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The Actions of Progesterone and Diethylstilboestrol on the Dehydrogenase and Esterase Activities of a Purified Aldehyde Dehydrogenase from Rabbit Liver

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A steroid-sensitive aldehyde dehydrogenase (EC 1.2.1.3) was purified from rabbit liver and is homogeneous by the criterion of electrophoresis in polyacrylamide gels with or without sodium dodecyl sulphate. The enzyme is tetrameric, of subunit mol.wt. 48300, and contains no tightly bound zinc. The fluorescence of the protein is decreased in the presence of progesterone, which is inhibitory to the reactions catalysed by the enzyme. When NADH is bound to the enzyme, the fluorescence of the coenzyme is augmented to an extent independent of the presence of steroids or acetaldehyde. The purified enzyme catalyses the oxidation of acetaldehyde and glucuronolactone, and the hydrolysis of 4-nitrophenyl acetate. Each of these reactions is inhibited by progesterone in such a manner as to suggest the formation of a catalytically active enzyme–hormone complex. Diethylstilboestrol inhibits the hydrolysis of esters by this enzyme, but stimulates the oxidation of aldehydes, except at low aldehyde concentrations; the ligand is then inhibitory. NADH inhibits the hydrolysis of 4-nitrophenyl acetate by the enzyme in a partially competitive fashion.

Maxwell & Topper (1961) discovered that an aldehyde dehydrogenase isolated from rabbit livers was sensitive to activation and inhibition by steroid hormones. More recently a number of other aldehyde dehydrogenases have been obtained in a highly purified state (Feldman & Weiner, 1972*a*; Takio *et al.*, 1974; Sidhu & Blair, 1975*a*,*b*), but have not been reported to be affected by steroids. A comparison of the properties of the steroid-sensitive and -insensitive aldehyde dehydrogenases may be of some help in the understanding of how these hormones interact with proteins.

It has been shown that one of the aldehyde dehydrogenases induced in rat liver by administration of phenobarbital is sensitive to steroids (Koivula & Koivusalo, 1975) when oxidizing acetaldehyde and that the induced enzyme will oxidize glucuronolactone (Marselos & Hänninen, 1974). Since urinary excretion of glucaric acid (the dehydrogenation product of glucuronolactone) is increased in man after administration of various drugs (Latham *et al.*, 1973), it is likely that this enzyme is inducible in humans, although the normal human liver aldehyde dehydrogenase is not sensitive to diethylstilboestrol (Kraemer & Deitrich, 1968).

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I undertook the purification of the steroid-sensitive rabbit liver aldehyde dehydrogenase in order to obtain a preparation to compare with the steroid-insensitive enzymes. The results presented here are a physical characterization of this enzyme and a limited investigation of the kinetics of the reactions catalysed by highly purified preparations of the enzyme.

Methods

Purification of the enzyme

A steroid-sensitive aldehyde dehydrogenase was purified from the soluble fraction of homogenates of rabbit liver by a modification and extension of the method proposed by Maxwell & Topper (1961). All steps were performed at 0–4°C, and all buffers contained 5mM-dithiothreitol. Two fresh rabbit livers (about 150g of tissue) were washed thoroughly in cold water and then homogenized by means of a Polytron homogenizer (Kinematica G.m.b.H, Lucerne, Switzerland) in 1.15% KCl (5ml/g of liver). The supernatant fraction from centrifugation of the homogenate at 30000*g* for 30min was filtered through glass wool, and then diluted with one-tenth of its volume of 500mM-sodium potassium phosphate, pH7.4. Fractionation with $(NH_4)_2SO_4$ was done as

