A New Subunit of Horse Liver Alcohol Dehydrogenase and Subunit Composition of the Polymorphic Form

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The most cathodal (on starch-gel electrophoresis), steroid-active band of horse liver alcohol dehydrogenase, whose catalytic properties were shown to be dependent on the livers used as a starting material [Pietruszko (1974) *Biochem. Biophys. Res. Commun.* **60**, 687–694], has been prepared from A-type and S-type horse livers by identical methods. Results presented here show that different isoenzymes are present in these preparations. Experimental evidence suggests that preparations from S-type livers consists of one component which has a subunit structure of SS (subunit S, active with steroids in addition to the classical substrates of alcohol dehydrogenase) and is identical with the SS isoenzyme described by Pietruszko & Theorell [(1969) *Arch. Biochem. Biophys.* **131**, 288–298]. Preparations from A-type livers consist of three components: one of these is the SS isoenzyme, the other two are previously unidentified isoenzymes of horse liver alcohol dehydrogenase. On the basis of the experimental evidence presented the preparations from A-type livers are composed of S and A subunits to form three isoenzymes: AA, AS and SS. Subunit A, a previously unidentified polypeptide, bears a catalytic site that does not catalyse interconversion of 3-oxo steroids or of 3 β -hydroxy steroids of A/B *cis* configuration. The A subunit, unlike the E and S subunits, occurs only in some horse livers and appears to be a polymorphic form of horse liver alcohol dehydrogenase.

Alcohol dehydrogenase from horse liver was one of the first enzymes crystallized (Bonnichsen & Wassen, 1948) and was considered for a long time to be structurally and catalytically homogeneous. With the advent of ion-exchange chromatography and zone electrophoresis it has become apparent that the enzyme is heterogeneous (Pietruszko *et al.*, 1966; Theorell *et al.*, 1966). In fact, commercial preparations consist of six electrophoretically separable components (Pietruszko *et al.*, 1966), and the separation pattern of horse liver alcohol dehydrogenase present in crude liver homogenates consists of nine (Pietruszko & Theorell, 1969), or more (Lutstorf *et al.*, 1970) distinct bands. The more cathodal bands, in addition to being active with classical alcohol dehydrogenase substrates, are also active with steroids (Pietruszko *et al.*, 1966). By using steroid activity as one of the experimental probes, three of the nine electrophoretic bands were found to be dimeric structures (Pietruszko *et al.*, 1966; Pietruszko & Theorell, 1969) composed of E and S polypeptide chains to form EE, ES and SS isoenzymes (subunit E, active with classical substrates of alcohol dehydrogenase; subunit S, also active with steroids). The identity of the remaining six or more electrophoretically separable bands of horse liver alcohol dehydrogenase is not yet understood, although it has been suggested that they may be conformational isomers of

the EE, ES and SS isoenzymes (Lutstorf & von Wartburg, 1969).

During investigations of the catalytic properties of the most cathodic, steroid-active component of horse liver alcohol dehydrogenase (isoenzyme SS), it became apparent (Pietruszko, 1974) that two kinds of 'isoenzyme SS' could be obtained from different horse livers by identical isolation techniques, the A-type and the S-type preparations differing in catalytic properties with respect to ethanol and acetaldehyde. These properties were strictly dependent on the livers from which these isoenzymes were purified. Thus A-type preparations of 'isoenzyme SS' [characterized by an acetaldehyde/steroid ketone (3-oxo-5 β -androstane-17 β -ol) activity ratio of about 20:1] were obtained from A-type livers and S-type preparations (characterized by an acetaldehyde/3-oxo-5 β -androstane-17 β -ol activity ratio of about 1:1) were obtained from S-type livers.

The results presented in the present paper demonstrate that whereas preparations from S-type livers consist of one isoenzyme (SS) preparations from A-type livers are heterogeneous and consist of three isoenzymes (AA, AS and SS) electrophoretically superimposable on starch gels. These isoenzymes are formed by combination of subunit S (Pietruszko & Theorell, 1969) with a previously unidentified polypeptide (subunit A) which is physicochemically

and catalytically distinct from either E or S subunits. The subunit composition of the nine electrophoretically separable (on starch-gel electrophoresis) isoenzyme bands of alcohol dehydrogenase from A-type and S-type horse livers is discussed.

Materials and Methods

Enzyme preparation

Fresh horse livers or livers stored frozen at -76°C were used. All preparative procedures were performed on ice or at 4°C . The horse liver (1.2 kg) was ground and suspended for 20 min in ice-cold 50 mM-sodium phosphate buffer, pH 7.5 (2 litres/kg of liver). The mixture was strained through cheesecloth, and $(\text{NH}_4)_2\text{SO}_4$ (enzyme grade, Schwarz-Mann, Orangeburg, N.Y., U.S.A.) was added to 50% saturation (310 g/l). The precipitated proteins were removed by centrifugation at 10000g for 45 min at 4°C . The $(\text{NH}_4)_2\text{SO}_4$ concentration in the supernatant was then brought to 80% saturation (210 g/litre of supernatant) and again centrifuged to recover the precipitate. The red precipitate was stored at 0°C .

