

## Selective Chemical Modification of Human Liver Aldehyde Dehydrogenases $E_1$ and $E_2$ by Iodoacetamide\*

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Human liver aldehyde dehydrogenase isoenzymes  $E_1$  and  $E_2$  are both irreversibly inactivated by iodoacetamide (but not by iodoacetic acid). The rate and degree of inactivation is potentiated by NAD while aldehyde substrates and chloral protect against inactivation. Saturation kinetics give  $y$  axis intercepts from which  $K_i$  values have been determined, and from which non-covalent recognition of iodoacetamide by each isoenzyme is indicated. Aldehyde substrates compete with iodoacetamide in steady state kinetics, from which  $K_i$  slope values are calculated that are comparable to those from saturation kinetics, indicating that the same process is studied in the steady state as during preincubation. Correlation of activity loss with incorporation reveals linear loss of 65-80% of catalytic activity as 1 eq of iodo[ $^{14}$ C]acetamide is incorporated. Further incorporation into either tetrameric isoenzyme, to a maximum of only 2 eq (in  $E_1$ ), causes further loss of residual activity with  $E_1$ , but not with  $E_2$ . Total loss of activity is never seen with either isoenzyme; residual activity of  $E_1$  is approximately 5% upon full incorporation of 2 eq, and with  $E_2$ , 20% activity remains even after long term incorporation of approximately 1.5 eq. Iodoacetamide-derivatized  $E_2$  is almost identical to control  $E_2$  with respect to  $K_m$  values for NAD, propanal, and glycolaldehyde; incorporation of iodoacetamide decreases only maximal velocity. Peptide mapping (CNBr and tryptic fragments) of iodo[ $^{14}$ C]acetamide-derivatized  $E_1$  and  $E_2$  indicates specific reaction at one site within either isoenzyme. Since complete inactivation is not seen with either isoenzyme, kinetic properties following modification remain essentially unchanged, and the mole fraction incorporated is less than the expected catalytic site number reaction with a hyperreactive sulfhydryl group at a modifier site of human liver aldehyde dehydrogenase is postulated.

NAD-linked oxidation of ethanol-derived acetaldehyde is now generally accepted as the primary route of acetaldehyde metabolism (Lindros, 1974; Parrilla *et al.*, 1974). Liver aldehyde dehydrogenases (E.C. 1.2.1.3) with low Michaelis con-

stants for acetaldehyde are recognized as being responsible for this metabolism, and two such aldehyde dehydrogenases,  $E_1$  and  $E_2$ , have been purified to homogeneity from human liver (Greenfield and Pietruszko, 1977). In other mammalian species, enzymes with properties similar to  $E_1$  are cytoplasmic and those with properties similar to  $E_2$  are found in the mitochondrial matrix, but due to unavailability of fresh material, subcellular fractionation has not been carried out on human tissues. The importance of  $E_1$  and  $E_2$  in acetaldehyde metabolism in humans is underscored by the potent inhibition of  $E_1$  by the alcohol aversive agent, disulfiram, which probably derives much of its therapeutic efficacy from blockage of acetaldehyde metabolism (Kitson, 1977). The recent finding that  $E_2$  is missing in those Japanese who display the well-known aversive "face-flush" reaction to ethanol also emphasizes the importance of  $E_2$  in acetaldehyde metabolism (Harada *et al.*, 1980).

Mammalian aldehyde dehydrogenases are tetramers of molecular weight of approximately 200,000, composed of subunits which are homogenous by the SDS-gel migration criteria. The catalytic site number (determined by titration with coenzyme) is usually less than the number of subunits. In addition to dehydrogenation of aldehydes, the enzymes also catalyze ester hydrolysis (Weiner, 1979) and their catalytic activity can be modified by small molecules such as bivalent and trivalent metals (Venteicher *et al.*, 1977) or steroids (Maxwell and Topper, 1960), suggesting the presence of regulatory sites. By analogy with glyceraldehyde-3-phosphate dehydrogenase, where the catalytic mechanism involves a covalent intermediate with a cysteine residue (reviewed by Harris and Waters, 1976), involvement of a cysteine residue in dehydrogenation of aldehydes by aldehyde dehydrogenase, including covalent bond formation, has long been suspected.

Selective chemical modification of proteins is possible with reagents that are not normally selective due to hyperreactivity of a group on the enzyme surface favoring reaction under conditions used. This may result in labeling of a group positioned at the active site, a region containing structural features responsible for both substrate specificity and for catalysis. In this paper we examine the chemical modification of  $E_1$  and  $E_2$  by iodoacetamide and demonstrate the specificity of modification. The position of modification with iodoacetamide relative to the active site is investigated and discussed.

### EXPERIMENTAL PROCEDURES

**Materials**—All chemicals were reagent grade. Propanal used for enzyme assay was redistilled before use. Iodoacetamide (Sigma) was recrystallized from water. Urea and guanidine HCl were ultrapure products of Schwarz/Mann, fluorescamine was manufactured by Roche, bovine serum albumin (A4378) and S-acetal coenzyme A were purchased from Sigma, Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone came from Worthington, and precoated thin layer cellulose plates were purchased from E. Merck (5611). Iodo[ $^{14}$ C]acetic acid, iodo[ $^{14}$ C]acetamide (lot number 1177-051, 13.1 mCi/mmol), and Liquifluor were products of New England Nuclear.

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Iodo[ $^{14}\text{C}$ ]acetamide was also purchased from Amersham (batch 48, 53 mCi/mmol).

**Enzymes**—Aldehyde dehydrogenases  $E_1$  and  $E_2$  were isolated by the method of Greenfield and Pietruszko (1977) from human liver obtained at autopsy, except that Sephadex G-100 chromatography was omitted. Purity was verified by starch and polyacrylamide gradient (Pharmacia 4/30) gel electrophoresis, both of which resolve  $E_1$  from  $E_2$ , and by specific activity. Enzyme isolated by this procedure is stored at  $-20^\circ\text{C}$  under nitrogen in 30 mM sodium phosphate, pH 6.0, containing 1 mM EDTA, 0.1% (v/v) mercaptoethanol, and 25% (v/v) glycerol. Prior to use, enzyme is dialyzed free of glycerol and thiol against degassed, nitrogen-saturated 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA.

**Enzyme Assay**—Unless otherwise specified, the standard assay conditions were similar to those of Feldman and Weiner (1972). The reaction system in 3 ml total volume contained 0.1 M sodium pyrophosphate, pH 9.0, 500  $\mu\text{M}$  NAD, 68  $\mu\text{M}$  propanal. Rates of enzyme reaction were determined on a Varian 635 recording spectrophotometer at  $25^\circ\text{C}$ , in cuvettes of 1-cm light path at 340 nm, by drawing tangents at initial velocity. The reaction progress curves were linear for the first few minutes.

