (26). All were found to be of broad substrate specificity including a wide spectrum of aldehydes. Retinaldehyde was recognized as substrate for E1, E2, and E3 isozymes (27). During this investigation an attempt was made to identify retinaldehyde dehydrogenases such as those purified from rat tissues (14, 15) in mature human liver and kidney utilizing the procedures and identification methods with which we have many years of experience. The results demonstrate that in human liver and kidney major retinaldehyde dehydrogenase activity is associated with aldehyde dehydrogenase of broad substrate.

* This work was supported by National Institute on Alcohol Abuse and Alcoholism Grant 1RO1 AA00186. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Center of Alcohol Studies, Rutgers, The State University of New Jersey, 607 Allison Rd., Piscataway, NJ 08854-8001. Tel.: 732-445-3643; Fax: 732-445-3500; E-mail: pietrusz@rci.rutgers.edu.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

The abbreviations used are: CRBP, cellular retinol-binding protein; HPLC, high performance liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.

Printed in U.S.A.

This paper is available on line at http://www.jbc.org

33366
specificty, which also recognizes CRBP-bound retinaldehyde as a substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**

Adult human autopsy livers and kidneys were from NDRI, Philadelphia, PA. NAD\(^+\) was from Roche Molecular Biochemicals, Indianapolis, IN. All-trans-retinaldehyde and all-trans-retinoic acid were obtained from Sigma-Aldrich; both were maintained under nitrogen at \(-70^\circ C\) and used in gold light illuminated rooms. Diethylaminobenzaldehyde was from Aldrich, disulfiram was from Ayerst, CM-Sephadex, DEAF-Sephadex, and 5'-AMP Sepharose 4B, Mono P column, Pharmalyte of 3–10 pH range, agarose, gradient gels PAA 4/30, and pI standards were from Amersham Pharmacia Biotech. All other chemicals were reagent grade.

**Enzyme Assays**

Aldehyde Dehydrogenase (EC 1.2.1.2)—Aldehyde dehydrogenase catalyzes the dehydrogenation of aldehydes in the presence of NAD\(^+\). Activity was determined by a standard assay, previously employed (24). The assay mixture in 100 mM sodium pyrophosphate buffer, pH 9.0, contained 500 \(\mu\)M NAD\(^+\), 1 mM propionaldehyde, and 1 mM EDTA. The reaction was started by addition of enzyme and followed by continuous recording at 340 nm and 25 °C. The reaction velocities were calculated from extinction coefficient of NADH of 6.22 cm\(^{-1}\)·M\(^{-1}\)·s\(^{-1}\). A variant of the above assay containing 0.2 mM octanaldehyde (added in 0.02 ml of acetonitrile/3 ml total volume) was used to determine the specific activity of enzyme. This ratio has been found to be 0.5–0.6 for the E1 isozyme and 0.92–0.97 for the E2 isozyme. The specific activity with propionaldehyde (micromol/min/mg of protein) was determined and adjusted to maximal specific activity, which for E1 is 0.6 \(\mu\)mol/min/mg, for E2, 1.6 \(\mu\)mol/min/mg, and for E3 0.6 \(\mu\)mol/min/mg.

**Retinaldehyde Dehydrogenase (EC 1.2.1.36)—**Catalyzes the NAD\(^+\)-linked dehydrogenation of retinaldehyde. Activity was assayed by HPLC by determining retinoic acid formed from the enzyme-catalyzed dehydrogenation of retinaldehyde. Determination of activity in chromatography fractions during purification, retinaldehyde was incubated with enzyme in 100 mM Tris glycine buffer, pH 9.0, containing 1 mM EDTA, 0.5 mM NAD\(^+\), and 20 \(\mu\)M all-trans-retinaldehyde in 1 ml total volume at 25 °C. The reaction was initiated by addition of 10 \(\mu\)l of retinaldehyde, dissolved in absolute ethanol. Retinaldehyde up to 20 \(\mu\)M concentration was completely soluble in these conditions, and no precipitation was observed at 50 \(\mu\)M retinaldehyde. After 20 min incubation the reaction was terminated by freezing in a dry ice-ethanol bath, which inactivates human aldehyde dehydrogenase, and then transferred to a –80 °C freezer for storage before the HPLC analysis. Total activity of the enzyme used per assay was in the range of 0.002–0.02 \(\mu\)mol/min for the E1 isozyme and the steady state reaction was observed for about 40 min and conversion of substrate after 20 min was not greater than 20%. Incubations were done in duplicate and appropriate controls without NAD\(^+\) and without enzyme for each experimental set were included. The incubation mixtures were rapidly thawed (about 30 s) in a water bath, and 200 \(\mu\)l were injected directly into the HPLC column. At the beginning of purification when protein concentrations were high, after thawing, the protein was precipitated with 50% v/v ethanol and removed by centrifugation. Reverse phase HPLC analysis (variation between duplicates below 5%) was carried out on a Waters µBondapak C18 column with isocratic elution of acetonitrile and 1% (v/v) ammonium acetate (80:20% v/v) at 1.25 ml/min flow rate and 340 nm detection. The quantitative measurement of retinoic acid was obtained by comparing sample peak areas with that of standard retinoic acid. Column performance and stability of enzymatic assay conditions during HPLC chromatography were checked by employing tetrathenylethylene (Aldrich) as an internal standard and retinoic acid during control enzymatic assay. In both cases variation was below 5% of the averages for controls used several times for each separate set of daily experiments. The progress of enzymatic reaction was also confirmed by measurement of retinaldehyde concentration, but variation in the range of 10–20% of average was not too high for the low flow rate used in this chromatography. The low detection limit with 340 nm detector and 200–200 \(\mu\)l sample loop was about 2 pmol for retinoic acid and 4 pmol for retinaldehyde. On the basis of HPLC/spectroscopy all-trans-retinaldehyde was free from retinoic acid with purity better than 96%. Purity of retinoic acid was better than 98%. At 0.02–0.5 detector sensitivity range (absorbance units at full scale) linear correlation (\(r = 0.999\)) of retinoic acid concentrations up to 50 \(\mu\)M and peak areas were observed. Magnesium chloride (150 \(\mu\)M) was employed in Pipers buffer, 25 mM, pH 7.6, in the presence of an appropriate substrate and 500 \(\mu\)M NAD\(^+\) and absence of EDTA to test its effect on reaction velocity. Diethylaminobenzaldehyde (10 \(\mu\)M) was used with the standard assay and added before the reaction was started with substrate. Disulfiram (50 \(\mu\)M) was also used in the standard assay and added either before or after the start of the reaction. Retinaldehyde Oxidase (Aldehyde Oxidase EC 1.2.3.1)—Retinaldehyde oxidase catalyzes the oxidation of retinaldehyde in the presence of atmospheric oxygen in the absence of NAD\(^+\). Retinaldehyde oxidase activity was determined by the same procedure as that used for retinaldehyde dehydrogenation, with NAD\(^+\) being omitted from the incubation mixture.