detailed by Maxwell & Topper (1961), but immediately after centrifugation, without incubation at 37°C. The precipitated enzyme was resuspended in about 40ml of 7.5mM-Tris hydrochloride/NaOH, pH8.5. Insoluble material was removed by centrifugation, and then the enzyme was gel-filtered into the Tris hydrochloride/NaOH buffer. The enzyme solution was passed over a column (2.5cm×20cm) of DEAE-cellulose (DE-52; Whatman, Maidstone, Kent, U.K.) equilibrated with the same buffer, then eluted with a linear gradient of Tris hydrochloride/NaOH, pH8.5, running from 7.5 to 120mM in 15 column volumes. Under these conditions the steroid-sensitive aldehyde dehydrogenase was eluted as a single peak of activity about half way along the gradient and was stable overnight at 0–4°C. Those fractions containing the enzyme were pooled, concentrated by precipitation with $(NH_4)_2SO_4$ (0.52g/ml) and resuspended in a minimum volume of 10mM-sodium/potassium phosphate, pH6.0. After gel filtration into the phosphate buffer, the enzyme solution was passed through a column (1.4cm×5cm) of CM-cellulose (CM-52; Whatman) equilibrated in the phosphate buffer. Under these conditions the steroid-sensitive aldehyde dehydrogenase was not retarded. The active effluent was passed directly through a column (2cm×2.5cm) of hydroxyapatite (Bio-Gel HT; Bio-Rad Laboratories, Richmond, CA, U.S.A.). The enzyme was eluted with a gradient of sodium/potassium phosphate, pH7.6 (10–200mM in 60 column volumes). After again concentrating by precipitation with $(NH_4)_2SO_4$ (0.52g/ml) the enzyme was passed through a column (2.5cm×90cm) of Sephadex G-150 equilibrated in 20mM-sodium/potassium phosphate, pH7.0. The enzyme, which was eluted along with a single peak of protein, was applied directly to an affinity column (5ml volume) of AMP immobilized on agarose (AGAMP, type 2; P–L Biochemicals, Milwaukee, WI, U.S.A.). After the column had been washed with a large volume of 55mM-sodium/potassium phosphate, pH7.6, the steroid-sensitive aldehyde dehydrogenase was eluted with NAD^+ (400μM in 10mM-sodium/potassium phosphate, pH7.6). If necessary, the enzyme could be concentrated, and NAD^+ removed, by adsorption of the enzyme to a small column (1.5cm×1.4cm) of hydroxyapatite, with subsequent elution by 200mM-sodium/potassium phosphate, pH7.6.

A summary of the purification procedure is given in Table 1.

Molecular weight of the enzyme and its subunits

The molecular weight of the purified steroid-sensitive aldehyde dehydrogenase was estimated by the method of Andrews (1964), by using a column (2.5cm×85cm) of Sephadex G-200, equilibrated with 50mM-sodium/potassium phosphate, pH7.0. The column was calibrated with rabbit muscle pyruvate kinase, yeast alcohol dehydrogenase, ox heart lactate dehydrogenase, bovine serum albumin and horseradish peroxidase, by using molecular weights taken from references quoted by Barman (1969) and by Klotz & Darnall (1969).

The molecular weight of the subunits of the steroid-sensitive aldehyde dehydrogenase was estimated by the method of Shapiro *et al.* (1967) by using polyacrylamide gels containing 0.1% sodium dodecyl sulphate. Two porosities of gels were used, containing either 5 or 10% acrylamide, with *NN'*-methylenebisacrylamide contributing 3.3% of the total acrylamide in each case. The molecular weight of the subunits was obtained by interpolation (Shapiro *et al.*, 1967) from the migration and molecular weights of the standards. For 10% gels the standards used were bovine cytochrome *c*, ribonuclease, chymotrypsinogen A, ox heart lactate dehydrogenase, yeast alcohol dehydrogenase, ovalbumin and catalase, and for 5% gels the standards were rabbit muscle phosphorylase *a*, bovine serum albumin, ox liver catalase, ovalbumin, yeast alcohol dehydrogenase and ox heart lactate dehydrogenase.

Each protein was incubated at 37°C for 60min with a final concentration of 1% 2-mercaptoethanol and 1% sodium dodecyl sulphate before about 20μg of protein was loaded on to the gel. Electrophoresis was at 10mA per gel for 4.5h for both porosities of gel. After electrophoresis the gels were stained for protein (Dunker & Rueckert, 1969).

