Biochemical Pharmacology, Vol. 30, No. 14, pp. 1931–1939, 1981.
Printed in Great Britain.

0006-2932/81/141931-09 $02.00/0, 1981.

STUDIES ON NADPH-DEPENDENT CHLORAL HYDRATE REDUCING ENZYMES IN RAT LIVER CYTOSOL

MIKIKO IKEDA, MIHO EZAKI, SUSUMU KOKEGUCHI and SHINJI OHMORI*
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1, Okayama 700, Japan

(*Received* 8 *July* 1980; *accepted* 31 *December* 1980)

Abstract—Chloral hydrate, a sedative hypnotic and also a major metabolite of trichloroethylene in higher animals, is reduced to trichloroethanol by liver extracts. The reducing activity in rat liver cytosol could be separated into four fractions [one NADH- (F_1) and three NADPH-dependent (F_2, F_3 and F_4)] by DEAE–cellulose column chromatography. By several procedures, F_2 was purified over 1000-fold and F_4 was purified over 600-fold from liver cytosol. As judged from polyacrylamide gel electrophoresis performed with and without the addition of sodium dodecylsulfate, the final preparations were essentially homogeneous. They differed in molecular weight, mobility on polyacrylamide gel electrophoresis, pH optimum, substrate specificity, and sensitivity to inhibitors. The molecular weights were estimated to be 36,000 and 32,500 for F_2 and F_4, respectively, by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. The estimation of molecular weights by thin-layer gel chromatography indicated that the enzymes were monomers. An examination of over thirty substrates revealed that both enzymes catalyzed the reduction of long-chain aliphatic, alicyclic and aromatic aldehydes as well as halogenated acetaldehyde. The F_2 enzyme acted on D-glucuronate, indicating that it was identical to the aldehyde reductase recently reported by other workers (L-gulonate:$NADP^+$ 1-oxidoreductase EC 1.1.1.19). The F_4 enzyme, on the other hand, preferentially acted on C_{24} 3-ketosteroids.

Chloral hydrade (CH), a known sedative hypnotic, has been shown to be an initial stable metabolite of trichloroethylene in higher animals [1]. Subsequent metabolism of CH in animals is either a reduction to trichloroethanol [2] or an oxidation to trichloroacetic acid [3], of which the former predominates [4, 5]. The reduction of CH to trichloroethanol is catalyzed by hepatic alcohol dehydrogenase of horse [1], rabbit [2], and human [6]. Tabakoff *et al.* [7] have postulated the existence of another enzyme in rat brain and liver that is capable of reducing CH. Previously, we studied the metabolism of trichloroethylene in rat liver and found that the reduction of CH is catalyzed by a cytosolic fraction [5]. In addition, the cytosolic enzymes were separated into four fractions, one NADH- and three NADPH-dependent, by DEAE–cellulose column chromatography [5].

In this paper, purification and characterization of two NADPH-dependent enzymes (F_2 and F_4) that were responsible for the major CH-reducing activity in the total extract, and their affinities with halogenated acetaldehyde, are described. A variety of aldehydes and ketones other than the halogenated acetaldehyde were also tested as substrates for both enzymes.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade. Pyridine nucleotides were purchased from the Oriental Yeast Co. Ltd. (Tokyo). Long-chain aliphatic aldehydes, aromatic aldehydes, and ketones were obtained from the Tokyo Chemical Industry Co. Ltd. (Tokyo). Octopamine, norepinephrine, tyramine, tryptamine, serotonin, testosterone, progesterone and daunorubicin were purchased from the Sigma Chemical Co. (St. Louis, MO). Biogenic aldehydes from biogenic amines were prepared by the method of Tabakoff *et al.* [8], and their concentrations in the preparations were determined spectrophotometrically using aldehyde dehydrogenase [8]. CM-Sephadex C-50 and Sephadex G-50 and G-100 were obtained from Pharmacia Fine Chemicals Ltd. (Uppsala, Sweden), and DEAE–cellulose (DE-52) was from the Whatman Co. (Maidstone, U.K.).