**Incubations with Iodoacetamide**—Investigations on the effect of iodoacetamide were conducted by incubating enzyme under nitrogen in 30 mM sodium phosphate, pH 7.0, containing 1 mM EDTA with the stated concentrations of iodoacetamide and NAD. All incubations were started by addition of iodoacetamide. Calculations of the amount of enzyme present were based on the known extinction of the isoenzymes at 280 nm, and used the tetrameric molecular weight of 216,000. For saturation kinetics, the concentrations of iodoacetamide used were 0.0625–4.0 mM for  $E_1$ , and 0.25–4.0 mM for  $E_2$ . Aliquots were removed for assay at different time intervals. The rate of inactivation was determined from semilogarithmic plots of the resultant reaction progress curves:  $k_{\text{app}}$  = negative slope of log per cent activity remaining versus time (Kitz and Wilson, 1962).

**Substrate Protection and Steady State Kinetics**—Steady state kinetic experiments with iodoacetamide as inhibitor were carried out in 0.1 M sodium phosphate, pH 7.0, containing 500  $\mu\text{M}$  NAD. Propanal was used as varied substrate for  $E_1$  at iodoacetamide concentrations of 0–7.2 mM. It was not practical to use propanal as substrate for  $E_2$  in such an experiment, owing to its low  $K_m$  (approximately 0.5  $\mu\text{M}$ ) for this substrate. Glycolaldehyde was therefore substituted, and fixed concentrations of iodoacetamide from 0–14.4 mM were used. Assays were started by the addition of enzyme, and initial velocities were taken. Lines were fit by Wilkinson (1961) regression analysis to the double reciprocal plots obtained.

**Protection by Chloral Hydrate**—The protective effect of chloral on inactivation by iodoacetamide was tested by assaying, over time, aliquots from incubations containing 1.33 mg/ml of  $E_1$ ; 0.6 mM NAD, 0–0.2 mM chloral, and 0.05 mM iodoacetamide. The pseudo-first order rate constant of inactivation,  $K_{\text{app}}$ , was determined as described above, and the results are presented as the reciprocal pseudo-first order rate versus chloral concentration.

The ability of chloral to block iodo[ $^{14}\text{C}$ ]acetamide incorporation was also tested as follows. Incubations of 1 ml containing  $E_1$  (1 mg/ml) or  $E_2$  (1.2 mg/ml) with iodo[ $^{14}\text{C}$ ]acetamide (18.5  $\mu\text{M}$  for  $E_1$  and 22.2  $\mu\text{M}$  for  $E_2$ ) in the presence or absence of 1.0 mM chloral were made in 30 mM sodium phosphate, pH 7.0, containing 0.7 mM NAD. Iodo[ $^{14}\text{C}$ ]acetamide was added last. After 3 h for  $E_1$  and 6 h for  $E_2$ , aliquots of each incubation were assayed to determine the loss of catalytic activity. Incorporation in the remainder of the incubation was stopped by addition of mercaptoethanol, after which the mixture was dialyzed against water ( $\times 4$ ) in Schleicher & Schuell collodion bag dialyzers. Incorporation of label was determined by counting the dialyzed protein in 10 ml of a mixture containing toluene/liquifluor/Triton X-100 (718:42:340) using an Intertechnique SL30 liquid scintillation counter. Counting efficiency was determined by the external standards technique as described by Lin and Lester (1975).

**Labeling of  $E_1$  and  $E_2$  by Iodo[ $^{14}\text{C}$ ]acetamide**— $E_1$  and  $E_2$  were incubated with 0.02–0.2 mM iodo[ $^{14}\text{C}$ ]acetamide in the presence of NAD. Aliquots were removed over time for assay, as well as for determination of incorporation of label. After labeling, the remaining enzyme SH groups were alkylated with unlabeled iodoacetic acid before fragmentation with CNBr or trypsin.

**Carboxymethylation**—The isoenzymes were precipitated using a boiling water bath, and centrifuged at  $5,000 \times g$  for 10 min. The precipitate was taken up at 2 mg/ml in 6 M guanidine HCl, 0.1 M Tris/HCl, 0.002 M EDTA, pH 8.1. Reduction and alkylation were effected by heating the mixture for 1 min in a boiling water bath, followed by

the addition of 5  $\mu\text{l}$  of mercaptoethanol with incubation for 5 min at  $37^\circ\text{C}$ . After flushing with nitrogen, another 5  $\mu\text{l}$  of mercaptoethanol was added under nitrogen, followed by incubation for 1 h at  $37^\circ\text{C}$ . Iodoacetic acid, enough to provide a 20% excess of reagent over SH groups contributed by enzyme protein and mercaptoethanol, was then added in dim light, with pH maintained at 8 by the addition of Tris. Incubation was continued for 1 h at room temperature, with excess reagent scavenged at the end of this period by the addition of excess mercaptoethanol. Carboxymethylated protein was then dialyzed versus 2 mM HCl (for CNBr) or versus 0.1 M  $\text{NH}_4\text{HCO}_3$  (for trypsin) to remove reagents. By using iodo[ $^{14}\text{C}$ ]acetate in the above procedure, enzyme SH groups were labeled as well as blocked.

**Cyanogen Bromide Fragmentation**—Carboxymethylated isoenzymes, lyophilized from 2 mM HCl, were fragmented with cyanogen bromide (10:1 versus protein, by weight) in 70% (v/v)  $\text{HCOOH}$  at  $37^\circ\text{C}$  for 4 h, at 2 mg of protein/ml. Cyanogen bromide fragments were mapped by polyacrylamide slab gel electrophoresis after the method of Laemmli (1970), by using a 16% (w/v) running and 6% stacking gel. SH-containing peptides were detected by autoradiography by the method of Bonner and Laskey (1974).

**Tryptic Digestion**—Carboxymethylated isoenzymes (2 mg/ml in 0.1 M  $\text{NH}_4\text{HCO}_3$ ) were digested with 1% (w/v) TPCK-trypsin for 8 h at  $37^\circ\text{C}$ . After lyophilization  $\times 3$  to remove  $\text{NH}_4\text{HCO}_3$ , the peptides were taken up in a small volume of chromatography buffer (see below) for peptide mapping. Approximately 50  $\mu\text{g}$  of peptides were applied to a cellulose thin layer chromatography plate (10  $\times$  10 cm) and chromatographed with water/acetic acid/pyridine/*n*-butyl alcohol (302:76:378:244). Electrophoresis in the second dimension was carried out at 450 V for 45 min in a Desaga thin layer electrophoresis apparatus, maintained at  $12^\circ\text{C}$  by a Lauda circulating water bath, with water/acetic acid/pyridine/*n*-butyl alcohol (900:25:25:50).

**Detection of Acyl Enzyme**—The presence of acyl enzyme was investigated using the method of Lipmann and Tuttle (1945) as modified by Bloch *et al.* (1971). Isoenzyme  $E_2$  (5–15 mg) was precipitated using cold 6% (w/v) trichloroacetic acid and rinsed once with 5% (w/v) trichloroacetic acid. The enzyme was then incubated with 0.2 ml of 14% (w/v) hydroxylamine HCl containing 7% (w/v) NaOH, and 0.2 ml of 0.2 M sodium acetate, pH 5.6, for 10 min at room temperature ( $25^\circ\text{C}$ ). Subsequently, 0.6 ml of 10% (w/v)  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 0.7 N HCl containing 3.3% (w/v) trichloroacetic acid was added, and after 10 min the absorbance was read at 530 nm. S-acetyl coenzyme A was used as a primary standard, and an equal weight of bovine serum albumin was used as a control.