In all experiments with pure enzyme the reactions were started by addition of enzyme. At pH 7.6, 50 mM Tris/HCl buffer, and for pH 9.0, 100 mM Tris glycine buffer, 1 mM EDTA was used; both buffers contained 500 \(\mu\)M NAD\(^+\). Reaction mixtures (1 ml) were set up in Eppendorf tubes and incubated for 20 min at 25 °C. When reactions were carried out in the presence of CRBP, retinaldehyde and CRBP were preincubated for 10 min before adding the enzyme. Kinetic constants were calculated by the Lineweaver-Burk (28), or a single line Yun and Suetler (29) procedures employing the statistical method of Cleland (30). All work was done under gold lights.

**Protein Determination**

Protein was determined by the microburet procedure (31) using bovine serum albumin as standard.

**Electrophoretic Procedures**

Isoelectric focusing was done employing 1% agarose gels prepared with Pharmalyte at pH 3–10 according to the manufacturer’s instructions. Propionaldehyde and retinaldehyde activities were detected on gels by employing 1 mM propionaldehyde and 20 \(\mu\)M retinaldehyde in the presence of NAD\(^+\) (1 mM), nitro blue tetrazolium (1 mM), and phenazine methosulfate (0.1 mM). The gels were stained for protein with Coomasie Brilliant Blue. Pl values were determined by comparison with those of pl standards (Amersham Pharmacia Biotech). Native molecular weights were determined by electrophoresis on a gradient gel (PAA 4/30) and comparison with molecular weight standards (Amersham Pharmacia Biotech). Subunit molecular weights were determined on 10% polyacrylamide gels containing SDS by comparison with known standards (Sigma).

**Enzyme Purification**

All purification steps were performed in anoxic conditions under nitrogen or argon at 4 °C. The tissues were extracted into 30 mM sodium phosphate buffer, pH 6.0, containing 0.1% (v/v) 2-mercaptoethanol and 1 mM EDTA (2 volumes of buffer per wet weight of tissue). Only the enzyme extracted into the buffer is accounted for during the subsequent purification steps. The purification procedure from 600 g of liver and about 300 g of human kidney employed consecutively CM-Sephadex and DEAE-Sephadex ion exchange chromatography followed by affinity chromatography on 5’-AMP Sepharose (Table I). All chromatographic steps routinely employed for aldehyde dehydrogenase purification are shown as steps 1–5 (Table I). Additional steps involving the same chromatographic column are shown as the step number with a letter suffix (e.g. steps 5A, 5B etc). The columns used for purification from kidney were decreased in size proportionately to the weight available. All buffers used were evacuated and exhaustively nitrogenated and contained 1 mM EDTA and 0.1% (v/v) of 2-mercaptoethanol. The dia- lyzed homogenate in 30 mM sodium phosphate buffer, pH 6.0, was loaded on CM-Sephadex column (20 g of CM-Sephadex for 600 g of liver) equilibrated with the same buffer. The proteins passing through the CM-Sephadex column, after adjustment of pH to 6.8, were applied to DEAE-Sephadex column (40 g of DE-Sephadex for 600 g of liver) equilibrated with the pH 6.8 buffer. The proteins passing through the CM-Sephadex column, after adjustment of pH to 6.8, were applied to 5’-AMP Sepharose 4B for 600 g of liver) equilibrated with the pH 6.8 buffer. The proteins passing through the CM-Sephadex column, after adjustment of pH to 6.8, were applied to 5’-AMP Sepharose 4B for 600 g of liver) equilibrated with the pH 6.8 buffer. The proteins passing through the CM-Sephadex column, after adjustment of pH to 6.8, were applied to 5’-AMP Sepharose 4B for 600 g of liver) equilibrated with the pH 6.8 buffer.
buffer, containing 0.3 mg/ml NADH. The procedure employed was essentially that described by Hempel et al. (32) except that additional high salt column washing steps were employed and the E2 isozyme was separated from E1 isozyme on 5'-AMP affinity column by using 1 M NaCl salt elution at pH 6.0, instead of previously described elution with phosphate buffer at pH 8.0. The currently employed procedure gives much cleaner separation of the E1 and E2 isozymes.