Electrophoresis of the enzyme in the presence of ligands

The purified enzyme was subjected to electrophoresis on a continuous polyacrylamide-gel system (5% total acrylamide) with 5mM-glycine/NaOH, pH9.0, as the gel buffer and the same buffer at a concentration of 25mM in the electrode wells. The

Table 1. *Purification of a steroid-sensitive aldehyde dehydrogenase from rabbit liver*

The enzyme activity was measured at pH7.4 in 100mM-phosphate in the presence of 10mM-pyrazole, 1.25mM-NAD^+ and 125μM-acetaldehyde. Formation of NADH at 30°C was measured at 340nm.

Fractionation step	Specific activity (μmol/min per mg)	Recovery (%)
$(NH_4)_2SO_4$, gel-filtered	0.046	100
DEAE-cellulose, chloride gradient	0.126	100
CM-cellulose	0.35	91
Hydroxyapatite, phosphate gradient	0.45	78
Sephadex G-150	0.5	53
Agarose–AMP, NAD^+ elution	0.5	25

enzyme was dialysed against well buffer for 2h just before electrophoresis, and approx. 25 μg of protein was loaded on the gel. To ascertain whether or not the migration of the enzyme was affected by the presence of diethylstilboestrol or progesterone, these compounds were added to the gel solutions before polymerization and to the enzyme solution after dialysis to give a final concentration of 5 μM (diethylstilboestrol) or 10 μM (progesterone) in separate gels. Control gels contained an appropriate volume of methanol (16 μl in 8ml). Electrophoresis was at 2.5mA per gel for 1.5h. Protein was stained with Amido Schwarz in 5% (v/v) acetic acid. Enzyme activity was detected by incubation for 1h at room temperature (22°C) with a Nitro Blue Tetrazolium stain (1.5mM-NAD^+, 250 μM-acetaldehyde, 0.03mg of phenazine methosulphate/ml and 0.3mg of Nitro Blue Tetrazolium/ml in 100mM-Tris/HCl, pH8.0). Control gels incubated with the staining mixture containing no NAD^+ developed no colour.

Zinc content of the enzyme

The zinc content of the purified enzyme was measured by means of a Perkin–Elmer model 403 atomic absorption spectrometer fitted with a model HGA 74 graphite furnace (instrument parameters used were: sample volume 25 μl; drying temperature 110°C; charring temperature 400°C; atomization at 2200°C). The absorption at the 214nm zinc line was recorded. Just before measurement the enzyme was gel-filtered into zinc-free ammonium acetate (20mM).

Enzyme activity assays

Aldehyde dehydrogenase was assayed as a routine at pH7.4, 30°C, in 100mM-sodium/potassium phosphate with 1.25mM-NAD^+ (Boehringer/Mannheim Corp., New York, NY, U.S.A.), 125 μM-acetaldehyde (freshly distilled) and 10mM-pyrazole. The rate of production of NADH was followed spectrophotometrically at 340nm. For kinetic work with purified enzyme the pyrazole was omitted from the assay solution, and in some cases acetaldehyde was replaced by D-glucuronolactone (Sigma, St. Louis, MO, U.S.A.).

The esterase activity of aldehyde dehydrogenase was measured at pH9.0, 30°C, in 100mM-sodium pyrophosphate with 4-nitrophenyl acetate. The rate of the reaction was proportional to enzyme concentration over at least the range 0–5 μg/ml. A millimolar extinction coefficient of 18 at 400nm under these conditions was assumed for 4-nitrophenol (Kezdy & Bender, 1962), and all results were corrected for the spontaneous hydrolysis of the ester, which was always less than 10% of the enzymic rate.

Progesterone and diethylstilboesterol were added to the assay solutions in a maximum of 5 μl of methanol/ml. Methanol at this concentration had no measurable effect on the enzyme acting as a dehydrogenase or as an esterase.

Before any kinetic or fluorimetric work the enzyme was gel-filtered into the assay buffer to remove dithiothreitol and NAD^+.

Alcohol dehydrogenase was assayed with acetaldehyde and NADH as described elsewhere (Duncan *et al.*, 1976).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) dried over silica gel, as the standard.