## RESULTS

**Inactivation of  $E_1$  and  $E_2$  by Iodoacetamide**—Fig. 1 illustrates the time course of inactivation of  $E_1$  (A) and  $E_2$  (B) by 0.3 mM iodoacetamide (4 moleq/tetrameric enzyme molecule). Under these conditions, the course of inactivation is slow, taking several hours. Both the rate and degree of inactivation are increased by the presence of NAD, but not NADH (0.35 mM). Iodoacetic acid (up to 0.37 mM, 40 moleq/tetrameric enzyme molecule) for 5 h in the presence or absence of NAD had negligible effect on either isoenzyme. With both isoenzymes, catalytic activity is never completely lost by treatment with iodoacetamide. Other experiments using increased iodoacetamide and NAD concentrations show that catalytic activity of  $E_1$  isoenzyme can be lowered slightly further, to a minimum of about 5% of initial activity, while that of  $E_2$  isoenzyme reaches a minimum of 20%. Losses of activity beyond these levels occur only with coincident precipitation of the isoenzymes.

**Saturation Kinetics (Varying Iodoacetamide)**—In this experiment, the effect of increasing concentrations of iodoacetamide on catalytic activity of  $E_1$  and  $E_2$  isoenzymes was systematically investigated by measuring the activity of aliquots withdrawn over time from incubations with NAD (0.7 mM), and varying iodoacetamide. The incubations were maintained at  $12^\circ\text{C}$  to slow the reaction rate and allow more accurate measurement. Plots of log per cent activity remaining versus time allow derivation of  $K_{\text{app}}$ , the pseudo-first order decay constant (negative change in log per cent activity remaining/min). In Fig. 2,  $1/K_{\text{app}}$  is plotted against the reciprocal

FIG. 1. Time course of inactivation of  $E_1$  and  $E_2$  by iodoacetamide. A,  $E_1$  (1.8 mg/ml), B,  $E_2$  (1.7 mg/ml), in incubations containing no additions ( $\circ$ — $\circ$ ); 350  $\mu$ M NAD ( $\bullet$ — $\bullet$ ); 33.3  $\mu$ M iodoacetamide for  $E_1$  or 31.4  $\mu$ M iodoacetamide for  $E_2$ , providing 4 moleq/tetramer ( $\square$ — $\square$ ); iodoacetamide concentrations as above, but in the presence of 350  $\mu$ M NAD ( $\blacksquare$ — $\blacksquare$ ).

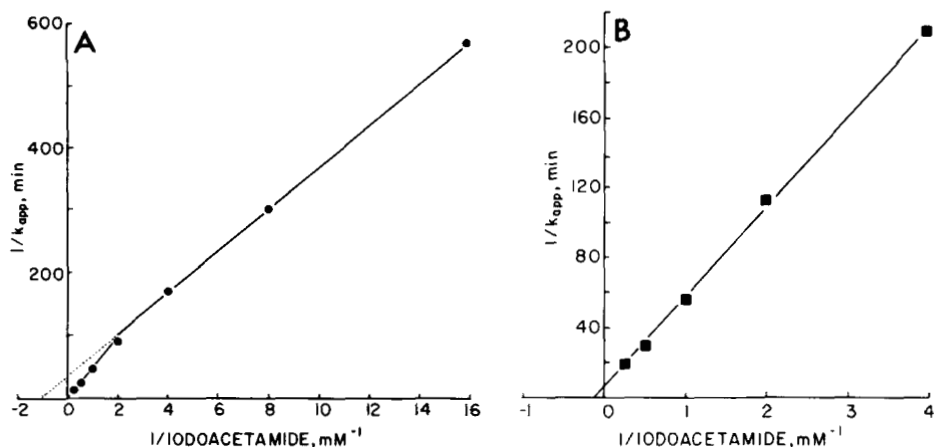
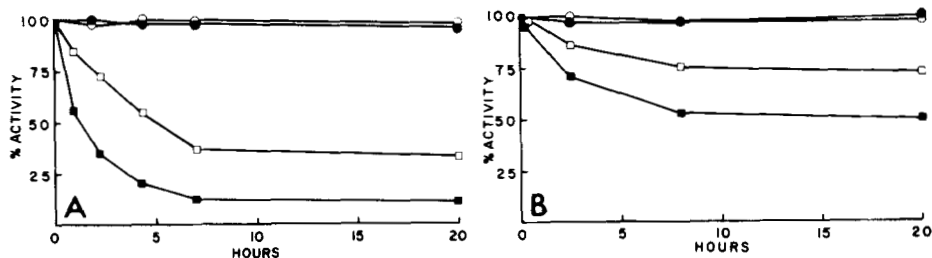


FIG. 2. Saturation effect of iodoacetamide on the rate of inactivation of  $E_1$  (A) and  $E_2$  (B). The isoenzymes were incubated at 12 °C with 0.7 mM NAD and iodoacetamide ranging from 0.0625–4.0 mM (with  $E_1$ ) or 0.25–4.0 mM (with  $E_2$ ). Enzyme concentrations were:  $E_1$ , 4.22 mg/ml (19.6  $\mu$ M); and  $E_2$ , 1.7 mg/ml (7.7  $\mu$ M). From plots of log per cent activity remaining *versus* time,  $K_{app}$ , the pseudo-first order decay constant was determined at each concentration. The

double reciprocal plot of this data for  $E_1$  (A) shows loss of the specific recognition of iodoacetamide at concentrations of iodoacetamide above 0.5 mM. The  $K_i$  of iodoacetamide for  $E_1$  is determined from this plot by extrapolation from the points obtained using iodoacetamide concentrations of  $\leq 0.5$  mM. All data points obtained from  $E_2$  (B) are used to determine the  $K_i$  of iodoacetamide for  $E_2$ . These values are listed in Table I.

TABLE I  
Summary of inhibition constants for iodoacetamide

Method	Isoenzyme $E_1$ ( $K_i \pm$ S.E.)	Isoenzyme $E_2$ ( $K_i \pm$ S.E.)
	mM	mM
Saturation kinetics	0.9 <sup>a</sup>	6.8 $\pm$ 1.2
Steady state kinetics (from slope replot)	1.3 $\pm$ 0.16	8.7 $\pm$ 1.4
Steady state kinetics (from intercept replot)	33.7 $\pm$ 8.6	12.1 $\pm$ 1.2

<sup>a</sup>  $K_i$  for iodoacetamide with  $E_1$  from saturation kinetics is obtained by manual extrapolation from lower iodoacetamide concentrations (see Fig. 2A); therefore, no standard error (S.E.) is available. All other values were obtained by Wilkinson (1961) regression analysis.

of iodoacetamide concentration. It should be noted that y axis intercepts are obtained from data of  $E_1$  at iodoacetamide concentration  $\leq 0.5$  mM, and from all data points of  $E_2$ .  $K_i$  values derived from these data (Kitz and Wilson, 1962) are listed in Table I.