Structural Analysis

Structural analysis was done by W. M. Keck Foundation, Yale University, New Haven, CT. The enzymes were carboxymethylated and digested with trypsin, prior to chromatography. Tryptic peptide maps were run analytically and preparatively on the microbore HPLC with the blank and transferrin control.

Purification of Human CRBP

CRBP was purified from human liver by combining the procedure of Ong and Chytil (33) with that of Pex and Johannesson (34). The crude liver homogenate in 100 mM Tris/HCl, pH 7.5, after centrifugation, was acidified to pH 5 with acetic acid. The precipitated proteins were removed by centrifugation and discarded. The supernatant was then mixed with CM52-cellulose (Whatman), equilibrated at pH 5 and filtered (4M, Whatman) after stirring 5 h at 4 °C. The filtrate was then concentrated by lyophilization and dialyzed against the gel filtration buffer (10 mM Tris acetate, pH 7.5, containing 0.2 M NaCl). The protein solution was loaded onto a gel filtration column (Sephadex G-50) and the fractions containing proteins of 15 kDa were combined, concentrated by lyophilization and dialyzed against the gel filtration buffer (10 mM Tris acetate, pH 7.5, containing 0.1 M EDTA and 0.1% (v/v) 2-mercaptoethanol and were thoroughly evacuated and nitrogen saturated before use at 4 °C.

| Original purification | Additional steps |
|-----------------------|------------------|
| 1. Homogenate         |                  |
| 2. Dialysis           |                  |
| 3. CM-Sephadex chromatoigraphy (E1, E2, and E3 do not bind) | 3A 1 M NaCl in 0.1 M phosphate, pH 8.0, elution of the proteins attached to CM-Sephadex |
| 4. DEAE-Sephadex chromatography (binding of E1, E2, E3 and their elution by NaCl gradient) | 4A Analysis of proteins not retained by DEAE-Sephadex |
| 5. 5'-AMP Sepharose chromatography Elution: (a) 1 M NaCl in 30 mM sodium phosphate, pH 6.0 to elute E2; 0.1 M phosphate, pH 8.0, containing 1 mg of NAD/m to elute E1 | 5A Analysis of proteins not retained by 5'-AMP Sepharose |
|                       | 5B Elution of proteins from 5'-AMP Sepharose by 1 M NaCl and 0.3 M NaCl in 0.1 M phosphate buffer, pH 8.0, after elution of E1 and E2 isozymes |

The resulting protein sample produced a single protein band of about 15 kDa on SDS-polyacrylamide gel electrophoresis. In the presence of CRBP the absorption spectrum of retinol showed the three characteristic peaks in the 320–350 nm range as described previously (35). The fluorescence emission spectrum obtained by excitation of the protein-retinol mixture at 350 nm showed a peak at about 470 nm, which disappeared upon exposure of the sample to UV light. The dissociation constant for retinol of human CRBP was previously reported to be 10 nM and that for retinol dehydrogenase to be 100 nM (34). During this investigation fluorometric titration with retinol was done on Perkin-Elmer Model MFP-2A fluorescence spectrophotometer following retinol fluorescence by the procedure described by Cogan et al. (36) at 350 nm excitation and 485 nm emission. The Kd for retinol calculated from titration was 14 nM. Fluorescence titration with retinaldehyde was done by quenching of CRBP fluorescence (37) at 280 nm excitation and 340 nm emission. The Kd for retinaldehyde calculated (36) from titration was 44 nM. It was determined that 62% of the purified CRBP protein was active in binding both retinol and retinaldehyde; the latter was used for calculations of the molar ratio of CRBP to retinaldehyde. Proportions of free and CRBP-bound retinaldehyde were calculated from the mass law equation: Kd = [(free retinaldehyde) (free CRBP)/(CRBP-retinaldehyde complex)]. At CRBP concentrations two times that of retinaldehyde, the mass law equation takes the following form: (CRBP-retinaldehyde complex)2 - (3(total retinaldehyde) + Kd) (CRBP-retinaldehyde complex) + 2 (total retinaldehyde)2 = 0.

RESULTS

Purification of Retinaldehyde and Aldehyde Dehydrogenases from Caucasian Human Livers and Kidneys—Four purifications were done from human liver and three from kidney; the results are summarized in Table II. Retinaldehyde activity was separated into three batches: (i) that absorbed to CM-Sephadex (about 10% of total retinaldehyde activity), (ii) that absorbed to DEAE-Sephadex and subsequently eluted by a salt gradient (the major retinaldehyde activity, about 2/3 of total), and (iii) that which did not absorb to DEAE-Sephadex (about 20% of total retinaldehyde activity).

(i) NAD+-independent retinaldehyde activity (presumably aldehyde oxidase) was retained on CM-Sephadex. It was eluted (see step 3A in Table II) by high salt concentrations and was present in both liver and kidney homogenates. In liver it constituted about 10% of total retinaldehyde activity, using the same HPLC assay as that used for dehydrogenase activity. The CM-Sephadex also retained a small amount of NAD+-dependant propionaldehyde activity, previously characterized as glutamic-y-semialdehyde dehydrogenase (25) found during this investigation to be inactive with retinaldehyde.

(ii) The majority of NAD+-dependent retinaldehyde activity of the human liver and kidney was absorbed to DEAE-Sephadex from which it was eluted by salt gradient (step 4 in Table II).