Purification of the CH-reducing enzymes from rat liver

Male Wistar strain albino rats (200–300 g body weight) were used. The livers were removed immediately after decapitation and processed as follows or, otherwise, stored frozen at −20° until use. They (100–150 g) were homogenized, using a Waring blender, in 3 vol. of 5 mM Tris–phosphate buffer (pH 8.0) containing 5 mM 2-mercaptoethanol (MSH), which is referred to as buffer A. The procedures for extraction, centrifugation, and ammonium sulfate extraction of the CH-reducing enzymes were similar to those described by Tulsiani and Touster [9]. The precipitate obtained from 0.4 to 0.7 saturated ammonium sulfate fractionation was dissolved in a small volume of buffer A, and then dialyzed against buffer A (10 litres) overnight. The desalted fraction was centrifuged at 140,000 *g* for 60 min, and the supernatant fraction was applied to a DE-52 column (45 × 4 cm) previously equilibrated with buffer A. The washing and the elution were done as described by Tulsiani and Touster [9], except that the pH of the buffer was adjusted to 8.0, and the elution was performed with a linear salt gradient (1 litre of buffer A and 1 litre of 0.1 M NaCl in

* Author to whom correspondence should be addressed.

buffer A). Each 20-ml fraction was monitored at 280 nm for protein, and the CH-reducing activity was assayed spectrophotometrically as described below. Fractions containing the CH-reducing activity (F_2 and F_4 in Fig. 1) were pooled, and ammonium sulfate was added to each fraction to 0.8 saturation. The precipitate was collected by centrifugation at 14,000 *g* for 30 min. After dissolving the precipitate in a small amount of 5 mM potassium phosphate buffer (pH 7.0) containing 5 mM MSH (referred to as buffer B), each fraction was purified further as described below.

The F_2 protein in buffer B solution was desalted by dialysis for 3 hr *in vacuo* against buffer B (1 litre × 3). The desalted fraction was applied to a CM-Sephadex C-50 column (45 × 2 cm) previously equilibrated with buffer B and was washed with buffer B (about 300 ml) until A_{280} of the effluent became zero. The enzyme was eluted with a linear salt gradient (300 ml of buffer B and 300 ml of 0.15 M NaCl in buffer B). The flow rate was adjusted to 30 ml/hr. Each 10-ml fraction was monitored for protein and the activity was assayed. The fractions containing the CH-reducing activity were combined, dialyzed against buffer B (5 litres × 3) for 3 hr, and then applied to a short DE-52 column (8 × 2.5 cm) from which the enzyme was eluted with a linear salt gradient (150 ml of buffer B and 150 ml of 0.05 M NaCl in buffer B). The enzyme-rich fractions were concentrated by dialysis *in vacuo* against buffer B. This final preparation was used for kinetic studies and gel chromatographic studies.

The buffer B solution of F_4 protein obtained by DE-52 column chromatography was similarly desalted, applied to a CM-Sephadex C-50 column (45 × 2 cm) previously equilibrated with buffer B, and eluted as described above. The fractions that contained most of the CH-reducing activity were combined, dialyzed, and applied to a short DE-52 column (8 × 2.5 cm) from which the enzyme was eluted with a linear salt gradient (150 ml of buffer B and 150 ml of 0.1 M NaCl in buffer B). The enzyme-rich fractions were concentrated by dialysis *in vacuo* and were applied to a Sephadex G-50 column (90 × 2.3 cm) previously equilibrated with buffer B. The enzyme-rich fractions were combined and concentrated as described above. This final preparation was used for kinetic studies and gel chromatographic studies.

Enzyme assay

The CH-reducing activity was assayed spectrophotometrically in a mixture containing NADPH or NADH (0.16 mM), enzyme solution, and CH (5 mM) in 50 mM potassium phosphate buffer (pH 7.0), in a total volume of 1.0 ml. The spectrophotometric assay was performed measuring the absorbance at 340 nm, at 25°, using a Hitachi 124 spectrophotometer. One unit of enzyme was defined as activity that caused a change in the absorbance of 0.01/min.

Oxidation of alcohols was also assayed spectrophotometrically at 340 nm using $NADP^+$ in the following mixture (1.0 ml): 50 mM glycine–NaOH (pH 9 ~ 10), 50 mM Tris–phosphate (pH 7.5 ~ 9) or potassium phosphate buffer (pH 6 ~ 7.5), 0.5 mM $NADP^+$, 100–400 mM alcohol and enzyme.

Stoichiometry

The stoichiometry was determined with 0.08 to 0.16 mM NADPH and 5 mM CH in 50 mM potassium phosphate buffer (pH 7.0) at 25°. The reaction was initiated with a large excess of enzyme (F_2 or F_4) to reach equilibrium within 1 hr. Equilibrium concentrations of $NADP^+$ were calculated from the changes in 340 nm absorbance. The reaction product was identified and quantitated by ECD-gas–liquid chromatography as described previously [5].

Analytical gel electrophoresis

Polyacrylamide gel (7.5%) electrophoresis was performed at 0–4° with an electric current of 2 mA/gel at pH 8.3 [10]. Polyacrylamide gel (10%) electrophoresis in the presence of sodium dodecylsulfate (0.4%) was performed according to the method of Laemmli [11]. Activity staining on the polyacrylamide gel was carried out by the method described by Tulsiani and Touster [9].