Incubation with iodoacetamide in the presence of propanal or hexanal, but in the absence of NAD, had no effect on the rate of inactivation of the isoenzymes. In the presence of NAD, however, protection from iodoacetamide by 0.1 mM hexanal was noted in preliminary experiments. It is interesting to note that the presence of 50 mM acetamide or chloroacetamide in the assay mixture has no effect on catalytic activity of either isoenzyme. Incubation with NAD and chloroacetamide prior to measurement of activity also produced no effect on catalytic activity.

**Steady State Kinetics**—The results presented in Fig. 3 show the effect of iodoacetamide with varied propanal on  $E_1$  (A), and varied glycolaldehyde on  $E_2$  (B). (The  $K_m$  of propanal for  $E_2$  is less than 0.001 mM; therefore, glycolaldehyde was used

with  $E_2$ .) Although the patterns obtained are not strictly competitive, the slope effect is pronounced in both cases, giving linear replots (C). The intercept effect with  $E_1$  is small, but with  $E_2$  is more pronounced. The intercept replots (D) from both primary patterns are also linear, which allows determination of  $K_{i\_intercept}$ , listed in Table I along with  $K_{i\_slope}$  and the  $K_i$  values from saturation kinetics. With the exception of  $K_{i\_intercept}$  for  $E_1$ , the  $K_i$  values obtained are similar to those obtained from saturation kinetics. Higher iodoacetamide levels could not be used because the enzyme activity decreases rapidly, presumably due to irreversible inhibition.

**Protection of  $E_1$  by Chloral from Iodoacetamide Inactivation**—The design of the experiment shown in Fig. 4 is similar to that for Fig. 2, except that the concentration of iodoacetamide is fixed in the incubations, while chloral is present at varied concentrations. In Fig. 4,  $1/K_{app}$  is plotted *versus* chloral present in each incubation, and a linear relationship is obtained. The data demonstrate that as chloral is increased, the rate of loss of activity effected by iodoacetamide continues to decrease. The  $K_i$  of chloral calculated from these data is 40  $\mu$ M which, when multiplied by the correction factor  $1/(1 + ([IAM]/K_{i\_IAM}))$  (derived by rearrangement of Equation 3 in Kitz and Wilson, 1962; IAM, iodoacetamide.), becomes 38  $\mu$ M, similar to the value obtained in steady state kinetics with  $E_1$  of 28  $\mu$ M by Vallari and Pietruszko (1981).

**Effect of pH on Inactivation of  $E_1$  and  $E_2$  by Iodoacetamide**—The catalytic activity of  $E_1$  and  $E_2$  isoenzymes as a function of pH is presented in Table II. A general increase in activity with pH is apparent. The rates of loss of catalytic activity with pH are also given in this table. A general increase in rate of loss of activity is seen in  $E_1$  between pH 6 and 8, but a sharp drop is seen at pH 9. With  $E_2$ , a general increase in rate of loss of activity with pH is seen across the range;

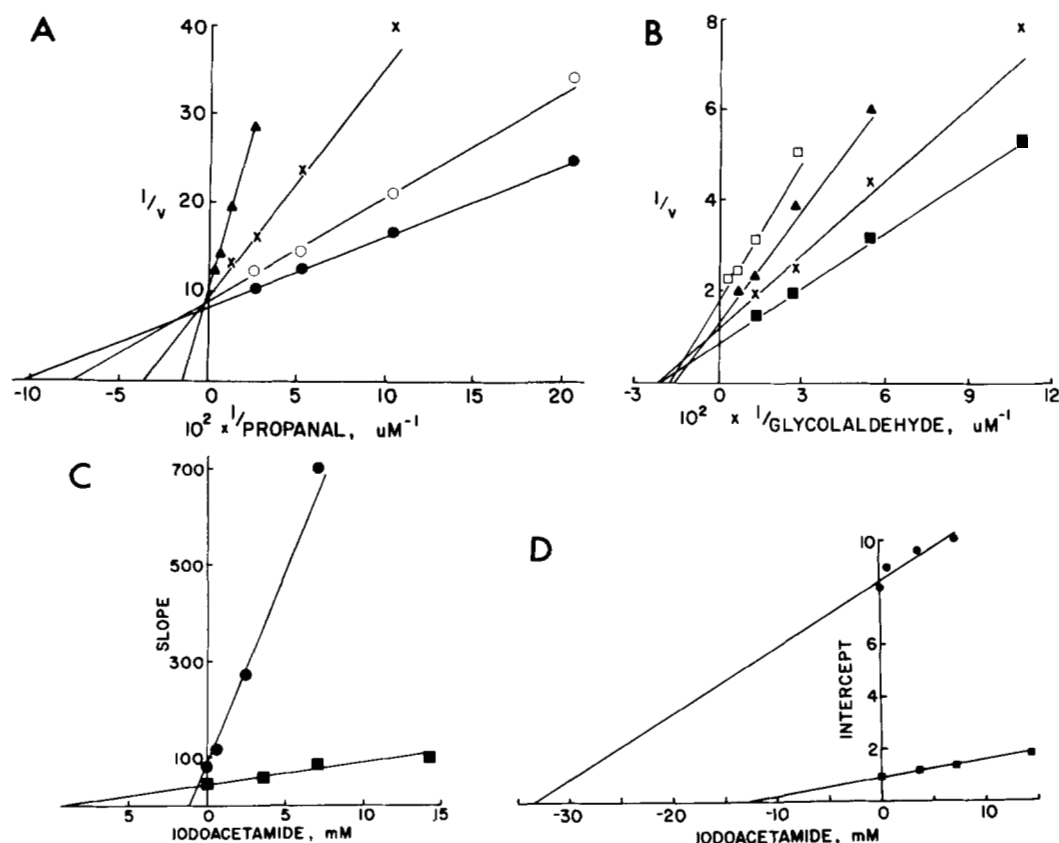


FIG. 3. Kinetic studies of  $E_1$  and  $E_2$  with iodoacetamide as inhibitor. A, Lineweaver-Burk plot of initial velocity of  $E_1$  with propanal in the presence of 0 (●—●), 0.54 (○—○), 3.6 (×—×), and 7.2 mM (▲—▲) iodoacetamide. Assays were performed at 25 °C in 0.1 M sodium phosphate, pH 7.0, containing 500  $\mu\text{M}$  NAD. The reaction was initiated by addition of enzyme.  $K_{m,app}$  of propanal is  $9.9 \pm 0.35 \mu\text{M}$ . B, Lineweaver-Burk plot of initial velocity

of  $E_2$  with glycolaldehyde in the presence of 0 (■—■), 3.6 (×—×), 7.2 (▲—▲), and 14.4 mM (□—□) iodoacetamide. Assay conditions as in (A). The  $K_{m,app}$  of glycolaldehyde is  $46.8 \pm 2.5 \mu\text{M}$ . C, replots of slopes, and D, replots of intercepts obtained with  $E_1$  (●—●) and  $E_2$  (■—■). Units of velocity are arbitrary.  $K_i$  values are listed in Table I.