(iii) Loss of retinaldehyde and propionaldehyde activities during loading and washing of DEAE-Sephadex (see step 4A in Table II) has been observed during these and previous aldehyde dehydrogenase purifications. Use of smaller loads, relative to column size, and adjustment of pH from 6.8 to pH 9 did not prevent the activity loss. Reloading of active fractions on new DEAE-Sephadex resulted in non-absorption of initially non-absorbing enzyme, demonstrating that the original loss of activity from DEAE was not due to an insufficient amount of DEAE-Sephadex used. Retinaldehyde was reabsorbed with propionaldehyde and retinaldehyde. The retinaldehyde active enzyme which did not bind to DEAE-Sephadex was also present in kidney. Chromatographic profiles with propionaldehyde and retinaldehyde as substrates of kidney homogenates were identical to those of liver.

Characterization of the Major Retinaldehyde Activity—The major retinaldehyde activity eluted by a salt gradient from DEAE-Sephadex (step 4 in Table II) was loaded on the 5'-AMP Sepharose column and eluted by NAD+ in pH 8.0 buffer, where...
The E1 isozyme is normally eluted. Isoelectric focusing showed a single band, active with both propionaldehyde and retinaldehyde, which was identified as the E1 isozyme. The ratio of retinaldehyde to propionaldehyde activity was 0.12:1.0 and was constant throughout the elution peak. No substrate inhibition with the E1 isozyme was observed up to 20 mM retinaldehyde. Its activity showing that this activity belonged to the mitochondrial E2 isozyme. Magnesium inhibited retinaldehyde activity of the E2 isozyme in the presence of CRBP never exceeded the velocity with free retinaldehyde which also contributed to the inhibition than bound retinaldehyde. The observed $K_m$ value for retinaldehyde was 450 nM and 27 nmol/min/mg, respectively. The presence of CRBP at a concentration two times higher than that of retinaldehyde resulted in an increase of the $K_m$ value for retinaldehyde (1.7 $\mu$M), showing that free retinaldehyde was utilized by the enzyme at lower concentration than bound retinaldehyde. The observed $K_m$ value for the CRBP-bound retinaldehyde is an approximation due to the presence of free retinaldehyde which also contributes to the reaction. The fact that CRBP has no effect on maximal velocity of retinaldehyde dehydrogenation (29 nmol/min/mg) by the E1 isozyme demonstrates that bound CRBP is a substrate for the E1 isozyme. Thus, the E1 isozyme can utilize CRBP-bound retinaldehyde.

The effect of CRBP on the retinaldehyde dehydrogenation of the E2 isozyme is shown in Fig. 3. In the absence of CRBP the $K_m$ and $V_{max}$ values of E1 for retinaldehyde were 450 nM and 27 nmol/min/mg, respectively. The presence of CRBP at a concentration two times higher than that of retinaldehyde resulted in an increase of the $K_m$ value for retinaldehyde (1.7 $\mu$M), showing that free retinaldehyde was utilized by the enzyme at lower concentration than bound retinaldehyde. The observed $K_m$ value for the CRBP-bound retinaldehyde is an approximation due to the presence of free retinaldehyde which also contributes to the reaction. The fact that CRBP has no effect on maximal velocity of retinaldehyde dehydrogenation (29 nmol/min/mg) by the E1 isozyme demonstrates that bound CRBP is a substrate for the E1 isozyme. Thus, the E1 isozyme can utilize CRBP-bound retinaldehyde.

The effect of CRBP on the retinaldehyde dehydrogenation of the E2 isozyme is shown in Fig. 3. In the absence of CRBP the $K_m$ and $V_{max}$ values of E2 were 450 nM and 27 nmol/min/mg, respectively. The presence of CRBP at twice the concentration of retinaldehyde resulted in the inhibition of the retinaldehyde dehydrogenation activity of the E2 isozyme. The $K_m$ for retinaldehyde in the presence of CRBP was determined as 310 nM. CRBP exerted its effect on $V_{max}$ which was less than 20% of that in the absence of CRBP. In fact, the maximal velocity with E2 isozyme in the presence of CRBP never exceeded the veloc-

### Table II

**Summary of purification of aldehyde dehydrogenase and retinaldehyde dehydrogenase from human liver and kidney**

Results are measurements of activity (IU = international units = micromoles of product formed per min) extracted by 200 ml of buffer from 100 g of liver or kidney and are presented as mean ± S.D. for n = 4 purifications (liver) and n = 3 purifications (kidney).