All fluorescence measurements were made at 30°C, by using buffer solutions of compositions described in the Results section and in the legends to the Figures, with an uncorrected Aminco–Bowman spectrophotofluorimeter.

Results

Purification and physical characteristics of the enzyme

Aldehyde dehydrogenase purified as described migrated as a single band of protein during electrophoresis in polyacrylamide gels at pH9.0, and in gels run at pH7.2 in the presence of 0.1% sodium dodecyl sulphate. Enzyme activity (detected by staining with the tetrazolium system) migrated precisely the same distance at pH9.0 as did the band of protein stained with Amido Schwarz. Fig. 1 shows a densitometric scan of a pH9.0 gel stained for protein. Although affinity chromatography caused a large loss of enzyme activity, and no increase in specific activity

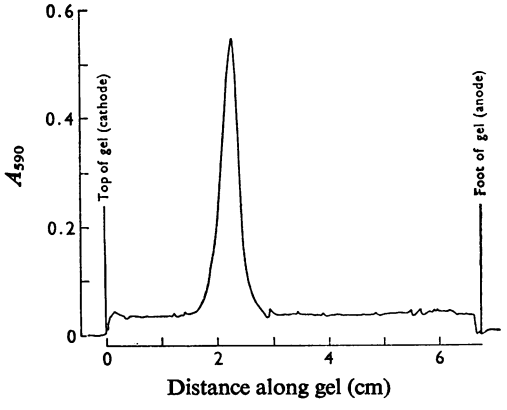

Fig. 1. *Polyacrylamide-gel electrophoretogram of purified aldehyde dehydrogenase*

About 25 μg of the enzyme preparation was loaded on to the 5% polyacrylamide gel. Electrophoresis at pH9.0 was for 1.5h at 2.5mA per gel. Protein was stained with Amido Schwarz and the gel scanned at 590nm.

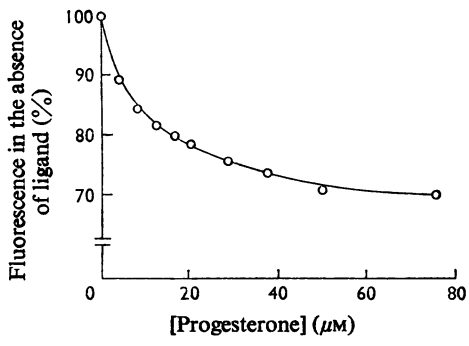

Fig. 2. *Quenching of the fluorescence of aldehyde dehydrogenase by progesterone*
The fluorescence (excitation 280nm; emission 340nm) of the enzyme (56pmol/ml) was measured at 30°C and pH7.4 in 100mM-phosphate in the presence of increasing concentrations of progesterone.

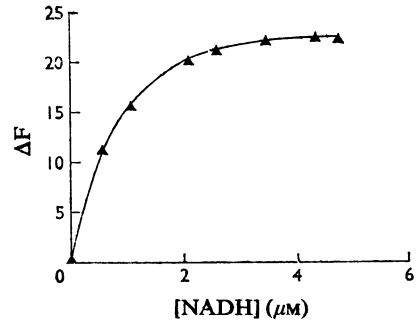

Fig. 3. *Augmentation of the fluorescence of NADH by aldehyde dehydrogenase*
The difference ΔF (fluorescence of NADH in the presence of enzyme) minus (fluorescence of the same concentration of NADH alone) in arbitrary fluorescence units is plotted against the NADH concentration. The fluorescence (excitation 340nm, emission 450nm) was measured at pH7.4 in 100mM-phosphate, 30°C, in the presence and absence of 90pmol of enzyme/ml.

(Table 1), this step was necessary in the purification of the enzyme, as it was found that several bands that stained with Amido Schwarz were present in the enzyme solution if it was subjected to gel electrophoresis before affinity chromatography. The electrophoretic migration of the enzyme was not changed by the addition of progesterone (10μM) or diethylstilboestrol (5μM).