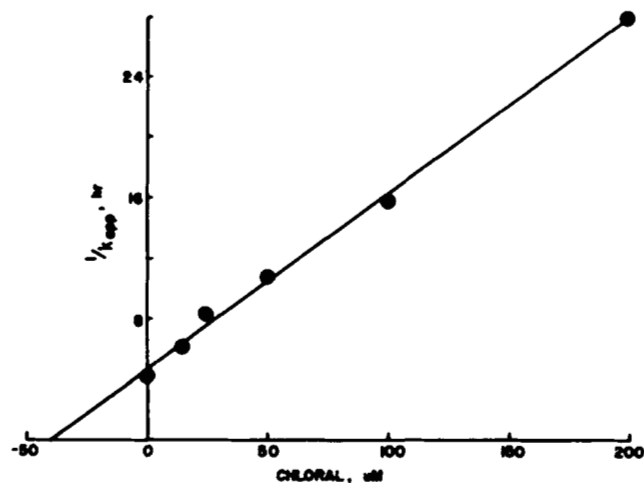


FIG. 4. Effect of chloral on rate of inactivation of  $E_1$  by iodoacetamide.  $E_1$ , 1.33 mg/ml (6.2  $\mu\text{M}$ ), was incubated with 0.6 mM NAD and 0.05 mM iodoacetamide in the presence of 0–0.2 mM chloral. Incubations were started by addition of iodoacetamide. Aliquots were removed from the incubations over time for assay, and from plots of log per cent activity remaining versus time,  $K_{app}$  was determined at each level of chloral. The above plot of  $-1/K_{app}$  versus chloral allows the determination of a  $K_i$  for chloral of 40  $\mu\text{M}$ .

however, the magnitude of the increase is only approximately 4-fold, while the increase in specific activity between pH 6 and 9 is 20-fold. The data are not readily fit to any  $pK_a$  value.

#### Relationship between Activity Loss and Incorporation of

TABLE II

#### Correlation of loss of catalytic activity with pH activity profiles

For determination of activity at different pH values using 680  $\mu\text{M}$  propanal and 500  $\mu\text{M}$  NAD, buffers, at pH 6.0, 7.0, and 8.0, of 50 mM sodium phosphate containing 1 mM EDTA were used. At pH 9.0, 50 mM sodium pyrophosphate containing 1 mM EDTA was used. Incubations from which aliquots were assayed to determine the rate of loss of activity at each pH contained  $E_1$ , 1.87 mg/ml in each buffer above, 0.67 mM NAD and 0.34 mM iodoacetamide; or,  $E_2$ , 1.26 mg/ml, 0.7 mM NAD and 0.23 mM iodoacetamide. In both cases, this is a 40-fold molar excess of iodoacetamide. All aliquots were assayed at 25 °C in the standard pH 9.0 assay mixture. Loss of activity is expressed as per cent lost in the 1st min; values are taken from semilogarithmic plots as in Figs. 2 and 4.

Isoenzyme	pH	Specific activity	% activity lost in first min
		$\mu\text{mol/mg} \cdot \text{min}$	
$E_1$	6.0	0.126	6.5
	7.0	0.174	16.0
	8.0	0.246	25.0
	9.0	0.343	11.5
$E_2$	6.0	0.063	4.0
	7.0	0.128	7.0
	8.0	0.370	11.0
	9.0	1.28	15.5

**Iodoacetamide**—Using iodo[ $^{14}\text{C}$ ]acetamide at two concentrations corresponding to 4- and 36-fold molar excess, the number of equivalents incorporated and the loss of catalytic activity were followed over time. In Fig. 5, time curves of incorporation and inactivation are presented. In Fig. 5A it can be seen that

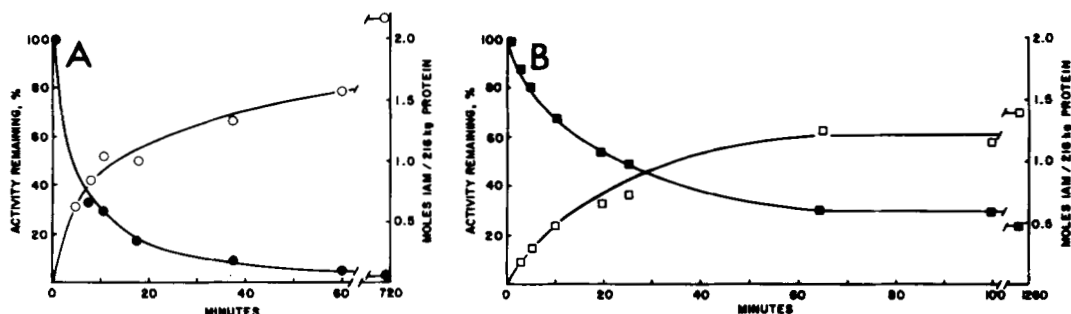


FIG. 5. Loss of catalytic activity and incorporation of iodo[ $^{14}\text{C}$ ]acetamide as a function of time. A, aliquots removed from incubations of isoenzyme  $E_1$ , 1.34 mg/ml ( $6.2\ \mu\text{M}$ ) in the presence of 0.6 mM NAD and 0.22 mM iodo[ $^{14}\text{C}$ ]acetamide (36-fold molar excess) for determination of activity remaining (●—●) and incor-

poration of iodo[ $^{14}\text{C}$ ]acetamide (○—○). B, aliquots removed from incubations of isoenzyme  $E_2$ , 1.15 mg/ml ( $5.3\ \mu\text{M}$ ) in the presence of 0.6 mM NAD and 0.19 mM iodo[ $^{14}\text{C}$ ]acetamide (36-fold molar excess) for determination of activity remaining (■—■) and incorporation of iodo[ $^{14}\text{C}$ ]acetamide (□—□).

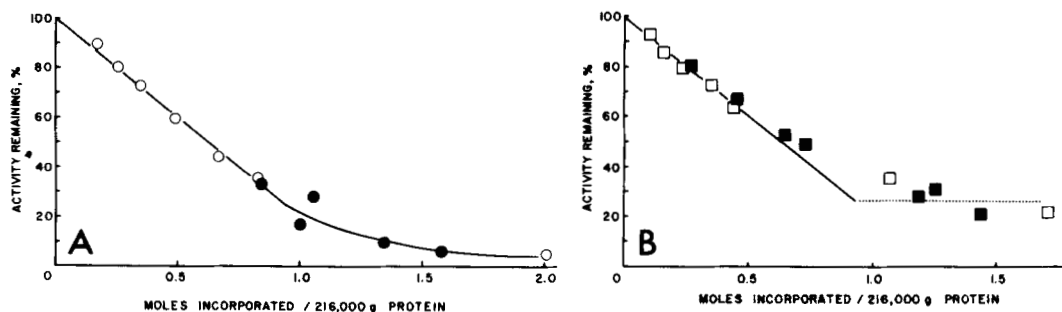


FIG. 6. Correlation of loss of catalytic activity with incorporation of iodo[ $^{14}\text{C}$ ]acetamide. A, isoenzyme  $E_1$ , 0.9 mg/ml ( $4.2\ \mu\text{M}$ ) incubated in the presence of 0.7 mM NAD and 0.017 mM iodo[ $^{14}\text{C}$ ]acetamide (4-fold molar excess), (○—○); isoenzyme  $E_1$ , 1.34 mg/ml ( $6.2\ \mu\text{M}$ ) in the presence of 0.6 mM NAD and 0.22 mM iodo[ $^{14}\text{C}$ ]acetamide (36-fold molar excess) (●—●). B, isoenzyme  $E_2$ , 1.34 mg/ml ( $6.2\ \mu\text{M}$ ) incubated in the presence of 0.7 mM NAD and 0.025