| Purification Step | Propionaldehyde | Retinaldehyde | Retinaldehyde/propionaldehyde $\times 10^9$ |
|------------------|-----------------|--------------|-----------------------------------------|
|                  | Liver           | Kidney       | Liver                                   | Kidney          |                      |
|                  | yield mean IU ± S.D. (%) | yield mean IU ± S.D. (%) | yield mean IU ± S.D. (%) | yield mean IU ± S.D. (%) |
| 1. Homogenate    | 161 ± 20        | 33 ± 13      | 7.1 ± 2.4                               | 1.7 ± 0.7       | 4.4 ± 0.0           | E1, E2, E3 |
|                  | (100)           | (100)        | (100)                                   | (100)           | (100)               |           |
| 2. Dialysis      | 138 ± 27        | 29 ± 13      | 6.3 ± 2.2                               | 1.5 ± 0.6       | 4.6 ± 0.0           | E1, E2, E3 |
|                  | (86)            | (88)         | (89)                                    | (88)            | (88)                |           |
| 3. CM-Sephadex   | 129 ± 28        | 27 ± 13      | 5.4 ± 1.5                               | 1.2 ± 0.5       | 4.2 ± 0.2           | E1, E2, E3 |
|                  | (80)            | (82)         | (76)                                    | (71)            | (71)                |           |
| 3A. CM-Sephadex (1 M NaCl, pH 8.0) | 2.2<sup>b</sup> | trace        | 0.9<sup>b</sup>                         | ND<sup>a</sup>  | ND                  |            |
|                  | (1)             |              | (13)                                    |                |                     |           |
| 4. DEAE-Sephadex gradient elution | 107 ± 29 | 24<sup>b</sup> | 4.3 ± 1.5                               | 1.0<sup>b</sup> | 4.0 ± 0.2           | E1, E2, E3 |
|                  | (66)            | (73)         | (61)                                    | (59)            | (59)                |           |
| 4A. DEAE loading and washing before elution | 13<sup>b</sup> | 2<sup>b</sup> | 1.8<sup>b</sup>                         | 0.3<sup>b</sup> | 13.8 ± 15.0         | E1, E2     |
|                  | (8)             | (6)          | (25)                                    | (18)            | (18)                |           |
| 4B. DEAE wash (1 M NaCl) after elution | Inactive | ND           | Inactive                               | ND              |                     |            |
| 5. 5'-AMP Sepharose elution<sup>a</sup> | 59 ± 15 | 12 ± 4       | 0.05 ± 0.02                             | 0.007 ± 0.002   | 0.08 ± 0.06         | E2         |
| (a) 1 M NaCl, pH 6.0 | (37) | (36) | (0.7) | (0.4) | 11.4 ± 13.0 | E1         |
| 5. 5'-AMP Sepharose elution<sup>a</sup> | 21 ± 4 | 5.4 ± 1.8   | 2.4 ± 0.7                               | 0.7 ± 0.3       | 14.6 ± 13.0         | E2         |
| (b) 0.1 M phosphate, pH 8.0, 1 mg/ml NAD | (13) | (16) | (34) | (41) | 14.6 ± 13.0 | E2         |
| 5A. 5'-AMP Sepharose loading and washing | 23<sup>b</sup> | 2.5<sup>b</sup> | Trace                                   | Trace           | E3                  |            |
|                  | (14)            | (8)          |                                         |                 |                     |            |
| 5B. Washing of 5'-AMP with 1 M NaCl + 0.3 mg/ml NADH after step 5 | 1.8 | ND | 0.34 | ND | 18.9 ± 19.8 | E1 |
|                  | (1)             |              | (5)                                     |                 |                     |            |

<sup>a</sup> Isozyme composition was identified by isoelectric focusing followed by staining with propionaldehyde and retinaldehyde as substrates.

<sup>b</sup> Mean from two purifications.

<sup>c</sup> ND, not determined.
Metabolism of Retinaldehyde and Other Aldehydes

Fig. 1. Binding of retinaldehyde to CRBP and its effect on retinaldehyde dehydrogenase activity of E1 and E2 isozymes. A, increasing concentrations of active human CRBP have different effects on the retinaldehyde dehydrogenase activity of isozymes E1 (circles) and E2 (triangles). Retinaldehyde concentration was 1 μM in 50 mM Tris/HCl buffer, pH 7.6, containing 500 μM NAD⁺. The proportion of free retinaldehyde out of total retinaldehyde (squares) was calculated from the mass law equation (see "Experimental Procedures") employing a \( K_d \) of 44 nM for the binding of retinaldehyde to CRBP. B, titration of human CRBP with all-trans-retinaldehyde by following quenching of protein fluorescence. Corrected intensity (%) of protein fluorescence is plotted as a function of total retinaldehyde concentration. Titration was carried out in 50 mM Tris/HCl buffer, pH 7.6, at 25 °C. Protein fluorescence was monitored at 340 nm with excitation at 280 nm. The concentration of active CRBP bound to retinaldehyde as a function of total retinaldehyde concentration.

Fig. 2. Dehydrogenation of retinaldehyde by the isozyme E1 of human aldehyde dehydrogenase in the absence or presence of CRBP. Production of retinoic acid in the absence of CRBP (circles) or presence of CRBP (triangles) plotted as a function of all-trans-retinaldehyde dehydrogenase concentration. Reaction conditions were 50 mM Tris/HCl buffer, pH 7.6, containing 500 μM NAD⁺ at 25 °C. The concentration of active CRBP at each reaction mixture was twice that of retinaldehyde. Employing the mass law equation and a \( K_d \) value for the binding of retinaldehyde to CRBP of 44 nM (see Fig. 1, panel B) the values for the percentage of total retinaldehyde bound to CRBP at each increasing retinaldehyde concentration point are: 74, 86, 92, 95, 97, 98, 99, 99, and 99, respectively.

Fig. 3. Dehydrogenation of retinaldehyde by the isozyme E2 of human aldehyde dehydrogenase in the absence or presence of CRBP. The reaction conditions were the same as described in the legend to Fig. 2. Plot of production of retinoic acid in the absence (circles) and presence (triangles) of CRBP.