Gel filtration

The molecular weights of F_2 and F_4 enzymes were estimated by thin-layer gel chromatography with Sephadex G-100 (superfine) according to the method of Determann and Michel [12]. The calibration curve was constructed by plotting the logarithm of molecular weight against R_{cyt}, where R_{cyt} was defined as mobility relative to cytochrome *c*.

Protein

Protein was determined by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

RESULTS

Enzyme purification

The F_2 fraction of the CM-Sephadex C-50 column chromatography comprised a large amount of a single protein and several minor proteins. The latter could be easily removed by subsequent DE-52 column chromatography. The activity of the F_4 fraction (Fig. 1a) was further separated into two peaks on a CM-Sephadex C-50 column (Fig. 1b), and the major peak was purified through gel filtration after a short DE-52 column, which effectively removed contaminating proteins. As summarized in Tables 1 and 2, F_2 was purified about 1000-fold with a 27.5 per cent yield from the individual activity in the crude extract, and F_4 was purified about 600-fold with a 41 per cent yield. Polyacrylamide gel electrophoresis of F_2 and F_4 at pH 8.3 [10] showed only one band when stained for either proteins or enzyme activity (Fig. 2).

Molecular weight determination

From the value of R_{cyt} on a thin layer with Sephadex G-100, the molecular weights of F_2 and F_4 were calculated to be 31,000 and 32,500 respectively (Fig. 3a). The molecular weights of F_2 and F_4 were calculated to be 36,000 and 32,500, respectively, from the electrophoresis carried out in the presence of sodium dodecylsulfate (Fig. 3b). Since the molecular weights found by these two methods were essentially identical, both F_2 and F_4 appear to have been single peptides.

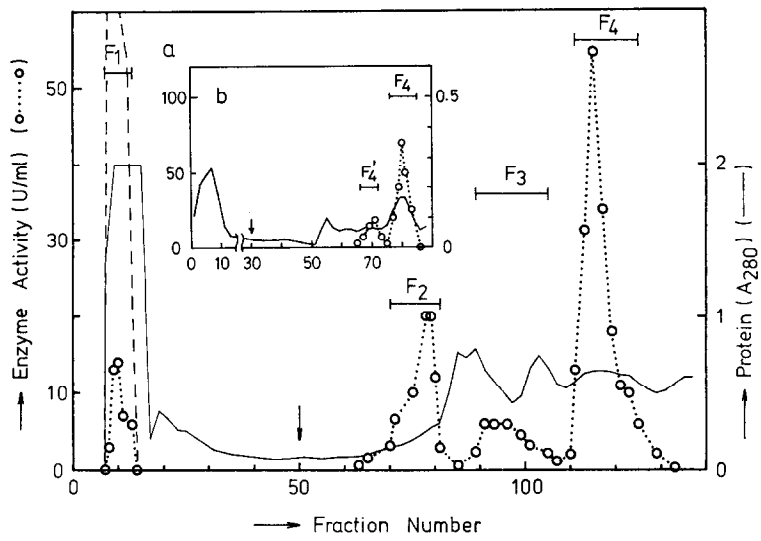

Fig. 1. Separation of the CH-reducing enzymes from rat liver on a DE-52 column (a) (45 × 4 cm) and that of F_4 on a CM-Sephadex C-50 column (b) (45 × 2 cm). The flow rate was 30 ml/hr for both columns. The arrow indicates the starting of a linear salt gradient. The enzyme activity in each fraction (20 ml for a DE-52 column and 10 ml for a CM-Sephadex column) was assayed at pH 7.0 using CH (5 mM) as substrate and NADH (– – – –) or NADPH (○· · · ·○) as cofactor as described in the text. Protein was monitored from A_{280}.

Table 1. Purification of rat liver CH-reducing enzyme (F_2)*

Step	Total volume (ml)	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg)	Enzyme recovery (%)	Purification (-fold)
(1) Supernatant	289	9248	4680*	0.506	100	1
(2) $(NH_4)_2SO_4$ fr.	61	3642	3150	0.975	67.3	1.9
(3) DE 52 column	335	37.9	3518	92.9	75.2	184
(4) CM-Sephadex C-50 column	79.5	3.02	2110	698	45.1	1380
(5) Short DE 52 column	20	2.59	1288	497	27.5	983

* Enzyme activity at each step was assayed at pH 7.0 using CH (5 mM) and NADPH (0.16 mM).
† The apparent total activity, 17,340 units, was multiplied by the "fraction" of F_2 activity (0.27) found in the DE 52 column chromatography. The "fraction", in turn, is that for the total of the activity eluted from the column.