Aldehyde dehydrogenase had a K_d (as defined by Marsden, 1965) of 0.25 on Sephadex G-200, and a mol.wt. of 194600±4300 (standard error of estimate) by interpolation from the standards. The subunit mol.wt. obtained by electrophoresis in the presence of 0.1% sodium dodecyl sulphate was 48200±1700 (standard error of estimate) (from 5% gels) or 48400±3000 (standard error of estimate) (from 10% gels).

The zinc contents of three samples of aldehyde dehydrogenase, purified separately, were 130, 99 and 174pmol of zinc/ml of enzyme solution. The same solutions contained 4.7, 1.9 and 6.2nmol of enzyme/ml respectively (assuming a mol.wt. of 194600), giving maximum values of 0.03, 0.05 and 0.03g-atom of zinc/mol of enzyme.

The solutions of purified aldehyde dehydrogenase fluoresced at 340nm when excited at 280nm in 100mM-sodium/potassium phosphate, pH7.4. Neither the intensity nor the wavelengths of this phenomenon were affected by the presence of 5μM-diethylstilboestrol, but progesterone had a marked effect on the intensity of the fluorescence, although not on the wavelengths of excitation or emission. Fig. 2 shows the decrease in fluorescence of aldehyde dehydrogenase induced by the hormone. At the concentrations of enzyme (56pmol/ml) and progesterone involved, the fluorescence is proportional to the protein concentration. The decrease in fluorescence cannot be ascribed to an 'inner filter effect' (Parker, 1968), but must be caused by an interaction of the protein and hormone. Various graphical procedures (Greenfield, 1975) to obtain the value of the dissociation constant of the presumed enzyme–ligand complex were unsuccessful, as the plots were markedly non-linear.

Fig. 3 shows the augmentation of the fluorescence of NADH (excitation 340nm; emission 450nm) in the presence of 23μg (118pmol) of enzyme at pH7.4 in 1.2ml of 100mM-sodium/potassium phosphate buffer. Graphical analysis (Greenfield, 1975) of these experimental values gives a value for the dissociation constant of the enzyme–NADH complex of 0.67±0.05 (standard error of estimate) μM. Neither the fluorescence of the enzyme–NADH complex nor its dissociation constant were changed by either 10μM-progesterone or 10μM-diethylstilboestrol. The addition of acetaldehyde to a final concentration of 250μM did not affect the enzyme–NADH fluorescence-titration curve in the presence or absence of diethylstilboestrol.

Some kinetic properties of the purified steroid-sensitive aldehyde dehydrogenase

The purified enzyme was free from alcohol dehydrogenase and there was no utilization of NADH by the enzyme preparation at either pH7.4 or 9.0 in the presence or absence of progesterone.

Table 2. *Some kinetic constants of purified rabbit liver aldehyde dehydrogenase*

The enzyme was assayed at 30°C in 100mM-pyrophosphate, pH9.0, or in 100mM-phosphate, pH7.4 (glucuronolactone as substrate), with 1.25mM-NAD^+ for the dehydrogenase reactions. K_m and $V_{max.}$ values and their standard errors were obtained from initial enzymic rates by the method of Wilkinson (1961). Progesterone and diethylstilboestrol were added in methanol. The formation of NADH or 4-nitrophenol was followed spectrophotometrically at 340 and 400nm respectively.

		No effector		20μM-Diethylstilboestrol		5μM-Progesterone	
Substrate	pH	K_m (μM)	$V_{max.}$ (μmol/min per mg)	K_m (μM)	$V_{max.}$ (μmol/min per mg)	K_m (μM)	$V_{max.}$ (μmol/min per mg)
4-Nitrophenyl acetate	9.0	2.2±0.27	2.07±0.15	1.64±0.3	0.68±0.1	2.6 ±0.35	1.02 ±0.02
DL-Glucuronolactone	7.4	3500±150	0.26±0.003	5130±3	1 ±0.04	8860±661	0.184±0.03
Acetaldehyde	9.0	61±7	0.57±0.03	214±14	1.6 ±0.1	107±10	0.25 ±0.08