Table III

| Step | Propionaldehyde IU | Retinaldehyde IU | Retinaldehyde/propionaldehyde × 100 | Enzyme ID |
|------|---------------------|------------------|------------------------------------|-----------|
| 5A   | Inactive            | Inactive         |                                    |           |
| b    | 7.0                 | Trace            |                                    | E2        |
| 5B   | 14.4                | 7.8              | 54.2                               | E1        |
| a    | 5.8                 | 2.8              | 48.4                               | E1        |

Purification and Characterization of the Enzyme Active with Retinaldehyde Which Did Not Bind to DEAE-Sepharose—It was found that both retinaldehyde and propionaldehyde activities which did not bind to DEAE-Sepharose (see step 4A in Table II) bound to 5′-AMP Sepharose in the same conditions as aldehyde dehydrogenase (Table III). The enzyme also behaved like E1 and E2 isozymes during elution. 1 M NaCl at pH 6.0 eluted the propionaldehyde-active enzyme (with almost no retinaldehyde activity) which on isoelectric focusing gels could be identified as the E2 isozyme. Elution from 5′-AMP Sepharose with NAD⁺ at pH 8.0 resulted in the recovery of most of the retinaldehyde activity and the remainder of propionaldehyde activity. As in the case of the E1 isozyme, the remainder was eluted with high salt and NADH. On isoelectric focusing both were identified as the E1 isozyme by staining with either propionaldehyde or...
Metabolism of Retinaldehyde and Other Aldehydes

### Table IV

Comparison of E1 which binds to DEAE-Sephadex with E1(4A), which passes through DEAE

| Property | E1 | E1(4A) |
|----------|----|--------|
| Absorption to DEAE (conditions as described) | A* | NA* |
| 5'-AMP | SA | SA |
| Isoelectric point | 5.3 | 5.3 |
| Specific activity (propionaldehyde)* | 0.6* | 0.8* |
| Specific activity (retinaldehyde)* | 0.06* | 0.3* |
| Retinaldehyde/propionaldehyde activity ratio | 0.1* | 0.5* |
| Octanaldehyde/propionaldehyde activity ratio | 0.5 | 0.5 |
| \( K_m \) acetaldelyde (\( \mu M \)) | 50 | 50 |
| Retinaldehyde substrate inhibition | None* | Strong* |
| MW gradient gel (kDa) | 240 | 240 |
| \( M_r \) SDS gel (kDa) | 54 | 54 |

* Standard assay employing 1 mM propionaldehyde, 0.5 mM NAD^+ in 0.1 M sodium pyrophosphate buffer, pH 9.0, containing 1 mM EDTA at 25 °C.

** HPLC assay for retinaldehyde as described under “Experimental Procedures.” Both E1 and E1(4A) were inhibited to the same extent by disulfitram (35 \( \mu M \)), diethylenetramine (10 \( \mu M \)), and magnesium chloride (150 \( \mu M \)). The comparison of properties was done following purification of E1 and E1(4A) on 5'-AMP Sepharose.

retinaldehyde. No other protein bands were detected. This E1 (separated in step 4A of DEAE-Sephadex and hence denoted as E1(4A)) differed from the E1 isozyme in retinaldehyde to propionaldehyde activity ratio which was about four times higher than that of the E1 isozyme. Its specific activity with propionaldehyde was also 25% higher (Table IV). Determination of its \( K_m \) value with retinaldehyde was attempted (Fig. 4). Unlike retinaldehyde activity of E1 isozyme (see Fig. 2) E1(4A) was subject to pronounced substrate inhibition with retinaldehyde. Although \( K_m \) and \( V_{\text{max}} \) values could not be accurately determined from data of Fig. 4, because of rapid curvature due to substrate inhibition, the results suggest \( K_m \) of about 1.6 \( \mu M \) and \( V_{\text{max}} \) of about 1.4 \( \mu M \) mol/min/mg. Both of these values are larger than those determined for the E1 isozyme. As shown in Table IV, other kinetic and physicochemical properties of E1(4A) were indistinguishable from those of E1. Structural comparison of E1 and E1(4A), by peptide mapping resulted in identical, completely superimposable peptide maps (Fig. 5).

Additional Experiments—Further investigation of DEAE elutes of liver proteins demonstrated that a large amount of protein with molecular mass of about 15,000 Da (which might have been CRBP) was eluted from DEAE during column loading and washing in the same fractions as the retinaldehyde-active E1(4A) enzyme. In our previous experiments, loss of propionaldehyde activity of 7–10% occurred during loading and washing of DEAE-Sephadex columns when human livers with no history of alcoholism were used, which compares with the results presented in step 4A of Table II. It is of interest to note that the loss of E1(4A) on DEAE-Sephadex, as measured by loss of propionaldehyde activity, was very small when purification was carried out from livers of one male and one female alcoholic (0.5 and 1.5%, respectively).

DISCUSSION

The procedure employed for purification of retinaldehyde dehydrogenase was similar to that used for purification of aldehyde dehydrogenases with additional steps included to analyze fractionated proteins with properties which differ from those of aldehyde dehydrogenase (Tables I and II). It was found that retinaldehyde dehydrogenase activity copurified with aldehyde dehydrogenase in both liver and kidney. Four livers and three kidneys (all from different Caucasian individuals) showed similar enzyme activity distribution in both of these tissues. Isoelectric focusing activity gels for liver and kidney extracts at different purification stages developed with retinaldehyde and propionaldehyde as substrates were also identical. During enzyme purification from both tissues (except in the case of aldehyde oxidase where propionaldehyde activity could not be determined by the assay employed) propionaldehyde activity paralleled retinaldehyde activity. Therefore, we state with confidence that NAD^+-linked retinaldehyde dehydrogenase in extracts of human liver and kidney is associated in small part with aldehyde oxidase and in large part with aldehyde dehydrogenase of broad substrate specificity. We also conclude that there is no separate or special retinaldehyde dehydrogenase, with detectable catalytic activity, in extracts of kidney that is absent from extracts of liver; aldehyde dehydrogenase composition of both tissues is also the same. No evidence could be detected for retinaldehyde dehydrogenases distinct from known aldehyde dehydrogenases.