Table 2. Purification of rat liver CH-reducing enzyme

Step	Total volume (ml)	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg)	Enzyme recovery (%)	Purification (-fold)
(1) Supernatant	289	9248	7509†	0.812	100	1
(2) $(NH_4)_2SO_4$ fr.	61	3642	5053	1.39	67.3	1.7
(3) DE 52 column	294	153	6152	40.2	81.9	49.5
(4) CM-Sephadex C-50 column	158	20.8	6401	308	85.2	379
(5) Short DE 52 column	20	17.8	4042	227	53.8	280
(6) Sephadex G-50 column	38	6.3	3077	488	41.0	601

*Enzyme activity at each step was assayed at pH 7.0 using CH (5 mM) and NADPH (0.16 mM).
† The apparent total activity, 17,340 units, was multiplied by the "fraction" of F_4 activity (0.52) found in the DE 52 column chromatography and the 'fraction' of F_4 activity (0.83) in the CM-Sephadex C-50 column chromatography. The "fraction" or 'fraction', in turn, is that for the total of the activity eluted from the respective column.

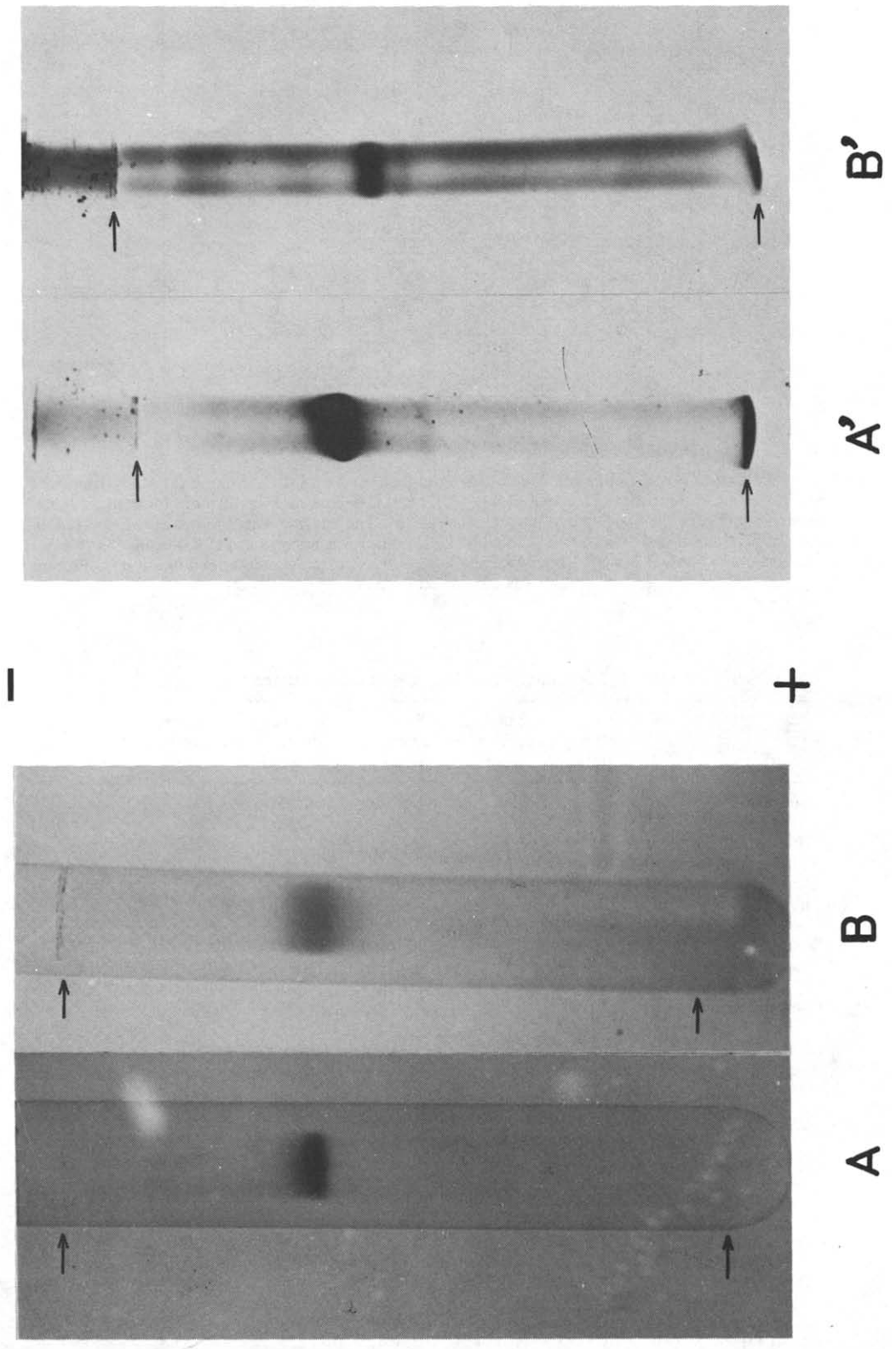

Fig. 2. Disc polyacrylamide gel electrophoresis of F_2 and F_4. Approximately 40 μg of protein was applied per gel. Electrophoresis was performed at pH 8.3 as described by Davis [10]. The photographs were taken after staining the gel with Coomassie brilliant blue (A and B) and after staining activity as described by Tulsiani and Touster [9] (A′ and B′). Arrows indicate front and starting point of gel.