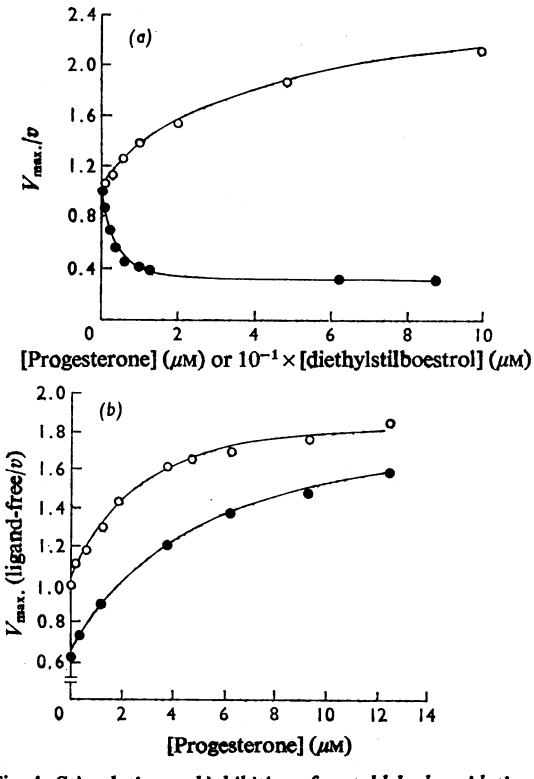

Fig. 4. *Stimulation and inhibition of acetaldehyde oxidation* (*a*) Dixon plot showing the effects of diethylstilboestrol (●) and progesterone (○) at pH7.4, 30°C, on aldehyde dehydrogenase. (*b*) Dixon plot showing that the stimulation by diethylstilboestrol may be overcome by progesterone. Rate of NADH production in the absence of diethylstilboestrol (○) or in the presence of 2μM-diethylstilboestrol (●). The NAD^+ concentration was 1.25mM for both (*a*) and (*b*). The acetaldehyde concentration was 100μM in (*a*) and 250μM in (*b*).

The apparent Michaelis constants and maximal velocities of the purified steroid-sensitive aldehyde dehydrogenase with a number of substrates are collected in Table 2, along with the values in the presence of 20μM-diethylstilboestrol or 5μM-progesterone. The actions of these effectors were reversible on dilution or gel filtration. Double-reciprocal plots of the values from which the constants were obtained were linear; the numerical values of the constants were derived by the method of Wilkinson (1961).

Both glucuronolactone and 4-nitrophenyl acetate, as well as acetaldehyde, are substrates for the enzyme. Each of the activities was eluted from Sephadex G-200 in the same peak, and each of the activities was affected by progesterone and by diethylstilboestrol. It was found that a second ester, 4-nitrophenyl phosphate, was not a substrate for the purified enzyme at either pH7.6 or 9.0.

The effects of diethylstilboestrol and of progesterone on acetaldehyde oxidation catalysed by the enzyme were not qualitatively affected by the concentration of phosphate in which the measurements were performed, although increasing the concentration had a marginal effect on the activity of the enzyme. In the presence of 250mM-phosphate the enzymic rate decreased to some 64% of the rate in 20mM-phosphate.

A Dixon (1953) plot (Fig. 4*a*) shows that both progesterone and diethylstilboestrol have an effect on aldehyde dehydrogenase which approaches a maximum value. The plot in Fig. 4(*b*) shows that the stimulation of the reaction produced by diethylstilboestrol can be overcome by progesterone in competitive fashion.

Inhibition of the dehydrogenase reaction by progesterone was observed at all concentrations of acetaldehyde and of glucuronolactone, but a change from inhibition to stimulation by diethylstilboestrol was found as the substrate concentration was increased. In the case of the enzyme acting as an esterase, inhibition was observed with both pro-