mM iodo[ $^{14}\text{C}$ ]acetamide (4-fold molar excess) (□—□); isoenzyme  $E_2$ , 1.15 mg/ml ( $5.3\ \mu\text{M}$ ) incubated in the presence of 0.6 mM NAD and 0.19 mM iodo[ $^{14}\text{C}$ ]acetamide (36-fold molar excess) (■—■). Aliquots were removed for assay and determination of incorporation over time. Dotted line indicates lack of further loss of catalytic activity.

a total of 2 eq of iodo[ $^{14}\text{C}$ ]acetamide are finally incorporated into  $E_1$ ; the 1st eq removes 80% of initial activity, while the 2nd eq removes only 15% (see also Fig. 6A). It can also be seen that the rate of incorporation of the 1st eq is much faster than that of the 2nd. In Fig. 5B it is apparent that only 1 eq is incorporated into  $E_2$ , as both incorporation and inactivation stop after incorporation of 1.2 eq, leaving 30% residual activity in this experiment. In repeated experiments,  $E_2$  is resistant to incorporation of more than approximately 1.5 eq (see Fig. 6B), and residual activity is never less than 20%.

From another experiment with a 4:1 molar excess of iodoacetamide over enzyme, second order rate constants of incorporation of  $0.69\ \text{mm}^{-1}\ \text{min}^{-1}$  and  $0.37\ \text{mm}^{-1}\ \text{min}^{-1}$  have been calculated for  $E_1$  and  $E_2$ , respectively. Second order constants of inactivation of  $0.53\ \text{mm}^{-1}\ \text{min}^{-1}$  and  $0.30\ \text{mm}^{-1}\ \text{min}^{-1}$  were also determined from these data.<sup>1</sup>

In Fig. 6 (A and B), loss of catalytic activity is plotted versus mol fraction of incorporation of iodo[ $^{14}\text{C}$ ]acetamide. Data from two separate experiments in which aliquots were removed from incubations at varying times, but which used

different levels of iodo[ $^{14}\text{C}$ ]acetamide in the incubations, are presented in each figure; the points fall on the same lines, independent of the iodo[ $^{14}\text{C}$ ]acetamide concentration used. Other experiments (not shown) in which iodo[ $^{14}\text{C}$ ]acetamide levels varied, but which were stopped at a constant time (10 min) also gave points which fell on the same lines. The figures illustrate that initial incorporation is linearly proportional to the loss of catalytic activity with both isoenzymes to at least 0.9 eq/mol of enzyme tetramer.

**Effects of Chloral on Incorporation of Iodo[ $^{14}\text{C}$ ]acetamide**—In Table III, data are presented demonstrating the protective effect of chloral against incorporation of iodo[ $^{14}\text{C}$ ]acetamide. Catalytic activity of both  $E_1$  and  $E_2$  is largely maintained by the presence of chloral in incubations, while incorporation is prevented.

**Specificity of Interaction of  $E_1$  and  $E_2$  with Iodoacetamide**—Mapping of iodo[ $^{14}\text{C}$ ]acetamide-inactivated, CNBr-digested  $E_1$  and  $E_2$  reveals carboxamidomethylation of one 16,000-dalton fragment in  $E_1$  and one 12,000-dalton fragment in  $E_2$  (Fig. 7). From the molecular weights of peptides in Fig. 7, it appears that the fainter radioactive bands are partial cleavage products (Hempel and Pietruszko<sup>2</sup>). Procedures which were successful in diminishing partial cleavage products of  $E_1$  failed to reduce the presence of the minor (approximately 15,000-dalton) doublet of  $E_2$ . As a further measure, superreduction of any methionine sulfoxide residues by the method of Adelstein and Kuehl (1970) in 50% HCOOH, 30% mercaptoethanol at 37 °C for 48 h was performed prior to cleavage, but also failed to diminish the presence of these two bands.

<sup>1</sup> The rate constants of inactivation are approximately 80% of those for incorporation in each case. The enzyme concentration used in calculating the constant for incorporation was enzyme tetramer unmodified,  $\mu\text{M}$ . At 1 eq incorporated, this value would be zero. For inactivation, the value used was arrived at by multiplying initial enzyme protein concentration, as  $\mu\text{M}$  tetramer, by % activity remaining. At 1 eq incorporated (Figs. 5 and 6) the % activity remaining is approximately 30%, so the active enzyme concentration would be  $0.3 \times$  initial enzyme protein concentration. Using this unitage, the rate of loss of activity would be expected to be about 0.7 times as fast as the rate of loss of unmodified tetramers if the processes are mutually dependent.

<sup>2</sup> J. Hempel and R. Pietruszko, manuscript in preparation.



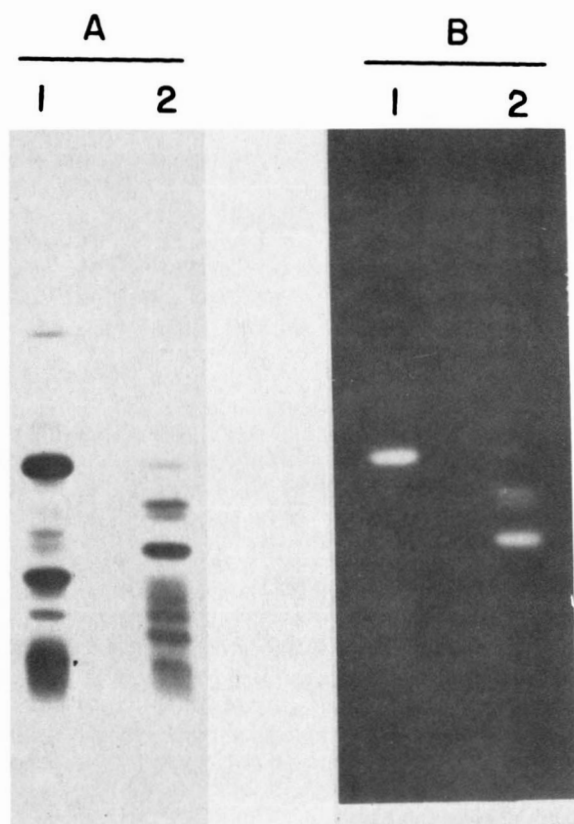
TABLE III

Prevention of loss of catalytic activity and incorporation of iodo[<sup>14</sup>C]acetamide by chloral

Incubations contained *E*<sub>1</sub> (1.0 mg/ml) or *E*<sub>2</sub> (1.2 mg/ml) in 30 mM sodium phosphate pH 7.0 with 0.7 mM NAD and 1 mM EDTA, and iodo[<sup>14</sup>C]acetamide to provide 4 equivalents/enzyme tetramer

Isoenzyme	Chloral μM	Incubation h	% Activity remaining %	Incorporation eq/tetramer
<i>E</i> <sub>1</sub>		3	30	0.61
	1000	3	93 <sup>a</sup>	0.11
<i>E</i> <sub>2</sub>		6	33	0.82
	1000	6	82 <sup>a</sup>	0.18

<sup>a</sup> Inhibition due to chloral introduced with enzyme to the assay system was negligible.