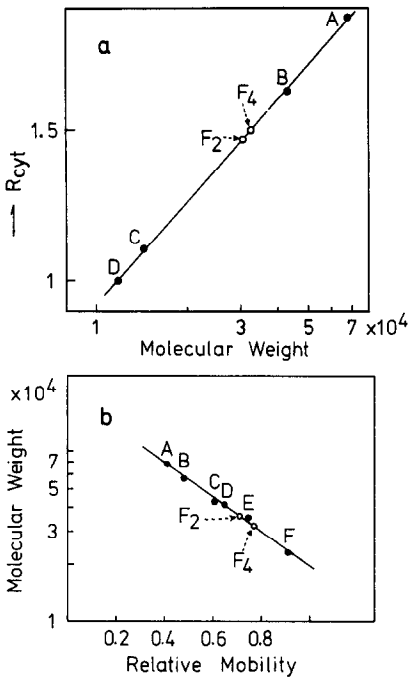

Fig. 3. Determination of molecular weights of F_2 and F_4 by (a) thin-layer gel chromatography and (b) by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Panel a: Thin-layer gel chromatography with Sephadex G-100 (superfine) was conducted as described by Determann and Michel [12]. Standard proteins for estimation of molecular weight were bovine serum albumin (A), ovalbumin (B), lysozyme (C), and cytochrome *c* (D). Potassium phosphate buffer (10 mM, pH 7.0) containing 5 mM MSH was used as equilibrating and developing medium. Panel b: Polyacrylamide gel (10%) electrophoresis was conducted in the presence of 0.4% sodium dodecylsulfate as described by Laemmli [11]. Standard proteins for estimation of molecular weight were bovine serum albumin (A), rabbit muscle pyruvate kinase (B), ovalbumin (C), horse liver alcohol dehydrogenase (D), pig heart lactate dehydrogenase (E), and trypsin (F).

pH Optima and buffer effects

The effects of pH on the enzyme activities are shown in Fig. 4. The pH optima for F_2 catalysis of CH and D-glucuronate reduction were between 6.0 and 6.5 (Fig. 4a). Although the pH optimum for F_4 reduction of CH and *m*-nitrobenzaldehyde was about pH 6.5, the optimum for *p*-nitroacetophenone was about pH 5.5 (Fig. 4b). Both F_2 and F_4 exhibited pH-dependent dehydrogenase activity, as expected from properties common to alcohol dehydrogenases, when alcohols were used as substrate (Fig. 4, c and d).

Stoichiometry for CH

At equilibrium, the ratio of $NADP^+$ to trichloroethanol formed was 0.999 ± 0.019 for F_2 and 1.004 ± 0.005 (mean ± S.D., N = 3) for F_4, which confirmed a 1:1 stoichiometry for the reduction.

Substrate specificity

A number of xenobiotic substances and naturally occurring compounds were tested as substrates for F_2 and F_4. The results are shown in Table 3.

Xenobiotic substances. Among halogenated acetaldehydes, bromal hydrate was the preferred substrate for both of the enzymes, and dichloroacetaldehyde was a better substrate for F_2 than CH or monochloroacetaldehyde. The K_m values for NADPH measured at the saturation level of CH (10 mM) were 5.3 μM for F_2 and 14 μM for F_4. Short-chain aliphatic aldehydes (C_1 to C_3) were poorer substrates than halogenated acetaldehydes for both enzymes. Longer-chain aliphatic aldehydes were better substrates than halogenated acetaldehydes for both enzymes, except for bromal hydrate. Alicyclic aldehyde was also reduced by the enzymes. The aromatic aldehydes, benzaldehyde, *m*-nitrobenzaldehyde, *p*-chlorobenzaldehyde, and pyridine-3-aldehyde, were preferred substrates. Several aliphatic and aromatic ketones were reduced by F_4, but little activity was observed for F_2; aliphatic ketones such as acetone and methyl ethyl ketone were poorer substrates than aromatic ketones such as *p*-nitro- and *p*-chloroacetophenone. However, 2-, 3-, or 4-benzoylpyridine and benzophenone were not reduced by either F_2 or F_4. Cyclohexanone was reduced by F_4.