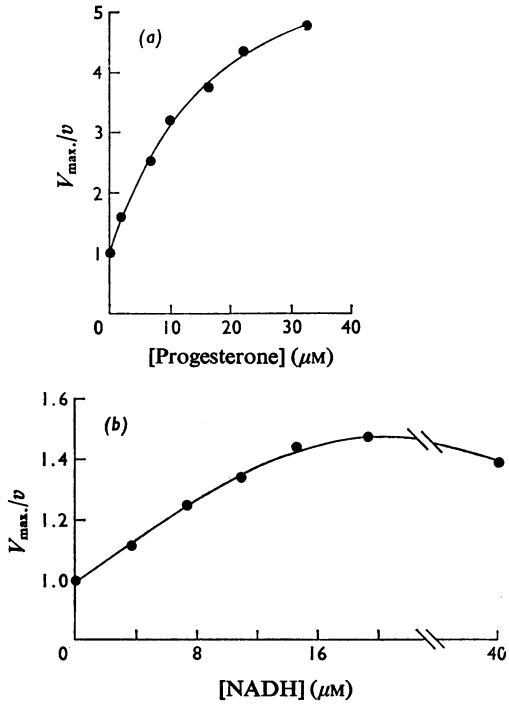

Fig. 5. *Action of progesterone and NADH on the hydrolysis of 4-nitrophenyl acetate by aldehyde dehydrogenase*
(*a*) Dixon plot showing the effect of progesterone on the esterase activity, at a concentration of 10μM-4-nitrophenyl acetate. (*b*) Dixon plot showing the effect of NADH on the esterase activity, at a concentration of 5μM-4-nitrophenyl acetate.

gesterone and diethylstilboestrol at all concentrations of 4-nitrophenyl acetate. Inhibition of the esterase activity, like that of the dehydrogenase, approaches a definite value as the concentration of the ligand is increased (Fig. 5*a*).

Hydrolysis of 4-nitrophenyl acetate by the enzyme is inhibited by NADH. Double-reciprocal plots and Dixon (1953) plots (Fig. 5*b*) show that the inhibition is of the partially competitive (Dixon & Webb, 1964) type, with a definite esterase activity in the presence of a large excess of NADH.

Discussion

It is evident from the molecular weight obtained for this aldehyde dehydrogenase by gel filtration (194600) and from the subunit molecular weight obtained by electrophoresis in the presence of sodium dodecyl sulphate (48300) that this enzyme is most probably a tetramer with all subunits of the same molecular weight. Other mammalian aldehyde dehydrogenases which have been obtained in a highly purified state have also proven to be tetrameric (Feldman & Weiner, 1972*a*; Eckfeldt *et al.*, 1976). The purified steroid-sensitive enzyme contains virtually no tightly bound zinc. Although chelating agents inhibit aldehyde dehydrogenases (Stoppani *et al.*, 1966), this has been shown to be due not to the chelating properties of these compounds but to other features of their molecular structure (Sidhu & Blair, 1975*a*).

Takio *et al.* (1974) have shown that aldehyde dehydrogenase activity in the cytosol of horse liver consists of at least three isoenzymes. One of these binds NADH with a concomitant augmentation of the fluorescence of the coenzyme, which is increased further in the presence of acetaldehyde, but the other isoenzymes do not greatly augment the fluorescence of the cofactor. The steroid-sensitive enzyme from rabbit liver increases the fluorescence of NADH slightly and to an extent independent of the presence or absence of acetaldehyde. From the results used to derive Fig. 3, the increase in fluorescence is 8-fold if only 1 mol of coenzyme binds per mol of tetrameric enzyme. The increase is less if each mol of enzyme binds more than 1 mol of coenzyme, but in the absence of detailed binding studies it is not possible to quantify this factor. Although progesterone interacts with the enzyme (as shown by the diminution of the fluorescence emission of the protein in the absence of any substrate), neither progesterone nor diethylstilboestrol affects the binding of NADH as revealed by the fluorescence-titration curve of the enzyme with NADH.