## ANODE

FIG. 7. Maps of CNBr fragments from iodo[<sup>14</sup>C]acetamide-inactivated *E*<sub>1</sub> and *E*<sub>2</sub> on sodium dodecyl sulfate-containing 16% polyacrylamide gels. A, gel stained with Coomassie blue. B, the same gel as revealed by the autoradiographic method of Bonner and Laskey (1974). Channel 1, fragments from *E*<sub>1</sub> which had incorporated the maximum of 2 eq of iodo[<sup>14</sup>C]acetamide/tetramer. Channel 2, fragments from *E*<sub>2</sub> which had incorporated the maximum of approximately 1.5 eq/tetramer.

On tryptic fingerprints, *E*<sub>1</sub> and *E*<sub>2</sub> each reveal a single labeled peptide, underscoring the specificity of alkylation of these isoenzymes by iodoacetamide.<sup>3</sup>

**Attempts to Detect Acyl-*E*<sub>2</sub>**—We were able to detect accurately, with the ferric chloride/hydroxylamine test, the presence of as little as 11 nmol of the primary standard, *S*-acetyl

<sup>3</sup> The tryptic peptide from *E*<sub>1</sub> labeled with iodo[<sup>14</sup>C]acetamide has been isolated. Compositional analysis of this peptide reveals the presence of a single residue of the acid hydrolysis product of *S*-carboxyamidomethyl cysteine, *S*-carboxymethyl cysteine (Hempel, Fietzek, and Pietruszko, work in progress).

coenzyme A. However, the A<sub>530</sub> (0.0035) observed with 15 mg of *E*<sub>2</sub> (67 nmol) indicated the presence of, at most, 4 nmol of acyl group (0.069 mol fraction/tetramer), even after extended incubation with hydroxylamine, elevation of the incubation temperature from 25 to 100 °C, or precipitation of the protein with ethanol instead of trichloroacetic acid.

**Kinetic Characterization of Iodoacetamide-derivatized *E*<sub>2</sub>**—Inactivation of both isoenzymes with iodoacetamide was followed by assay systems containing 500 μM NAD and 68 μM propanal. Similar inactivation curves were obtained using 1.3 or 13 mM propanal, indicating no major change in the *K*<sub>m</sub> of propanal. Following incorporation of 2 eq of iodoacetamide, the activity of *E*<sub>1</sub> is low and therefore difficult to study. With *E*<sub>2</sub>, however, the 20–30% activity remaining after full incorporation of iodoacetamide allowed study of the general kinetic properties of iodoacetamide-inactivated *E*<sub>2</sub>. All assays were performed in 0.1 M sodium pyrophosphate, pH 9.0, containing 1 mM EDTA. The *K*<sub>m</sub> of propanal (using 500 μM NAD) was found to be 1.0 μM for the iodoacetamide-derivatized isoenzyme, compared to 0.4 μM for unmodified *E*<sub>2</sub>. Similarly, the *K*<sub>m</sub> of glycolaldehyde was 59 μM for derivatized *E*<sub>2</sub>, compared to 25 μM for the unmodified isoenzyme. The *K*<sub>m</sub> of NAD (in the presence of 680 μM propanal) for the modified isoenzyme was found to be 240 μM, while that for native *E*<sub>2</sub> was 150 μM.

**Attempts to determine Coenzyme Binding Site Number**—Approaches employed to determine the number of coenzyme binding sites of these isoenzymes, including fluorescence enhancement of NADH and nicotinamide-1, *N*<sup>6</sup>-ethenoadenine dinucleotide (Luisi *et al.*, 1975), and quenching of protein fluorescence by NAD in the presence and absence of chloral or *p*-methoxyacetophenone (Takahashi *et al.*, 1980) were unsuccessful.

## DISCUSSION

We show here that two isoenzymes of human liver aldehyde dehydrogenase interact with iodoacetamide with a high degree of specificity. Either isoenzyme contains 36 SH groups, yet the reaction is selective for only two of these in *E*<sub>1</sub> and for only one in *E*<sub>2</sub>, covalently modifying single CNBr (Fig. 7) and single tryptic fragments from each isoenzyme. These isoenzymes are apparent homotetramers from examination of CNBr and tryptic maps; however, differences among the subunits of either isoenzyme due to microheterogeneity cannot be excluded (Hempel and Pietruszko<sup>2</sup>). It is especially noteworthy, therefore, that the stoichiometry of incorporation is considerably less than the number of subunits. Several explanations of this phenomenon are possible, the most obvious of which could be the presence of contaminating foreign proteins in the preparations. We have repeatedly checked various preparations by procedures including tryptic and CNBr peptide mapping, but have found no evidence for the presence of foreign protein. Human aldehyde dehydrogenases *E*<sub>1</sub> and *E*<sub>2</sub> are, however, extremely sensitive to air oxidation, and despite considerable precautions to prevent oxidation during purification, we are unable to rule out the possibility that some oxidation occurs at potential sites of iodoacetamide modification. Even if this happens, only the stoichiometry and not the specificity would be affected. This specificity of interaction suggests possible functional significance of the modified SH group.

Iodoacetamide may be treated both as a covalent inactivator (as seen in the loss of activity during incubations) and as a noncovalent inhibitor (as seen in steady state kinetics). By studying the covalent interaction through saturation kinetics (Fig. 2), a prior noncovalent recognition is indicated with both *E*<sub>1</sub> and *E*<sub>2</sub> isoenzymes by the presence of *y* axis intercepts. This situation is to be contrasted with modification without

prior formation of a noncovalent complex, which would give points on a line intersecting the origin. At high iodoacetamide concentration,  $\geq 0.5$  mM, with  $E_1$  (Fig. 2A) the reaction does apparently become nonspecific as inactivation appears to proceed as a bireactant chemical reaction. In Fig. 2, the data obtained with  $E_2$ , and with  $E_1$  using iodoacetamide at a concentration of  $\leq 0.5$  mM, both of which give y axis intercepts, allows  $K_i$  values to be determined (Table I).  $K_i$  values were also obtained from steady state kinetics (Fig. 3 and Table I). The close similarity of the respective  $K_i$  values for  $E_1$  and  $E_2$ , derived from saturation kinetics, to the  $K_i$  (slope) values from steady state kinetics indicates that the same process is being examined in the steady state as during preincubation with iodoacetamide.