Naturally occurring compounds. Naturally occurring keto compounds were tested as possible alternative substrates for both enzymes. F_2 readily reduced D-glucuronate, D-glucuronolactone and DL-glyceraldehyde, but a lower level of activity was exhibited toward D-glucose, D-galactose and arabinose. On the other hand, F_4 exhibited no detectable activity toward the substrates described above except for DL-glyceraldehyde. Biogenic aldehydes derived from serotonin, tryptamine, tyramine, octopamine and norepinephrine were preferred substrates for both enzymes. Among substrates tested, C_{24} 3-keto-

Table 4. Effect of inhibitors on F_2 and F_4 of the rat liver*

Inhibitors	Conc (mM)	% Inhibition F_2	% Inhibition F_4
$CuSO_4$	0.1	35.7	95
$HgCl_2$	0.005	26.8	21.1
	0.01	100	100
LiCl	1.0	0	0
KF	1.0	23.2	0
	5.0	96.4	—†
KCN	1.0	10.7	0
Phenobarbital	1.0	53.6	0
	5.0	88.4	26.5
2-Mercaptoethanol	1.0	−19.6	−10.6
	5.0	−7.1	−8.0
Iodoacetic acid	1.0	17.9	7.08
	5.0	46.4	44.2
p-Chloromercuribenzoate	0.1	65.2	72.0
	1.0	100	100
Pyrazole	5.0	0	0
	10.0	0	0

* Aqueous solutions of the inhibitors were added to the reaction mixture with the final concentration shown above. The system was preincubated at 25° for 2 min prior to the addition of substrate. The remaining activity was assayed for reduction of D-glucuronate (1 mM) with F_2 or for reduction of 3-ketocholanic acid (50 μM) with F_4.

† Not tested.

Table 3. Substrate specificity of F_2 and F_4 purified from rat liver*

Substrates	F_2				F_4			
	K_m (mM)	V_{max} (μmoles·min^{-1}·ml^{-1})	k_{cat} (sec^{-1})	k_{cat}/k_m (sec^{-1}·M^{-1})	K_m (mM)	V_{max} (μmoles·min^{-1}·ml^{-1})	k_{cat} (sec^{-1})	k_{cat}/k_m (sec^{-1}·M^{-1})
Acetaldehyde	ND*				ND			
Monochloroacethaldehyde	16.4	7.14	2.33	142	10.9	0.847	0.232	21.3
Dichloroacetaldehyde	3.32	18.8	6.12	1,842	10.6	2.77	0.760	71.7
Chloral hydrate	16.1	6.81	2.22	138	11.3	3.52	0.965	85.8
Bromal hydrate	0.29	9.75	3.18	10,949	0.43	3.67	1.01	2,351
Propionaldehyde	95.2	10.5	3.43	36	300	3.82	1.05	3.5
n-Butyraldehyde	18.5	6.10	1.99	108	60.5	3.19	0.874	14.4
iso-Butyraldehyde	9.92	5.62	1.83	185	28.6	3.14	0.861	301
n-Hexyaldehyde	2.30	12.1	3.98	1,730	6.13	1.96	0.537	87.6
n-Octylaldehyde	1.49	18.7	6.11	4,098	0.22	1.15	0.314	1,427
n-Decylaldehyde	0.60	5.56	1.81	3,022	0.144	2.03	0.556	3,861
Cyclohexene-4-carboxyaldehyde	2.70	10.0	6.35	2,350	2.12	1.60	0.437	206
Benzaldehyde	12.4	14.6	4.76	384	11.4	2.89	0.792	69.5
m-Nitrobenzaldehyde	1.62	5.50	1.79	1,106	0.44	3.17	0.87	1,977
p-Chlorobenzaldehyde	12.6	71.1	23.2	1,841	4.36	6.11	1.68	385
Pyridine-3-aldehyde	0.93	19.3	6.28	6,752	8.53	2.21	0.606	71.0
DL-Glyceraldehyde	8.09	11.9	3.87	478	113.5	3.77	1.03	9.11
D-Glucuronate	4.55	19.5	6.35	1,395	ND			
D-Glucuronolactone	6.63	6.39	2.08	314	ND			