The ratios of retinaldehyde to propionaldehyde activity varied greatly with the isozymes. The major retinaldehyde activity comprising 2/3 of the total starting was found to be associated with the E1 isozyme of human aldehyde dehydrogenase. Its Michaelis constant for retinaldehyde was low and so was its maximal velocity. The \( K_m \) values agreed with those previously determined (12, 13); the maximal velocity was, however, much lower than that previously reported. The maximal velocity of the E1 isozyme with retinaldehyde as substrate at pH 9.0 was only about 12% of its maximal velocity with propionaldehyde. However, the enzyme occurs at a large concentration in human liver (about 1 g/kg); thus, an average human liver of 1.4 kg should be capable of metabolizing about 38 \( \mu M \) min of retinaldehyde at maximal velocity determined here to be 0.027 \( \mu M \) min/mg at pH 7.6.

The E2 isozyme was also found to have some activity with retinaldehyde. Its Michaelis constant for retinaldehyde was similar to that of the E1 isozyme, the maximal velocity, however, was considerably lower than that of the E1 isozyme. However, since this enzyme occurs in human livers at protein concentrations similar to that of the E1 isozyme, even at this low velocity 1.5 \( \mu M \) min of retinoic acid could be produced by this enzyme from retinaldehyde by an average human liver. The activity of the E3 isozyme with retinaldehyde has been found to be so small that it could not be accurately determined via HPLC. Thus, the E1 isozyme of human aldehyde dehydrogenase appears to be a major contributor to retinaldehyde metabolism in human liver and kidney.

Retinaldehyde is inherently unstable and is known to be present in tissues in a bound form, bound inside the cell to CRBP. Utilization of CRBP-bound retinaldehyde by retinalde-
Hyde dehydrogenase has been described (14) as a distinguishing characteristic for retinaldehyde dehydrogenases. It was important, therefore, during this investigation to find out if aldehyde dehydrogenases could utilize retinaldehyde-bound to CRBP. Human CRBP purified from liver was used for studying retinaldehyde activity of the E1 and E2 isozymes. As shown in Fig. 2, CRBP-bound retinaldehyde is utilized by the E1 isozyme as substrate. What makes this experiment especially convincing is that the E2 isozyme apparently cannot utilize CRBP-bound retinaldehyde (Fig. 3), only free retinaldehyde can be utilized by the E2 isozyme. Thus, the E1 isozyme exhibits features previously attributed to specific retinaldehyde dehydrogenases. In view of the above results, it can be stated with confidence that retinaldehyde dehydrogenase 1 (14, 15) and E1 are the same enzyme.

The second major retinaldehyde activity (constituting about 15–20% of starting retinaldehyde activity, Table II) was eluted during washing of DEAE-Sephadex (step 4A in Table II) and called E1(4A), because of its similarity to the E1 isozyme (Table IV). Although E1(4A) appeared to be the same as the E1 isozyme on isoelectric focusing gels and in behavior on 5′-AMP Sepharose, as well as in the majority of properties (see Table IV), its retinaldehyde:propionaldehyde activity ratio was considerably higher, about four times higher than that of the E1 isozyme (Table III). The ratio of retinaldehyde to propionaldehyde activity was determined at 20 μM retinaldehyde where substrate inhibition is considerable (see Fig. 4); thus this ratio is even higher at lower retinaldehyde concentrations. The maximal velocity of 1.4 μmol/min/mg, obtained by extrapolation of data in Fig. 4, is higher than those reported for rat retinaldehyde dehydrogenases (14, 15). The enzyme was also subject to substrate inhibition with retinaldehyde (Fig. 4), which was not observed with the E1 isozyme (Fig. 2). Thus, it appeared that this enzyme might be the human equivalent of animal retinaldehyde dehydrogenases. However, when E1(4A) was subjected to careful peptide mapping to compare it with the E1 isozyme no structural differences could be detected (Fig. 5). If there are only a few substitutions, they may not be visible in tryptic peptide maps, where symmetrical peaks usually represent mixtures of several peptides. Thus, the question of primary structure of E1(4A) cannot be finally resolved without complete sequencing of the E1(4A) protein. This may be important in view of the fact that further investigations indicated that E1(4A) was present at much lower concentrations in DEAE-Sephadex eluates from livers of alcoholics. There are also other possibilities. The enzyme that does not attach to DEAE might be E1 which is bound to CRBP or some other small ligand. Large amounts of protein of molecular mass of about 15,000 Da was visualized in chromatography eluates containing E1(4A). It has been recently demonstrated that binding of metals can alter substrate specificity of an enzyme involved in the methionine salvage pathway (39). Thus, higher activity with retinaldehyde, achieved in other mammals by gene duplication, may have been achieved in humans by ligand binding. E1 is extremely unstable and sensitive to atmospheric oxygen. If E1(4A) represents the E1 which is bound to CRBP, its higher specific activity with propionaldehyde could be the result of the protecting effect by CRBP. Similarity of E1(4A) to E1 isozyme, however, argues against E1(4A) being a specific retinaldehyde dehydrogenase. Even if some structural differences are detected, E1(4A) has to be considered as a variant of the E1 isozyme (see Table IV) and is, therefore, an aldehyde dehydrogenase of broad substrate specificity.