The highest specific activity of oxidation of acetaldehyde by this aldehyde dehydrogenase was 0.57μmol/min per mg of protein at 30°C and pH9.0 in the absence of any effectors. This corresponds to a turnover of 1.8mol/s per mol of enzyme. In the presence of diethylstilboestrol this rises to a maximum of 5.15mol/s per mol of enzyme. For the enzyme acting as an esterase the corresponding values are 6.7 and 2.2mol/s per mol of enzyme with 4-nitrophenyl acetate as the substrate. With either aldehyde or 4-nitrophenyl acetate as substrate, inhibition in the presence of a high concentration of progesterone approached a finite value (Figs. 4*a*, 4*b* and 5*a*). This is in agreement with the observations by Maxwell & Topper (1961), but not with those by Donville & Warren (1968), who found inhibition to increase indefinitely with glyceraldehyde as substrate. The reason for these discrepancies is not known. Stimulation by diethylstilboestrol also tends to a finite value as the effector concentration is increased. From these results, and the electrophoretic and gel-chromatographic homogeneity of the enzyme, it is apparent that the effect of ligands is to produce a new form of the enzyme with modified kinetic properties.

Maxwell (1962) showed that the sedimentation rate of rabbit liver aldehyde dehydrogenase in the presence of aldehyde and NAD^+ was not altered by effector ligands. Although the effectors interact with the free enzyme [Fig. 2 and unpublished work on gel filtration of the enzyme in the presence of radioactive progesterone (R. J. S. Duncan)], they do not alter its electrophoretic mobility. Any conformational change brought about by these effectors cannot be great, and certainly does not extend to dissociation of the subunits either in the presence of substrates (Maxwell, 1962) or in their absence.

The esterase activity of purified aldehyde dehydrogenases is usually less than the dehydrogenase activity (Feldman & Weiner, 1972*b*; Sidhu & Blair, 1975*b*; Eckfeldt & Yonetani, 1976) and NAD usually stimulates the esterase activity. This steroid-sensitive aldehyde dehydrogenase is an exception to this in that the esterase activity is greater than the dehydrogenase activity, and in that NAD inhibits hydrolysis of esters by the enzyme. The inhibition by NADH is, within experimental error, competitive with respect to ester and approaches a definite limit at high NADH concentrations with a non-saturating concentration of ester (Fig. 5*b*). The enzyme–NADH complex must hence be catalytically active in ester hydrolysis with certain rate constants modified relative to those of the enzyme free from coenzyme. It is often assumed that a thiohemiacetal is formed between an active-centre thiol group of aldehyde dehydrogenase and an aldehyde substrate (Jakoby, 1963). The thiohemiacetal, it is suggested, is then oxidized to give a thioacylated enzyme–NADH complex, which is then hydrolysed to release the reaction products. Hydrolysis of such an acylated intermediate would be expected to result in a large release of Gibbs free energy, and perhaps underly the experimental irreversibility of the overall reaction. It has been proposed that hydrolysis of 4-nitrophenyl acetate by aldehyde dehydrogenase may proceed through a similar acylated (thioacetyl) enzyme (Feldman & Weiner, 1972*b*). As the results above indicate that the enzyme–NADH complex also catalyses the hydrolysis of esters, a thioacetyl-enzyme–NADH complex must be formed during ester hydrolysis if the acetylated enzyme is, in fact, an intermediate. As the stage of major free-energy gain in the reversal of aldehyde oxidation would have been by-passed in the formation of this postulated complex from 4-nitrophenyl acetate, a strong possibility should exist of the oxidation of NADH, with subsequent release of acetaldehyde and NAD^+ from the enzyme. As yet it has not been possible to demonstrate such a reaction, nor was it possible to observe oxidation of NADH by a semipurified aldehyde dehydrogenase from pig brain in the presence of ethyl thioacetate, which might also be expected to form the crucial acetyl-thioenzyme (Duncan, 1970). Perhaps some other barrier to the reversal of this enzyme's reaction pathway exists.

The physiological role of this enzyme is unknown, but with the concentrations of steroids that occur in liver it is not certain that these phenomena are of any significance *in vivo*. Preliminary results show that the aldehyde dehydrogenase from rabbit brain is unaffected by diethylstilboestrol or by progesterone *in vitro*, although the oxidation of biogenic aldehydes (Duncan & Sourkes, 1974; Duncan, 1975) by the purified liver enzyme is markedly affected.

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