The slope effects seen in Figs. 3A and 3B also indicate that iodoacetamide interacts upstream from aldehyde binding, and that aldehyde protects against inhibition by iodoacetamide. The observation of a linear intercept effect (Fig. 3D) may be explained simply on the basis that some covalent interaction occurs during mixing of the enzyme with the assay mixture, removing increasing amounts of active species as the iodoacetamide concentration is increased. This possibility probably occurs with  $E_1$  since  $K_i$  (intercept) differs considerably from that obtained from slopes and saturation kinetics, and the primary plots (Figure 3A) resemble a competitive pattern. The intercept effects with  $E_2$  (Fig. 3B) are more pronounced than with  $E_1$ . Glycolaldehyde was used with  $E_2$  for this experiment, and since the  $K_m$  of  $E_2$  for this substrate is 4-fold larger than the  $K_m$  of  $E_1$  for propanal, the possibility was considered that glycolaldehyde simply provides less protection. Intercept effect was also seen, however, in preliminary experiments with propanal as substrate ( $K_m$  less than  $1 \mu\text{M}$ ). It appears, therefore, that the intercept effect is real and that iodoacetamide may also react after binding of aldehyde to divert the catalytic pathway to an alternate, slower pathway.

In view of the fact that iodoacetamide is a general alkylator of enzyme sulfhydryls, the strict specificity of its interaction with  $E_1$  and  $E_2$  is striking. Since the chemical structure of iodoacetamide is not too greatly different from that of the substrate, acetaldehyde, the possibility was considered that iodoacetamide functions as an affinity reagent. The noncovalent recognition, discussed above, as well as the protection by chloral (Fig. 4 and Table III) also suggest such a role for iodoacetamide. The complete lack of effect by acetamide and chloracetamide, however, indicates that reactivity of both iodoacetamide and the enzyme SH are important in the selectivity of interaction. The environment surrounding the alkylated cysteine must also be important, as indicated by the lack of effect of iodoacetic acid.

With both isoenzymes, incorporation of the first equivalent of iodoacetamide results in loss of 65–80% of catalytic activity (see Figs. 5 and 6). Incorporation of iodoacetamide and initial activity loss show similar time dependencies, as evidenced by the similar second order rate constants for incorporation and inactivation. Both processes are faster with  $E_1$  than with  $E_2$ . With  $E_1$ , 2 eq of iodoacetamide are finally incorporated, but the slow incorporation of the 2nd eq (Fig. 5A) results in further loss of only approximately 15% of activity. Incorporation beyond 1 eq is obtained with  $E_1$  on long term incubation, but only the 1st eq (Figs. 5B and 6B) effects loss of activity. Therefore,  $E_1$  and  $E_2$  resemble each other in the amount of catalytic activity lost upon incorporation of the 1st eq, but differ in the mole fraction incorporated and in the amount of activity finally lost. With both isoenzymes, a linear relationship between mole fraction of incorporation and initial activity loss is obtained up to at least 0.9 eq incorporated (Figs. 6A and 6B), as would be expected for a reagent acting at a single

class of sites. With  $E_1$ , incorporation of the 2nd eq does not follow the same line, indicating that iodoacetamide acts rapidly at one class of sites and more slowly at the second class of sites. Since peptide mapping indicates selective modification of only one peptide in  $E_1$  after incorporation of 2 eq of iodo[ $^{14}\text{C}$ ]acetamide, the second class of sites must be identical to the first class in primary structure.

The catalytic mechanism of aldehyde dehydrogenases is thought to involve a specific cysteine (catalytic sulfhydryl) which forms a covalent intermediate with aldehyde substrates (thiohemiacetal oxidized to thioester). Such a mechanism has been demonstrated in glyceraldehyde-3-phosphate dehydrogenase (see review by Harris and Waters, 1976). However, no direct, unequivocal proof of the existence of a catalytic cysteine in aldehyde dehydrogenase has yet been presented (see review by Weiner, 1979).

To explain our results on the basis of alkylation of a catalytic sulfhydryl, it is necessary to postulate that either 1) there are different classes of active sites on a single aldehyde dehydrogenase molecule (both more catalytically active and reactive with iodoacetamide, and less catalytically active and not reactive with iodoacetamide) as a result of differences in primary structure, or 2) that modification of one site allosterically affects the catalytic activity of the other sites. The fact that  $K_m$  values of iodoacetamide-derivatized  $E_2$  are essentially unaltered argues against the presence of structurally different sites. Furthermore, the fact that identical regions of  $E_1$  are apparently alkylated by the 1st and 2nd eq of iodo[ $^{14}\text{C}$ ]acetamide argues against the presence of sites with different primary structures, as does peptide mapping which indicates that the isoenzymes, in all probability, are homotetramers. The second of the above postulates could be consistent with our data if we were able to eventually abolish total catalytic activity. Moreover, since iodoacetamide and propanal (an excellent substrate) are of approximately the same size and charge, it is difficult to understand why iodoacetamide would not derivatize sites where propanal is oxidized in a manner essentially unchanged from control, except for maximal velocity. We have also considered a possibility that this could occur by blockage of the catalytic residue by acylation, which is reversible by binding of aldehyde, as in the case of glyceraldehyde-3-phosphate dehydrogenase (Bloch *et al.*, 1971). In this scheme, the "low activity" site would remain acylated at the end of each catalytic cycle until the binding of aldehyde in the next cycle. Failure to detect any acyl groups (see "Results") argues against this scheme.

The lack of strict correlation between pH activity profiles (Table II) and loss of catalytic activity at different pH values does not support reaction with the catalytic residue, but neither does it detract from this possibility since the general trends are the same (except at pH 9.0 with  $E_1$ ). The facts, however 1) that catalytic activity is never lost completely, even in the case of  $E_1$ , where it falls as low as 5% of initial activity, 2) that inactivation is promoted by NAD, and 3) that  $K_m$  values for propanal, glycolaldehyde, and NAD are essentially unchanged argue, respectively, against involvement of the catalytic, NAD-, and substrate-binding areas of the active site.

The explanation most consistent with the results presented is in terms of a hyperreactive SH located in a modifier site. The fact that aldehyde and chloral protect against iodoacetamide is not inconsistent with this scheme, since substrates or substrate-competitive inhibitors could simply alter conformation to exclude iodoacetamide, or could themselves compete directly with iodoacetamide for the modifier site. Recent experiments with chloral (Vallari and Pietruszko, 1981) are consistent with this scheme. The presence of pronounced

intercepts in Fig. 3B may be an indication that increasing iodoacetamide directs the catalytic pathway to a slower, alternate one by reaction with the modifier site. The existence of regulatory sites in aldehyde dehydrogenase has been suggested by Hart and Dickinson (1978), who show that mitochondrial sheep liver aldehyde dehydrogenase is a hysteretic enzyme (Frieden, 1979), the activity of which is modified by substrate. If a modifier site is involved, the active site number in the enzyme derivatized by iodoacetamide should remain the same as in the control. More information about the site of iodoacetamide interaction will be obtained after a stopped-flow spectrophotometer (which is the only means we know to determine the active site number of these isoenzymes) becomes available to us. The strict specificity of the reaction established here should, in the meantime, prove useful as a benchmark with which the location of other ligands may be correlated.

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