The third major activity was associated with aldehyde oxidase as previously observed by Chen and Juchau (40) in rat conceptual homogenates. This enzyme could be easily separated from dehydrogenases by chromatography on CM-Sephadex. In one liver less than 1% of total propionaldehyde or octanaldehyde activity separated on DEAE from E1, E2, and E3 isozymes, suggesting that it may be yet another, hitherto unidentified, aldehyde dehydrogenase; no activity with retinaldehyde was detected in this fraction. Retinaldehyde dehydrogenase 2, which has been reported to occur in response to developmental stimuli (16, 17) has not yet been described in human organism. This enzyme has been recently cloned from mouse developing eye (16) and rat testis (17). Published amino acid sequence and properties reported (17) are so similar to those of aldehyde dehydrogenase that there is no reason to
assume that even this enzyme is not an aldehyde dehydrogenase of broad substrate specificity, since it can apparently utilize short and long chain aldehydes as substrates (17).

REFERENCES
1. Gudas, L. (1994) J. Biol. Chem. 269, 15399–15402
2. Niederreither, K., Subbarayan, V., and Chambon, P. (1999) Nat. Genet. 21, 444–448
3. Hong, W. K., and Itri, L. M. (1994) in The Retinoids Biology, Chemistry and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 597–630, Raven Press, New York
4. Goodman, A. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 95, 7240–7244
5. Niederreither, K., Subbarayan, V., and Chambon, P. (1999) Nat. Genet. 21, 444–448
6. Chinese, W. K., and Itri, L. M. (1994) in The Retinoids Biology, Chemistry and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 597–630, Raven Press, New York
7. Wang, X. D., Russell, R. M., Liu, C., Stickel, F., Smith, D. E., and Krinsky, N. L. (1996) J. Biol. Chem. 271, 26490–26498
8. Bliss, A. F. (1951) Arch. Biochem. Biophys. 31, 197–204
9. Futterman, S. (1962) J. Biol. Chem. 237, 677–680
10. Elder, T. D., and Topper, Y. J (1962) Biochim. Biophys. Acta 64, 430–437
11. Lee, M-O., Manthey, C. L., and Sladek, N. E. (1991) Biochem. Pharmacol. 42, 1279–1285
12. Dockham, P. A., Lee, M-O., and Sladek, N. E. (1992) Biochem. Pharmacol. 43, 2453–2469
13. Yoshida, A., Hsu, L. C., and Dave, V. (1992) Enzyme 46, 239–244
14. Posch, K. C., Burns, R. D., and Napoli, J. L. (1992) J. Biol. Chem. 267, 19676–19682
15. Labreque, J., Bhat, P. V., and Lacroix, A. (1993) Biochem. Cell Biol. 71, 85–89
16. Zhao, D., McCaffery, P., Ivins, F. C., Hogan, P., Chin, W. W., and Drager, U. C. (1996) Eur. J. Biochem. 240, 15–22
17. Wang, X., Penzes, P., and Napoli, J. L. (1996) J. Biol. Chem. 271, 16288–16293
18. Tottrn, S. O. C., Petterson, H., and Kiessling, K.-H. (1973) Biochem. J. 135, 577–586
19. Cao, Q.-N., Tu, G.-C., and Weiner, H. (1989) Biochem. Pharmacol. 38, 77–83
20. Dunn, T. S., Koleske, A. J., Lindahl, R., and Pitot, H. C. (1989) J. Biol. Chem. 264, 13057–13065
21. Kathmann, E. C., and Lipsky, J. (1997) Biochem. Biophys. Res. Commun. 236, 527–531
22. Bhat, P. V., Labreque, J., Boutin, J.-M., Lacroix, A., and Yoshida, A. (1995) Gene (Amst.) 166, 303–306
23. Graham, C., Hodin, J., and Wistow, G. (1996) J. Biol. Chem. 271, 15623–15628
24. Greenfield, N. J., and Pietruszko, R. (1977) Biochim. Biophys. Acta 483, 35–45
25. Forte-McRobbie C. M., and Pietruszko, R. (1986) J. Biol. Chem. 261, 2154–2163
26. Kury, G., Ambroziak, W., and Pietruszko, R. (1989) J. Biol. Chem. 264, 4715–4721
27. Ambroziak, W., and Pietruszko, R. (1991) J. Biol. Chem. 266, 13011–13018
28. Lineweaver, H., and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–667
29. Yun, S. L., and Switzer, C. H. (1977) Biochim. Biophys. Acta 480, 324–337
30. Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
31. Goa, J. (1953) Scand. J. Clin. Lab. Invest. 5, 218–222
32. Hempel, J., Reed, D., and Pietruszko, R. (1982) Clin. Exp. Res. 6, 417–425
33. Ong, D. E., and Chytíl, P. (1978) J. Biol. Chem. 253, 828–832
34. Fex, G., and Johannesson, G. (1982) Biochim. Biophys. Acta 714, 536–542
35. Ong, D. E. (1982) Cancer Res. 42, 1033–1037
36. Cogan, U., Kopelman, M., Mukady, S., and Shnitzky, M. (1976) Eur. J. Biochem. 65, 71–78
37. Malpelí, G., Stoppini, M., Zapponi, M. C., Folli, C., and Berni, R. (1995) Eur. J. Biochem. 228, 486–493
38. Venteicher, R., Mope, L., and Yonetani, T. (1977) in Alcohol and Aldehyde Metabolizing Systems (Thurman, R. G., Williamson, J. R., Drott, H. R., and Chance, B., eds) Vol. 2, pp. 157–166, Academic Press, New York
39. Dai, Y., Wensink, P. C., and Abeles, R. H. (1999) J. Biol. Chem. 274, 1193–1195
40. Chen, H., Numkung, M. J., and Juchau, M. R. (1995) Biochem. Pharmacol. 50, 1257–1264