D-Glucose	338	0.365	0.422	1.25	ND			
D-Galactose	285	0.189	0.219	0.768	ND			
D-Arabinose	351	0.472	0.546	1.56	ND			
Acetone	ND				+†			
p-Nitroacetophenone	±				2.80	3.14	0.861	308
p-Chloroacetophenone	±				2.96	0.492	0.135	45.6
Cyclohexanone	+‡				13.0	2.19	0.601	46.2
3-Ketocholanic acid					0.00722	4.40	0.121	167,590
Glycodehydrocholanic acid					0.020	6.64	0.182	91,000
p-Hydroxyphenylacetaldehyde	0.33	0.671	0.218	666	0.35	0.46	0.126	360
3,4-Dihydroxyphenylacetaldehyde	0.073	0.220	0.072	983	1.37	1.53	0.419	306
p-Hydroxyphenylglycolaldehyde	0.183	0.243	0.079	432	1.53	1.38	0.378	247
3,4-Dihydroxyphenylglycolaldehyde	0.577	1.41	0.458	822	2.50	4.49	1.23	492
Indoleacetaldehyde	0.170	0.208	0.068	398	4.73	5.91	1.62	342
5-Hydroxyindoleacetaldehyde	0.470	0.409	0.133	284	0.54	0.515	0.141	261
Daunorubicin	+§							
NADPH	0.0053				0.014			

* Various concentrations of substrates were added to the reaction mixture containing 0.16 mM NADPH, enzyme, and buffer at optimal pH (50 mM) with a total volume of 1.0 ml. Aromatic ketones were assayed at pH 5.5, 3-ketocholanic acid at pH 7.0, daunorubicin at pH 8.5, and other aldehydes at pH 6.5. The K_m values for NADPH were determined using 10 mM CH as substrate. When substrates slightly soluble in water were used, methanolic solutions were prepared, and methanol itself affected the enzyme activity up to 5% (v/v) in an incubation mixture. Incubation was conducted at 25°.

K_m and V_{max} values were determined by Lineweaver–Burk plots, and V_{max} values were converted to K_{cat} values by dividing by E_0. E_0 was determined by dividing the protein concentration by the assumed molecular weight of 36,000 (F_2) or 32,500 (F_4).

† Not detectable under our assay condition.

‡ Not linear as the result of Lineweaver–Burk plots.

§ Catalytic constants were not determined.

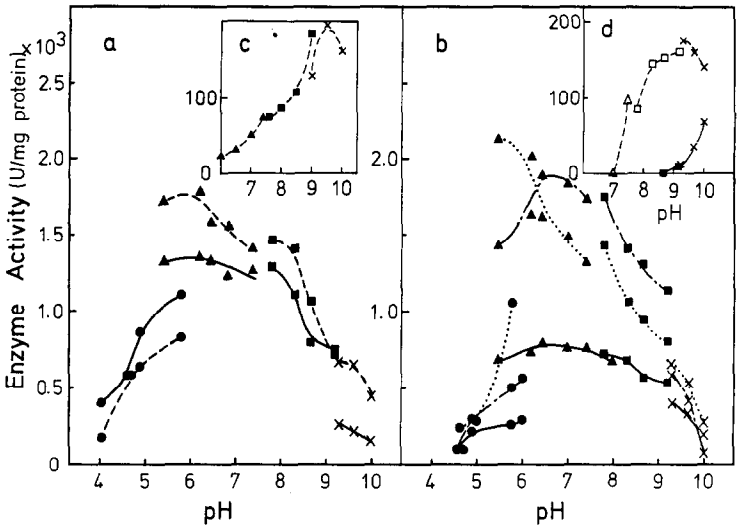

Fig. 4. Activities of F_2 and F_4 as a function of pH and the buffer effects. Activities of F_2 (left) and F_4 (right) were determined with NADP (H) as cofactor in each buffer (50 mM) as described in the text; sodium acetate buffer (●), potassium phosphate buffer (▲), Tris–phosphate buffer (■) and glycine–NaOH buffer (×) were used. Key:(———) represents the activity toward CH (10 mM), (– – –) toward D-glucuronate (1 mM), (— – —) toward *m*-nitrobenzaldehyde (1 mM), and F_4 activity toward oxidation of benzylalcohol (0.2 M, – – –) and toward *p*-nitroacetophenone (1 mM) in panels a and b. Panels c and d show F_2 activity toward oxidation of benzylalcohol (0.2 M, – – –), and ethylenebromohydrin (0.4 M, ——) respectively.

steroids were the best substrates for F_4. Testosterone and progesterone were also tested for F_4, but little activity was observed.

Inhibitors

A number of chemicals were tested for their abilities to inhibit F_2 and F_4, using D-glucuronate as substrate for the former and dehydrolithocholic acid (3-ketocholanic acid) for the latter (Table 4). *p*-Chloromercuribenzoate and Hg^{2+} were potent inhibitors of both enzymes. Phenobarbital was a potent inhibitor of F_2, but a less potent inhibitor of F_4. Pyrazole, a classical inhibitor of alcohol dehydrogenase, had no effect on either enzyme. MSH had a slight stimulatory effect on the enzymes. Most of the other inhibitors listed in Table 4 affected these enzymes to various extents.

Stability

The purified enzymes in concentrated states were stable for at least 1 week at 4°. Freezing, however, resulted in denaturation of the proteins.

DISCUSSION

The results in the present paper demonstrate that pyridine nucleotide-dependent reduction of CH in rat liver is due to at least four proteins (F_1 to F_4). Two of them (F_2 and F_4) could be distinguished by pH optimum, substrate specificity, inhibitor sensitivity, and molecular weight as determined by gel electrophoresis and thin-layer gel chromatography. Both enzymes acted more effectively on longer-chain aliphatic aldehydes than on short-chain aliphatic aldehydes, and aromatic aldehydes and aromatic ketones were good substrates. From these findings, both enzymes can be classified as the NADPH-dependent aldo-keto reductases according to Bachur [14]. The present study also demonstrates that halogenated acetaldehydes are substrates for F_2 and F_4, the enzyme activity depending on the number of substituted halogens and species of halogen atoms. Substitution with bromine markedly enhanced the affinity of the substrate for both enzymes. These results contrast with those observed using human liver alcohol dehydrogenase, where an increase in the number of chlorines on acetaldehyde resulted in a decrease in affinity [6].

In 1972, Erwin and Deitrich [15] reported that enzymes that catalyze aldehyde reduction in livers of Sprague–Dawley rats were eluted in three peaks, using a calcium phosphate gel-cellulose column, one peak being alcohol dehydrogenase (EC 1.1.1.1). Since then a number of studies of the enzyme have been published [16–20]. Felsted *et al.* [16] purified from rat liver cytosol an aldo-keto reductase designated as daunorubicin reductase, which was reported to be identical with L-gulonate:$NADP^+$ 1-oxidoreductase (EC 1.1.1.19). F_2 in the present study is most likely identical to daunorubicin reductase, as judged by characteristics such as substrate specificity, inhibitor sensitivity, and molecular weight. Later, workers in the same laboratory [21] reported the broad substrate range of the reductase and that daunorubicin was the only ketone reduced significantly by the enzyme. Our experiments gave the same results. Recently, two isozymes of aldehyde reductases, AR I and AR II, were isolated from mouse liver by Tulsiani and Touster [9]. AR II, which was purified to homogeneity, appears to be comparable to F_2 in rat liver, judging from substrate specificity, inhibitor sensitivity, and molecular weight. Sawada *et al.* [20] also reported the purification and the properties of reductases for aromatic

aldehydes and ketones from guinea pig liver. They separated enzyme activities toward pyridine-4-aldehyde into three fractions (AR 1, 2 and 3) by DEAE–cellulose column chromatography, and they purified AR 2 and 3 to homogeneity. On the basis of substrate specificity and inhibitor sensitivity, AR 3 is comparable to F_2. It is noteworthy that the best substrate for F_4 was C_{24} 3-ketosteroids as demonstrated in the present paper. Pietruszko and Chen [17] suggested that one of the aldehyde reductases from rat liver that were separated by Erwin and Deitrich [15] was 3α-hydroxysteroid dehydrogenase, catalyzing reversible oxido-reduction of 3α-hydroxysteroids of the A/B *cis* configuration. However, we obtained results contradictory to their findings, i.e. F_4 also catalyzed the oxido-reduction of 3α-hydroxysteroids of the A/B *trans* configuration (data not shown). The details in the oxido-reduction of 3α-hydroxysteroids, especially of bile acid analogues, by F_4 will be presented in a subsequent paper.

In the present work, the purification and characterization of two enzymes of the four fractions (F_1 to F_4) were achieved. We believe that this is the first report describing the properties of F_4. The F_1 fraction catalyzed both NADH- (main) and NADPH-dependent (minor) reduction of CH. Since NADH-dependent reduction was completely inhibited by pyrazole, a specific inhibitor for alcohol dehydrogenase, the activity of the F_1 fraction was probably due to alcohol dehydrogenase in the fraction. As for the F_3 fraction, the CH-reducing activity was lower than in other fractions, but we found that C_{24} 3-ketosteroid was also reduced by the fraction. Purification and characterization of F_3 are in progress to elucidate its role in the oxido-reduction of the steroid moiety as well as in the reduction of aldehydes or ketones.

CH is also reduced to trichloroethanol by NADH-dependent alcohol dehydrogenase (EC 1.1.1.1), which corresponds to the F_1 fraction in Fig. 1a. Using substrate-saturated conditions, the ratio of the velocity of CH reduction by the F_1 fraction with NADH to that by the F_2, F_3 and F_4 fractions with NADPH was about 3 to 1. As reported previously, however, the reduction of CH by rat liver cytosolic fraction took place at the same rate in the presence of either NADH or NADPH. We attribute this difference to the substrate-unsaturating conditions and the long reaction time employed in the latter experiments. Probably, the metabolism of CH *in vivo* is similar to that observed for the liver cytosolic fraction.

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