The $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in a minimum volume of 20 mM-Tris/HCl buffer, pH 9.0, (conductivity 0.235 mho at 25°C), and dialysed against at least four changes of 16 times its volume of 20 mM-Tris/HCl buffer, pH 9.0. The red solution was centrifuged at 10000g for 20 min at 4°C to remove insoluble materials before column loading.

A column (40 cm $\times$ 3.4 cm) of ion-exchange DEAE-cellulose (DE-11; H. Reeve Angel and Co., Clifton, N.J., U.S.A.) was equilibrated with the 20 mM-Tris/HCl buffer, pH 9.0. The same buffer was used to elute the column; fractions (35–40 ml) were collected. Protein concentration of the eluted fractions was monitored at 280 nm (Instrumentation Specialties Co. model UA4 absorbance monitor). Enzyme activity was determined spectrophotometrically at 340 nm with cyclohexanone and/or a steroid ketone (3-oxo-5 β -androstane-17 β -ol) as substrates. The eluted enzymes were identified by starch-gel electrophoresis, with 0.3 M-Tris/HCl, pH 8.6, as the well buffer and 0.025 M-Tris/HCl, pH 8.6, as the gel buffer. The gel dimensions were 242 mm $\times$ 97 mm $\times$ 6 mm. Electrophoresis was performed at 200 V for 12–14 h at 4°C . Alcohol dehydrogenase was detected on gels by activity staining (see below).

Although the enzyme was 100% pure with respect to other starch-gel-identifiable isoenzymes of horse liver alcohol dehydrogenase, with respect to other protein its purity was 17% as determined by spectrophotometric (Theorell & Yonetani, 1963) and fluorimetric (Winer & Theorell, 1960) titration and comparison of the results with those obtained by the Lowry *et al.* (1951) procedure (see below). The contaminating protein was colourless and inert; it had no NAD^{+} -reducing or NADH -oxidizing activity

under the conditions of our assay system in the absence of substrate, nor did it have any aldehyde dehydrogenase activity (Feldman & Weiner, 1972).

Protein determination

Total protein concentration was determined by the method of Lowry *et al.* (1951) as described by Layne (1957) with bovine serum albumin [fraction V, powder, 96–99% (w/w) albumin; Sigma Chemical Co., St. Louis, Mo., U.S.A.] as a primary standard.

Titration of the enzyme

Concentration of alcohol dehydrogenase active sites was determined by fluorimetric titration with standardized NADH (Winer & Theorell, 1960) in the presence of 0.1 M-isobutyramide in 0.1 M-sodium phosphate buffer, pH 7.0, in an Aminco-Bowman recording spectrofluorimeter at an excitation wavelength of 330 nm and emission wavelength of 410 nm and by spectrophotometric titration with standardized NAD^{+} (Theorell & Yonetani, 1963) in the presence of 0.2 M-pyrazole (Ortho Research Foundation, Raritan, N.J., U.S.A.) in 0.1 M-sodium phosphate buffer, pH 7.0, in a Beckman DB-GT recording spectrophotometer at 300 nm over 0–0.1 absorbance unit ranges. Both methods gave identical results. The alcohol dehydrogenase protein concentration (g/l) was calculated from the active-site concentration and the subunit weight.

Enzyme activity assays

Assays were performed at 25°C in cuvettes of 1 cm light-path by monitoring changes in E_{340} with a Beckman DB-GT recording spectrophotometer. The reaction was started by the addition of enzyme. The assay system contained: 0.1 M-sodium phosphate buffer, pH 7.0; 170 μM - NADH (grade III, Sigma Chemical Co.); 1.2 mM-acetaldehyde (freshly distilled); or 12.7 mM-cyclohexanone; or 114.6 μM -3-oxo-5 β -androstane-17 β -ol (Sigma Chemical Co.). The steroids were dissolved in double-distilled dioxan before being added (10 μl to the 3 ml assay volume).

The rates of reaction with 3-oxo-5 β -androstane-17 β -ol (114.6 μM) and cyclohexanone (114.6 μM) individually and together were determined in 0.1 M-sodium phosphate buffer, pH 7.0, with 170 μM - NADH at 25°C . Since 3-oxo-5 β -androstane-17 β -ol was added as a solution in dioxan, cyclohexanone activity was determined in the presence of the same concentration of dioxan. When 3-oxo-5 β -androstane-17 β -ol and cyclohexanone were used together, only 3-oxo-5 β -androstane-17 β -ol was added in dioxan.

Kinetics

The Michaelis constants and turnover numbers at maximum velocity were calculated from Lineweaver-Burk plots of duplicate determinations from at least

four concentrations, each well below the substrate inhibition concentrations. Slopes and intercepts were calculated by the method of least squares. In the reductive direction the reaction was carried out in 0.1M-sodium phosphate buffer, pH7.0, containing 170 μ M-NADH. In the oxidative direction the reaction was carried out in 62mM-glycine buffer, pH9.5, containing 500 μ M-NAD⁺.

Heat-treatment experiments

Before heat treatment enzymes were dialysed against 50mM-sodium phosphate buffer, pH7.5, at 0°C. The concentrations were adjusted by the addition of 50mM-sodium phosphate buffer, pH7.5, until the active-site concentrations of both solutions were approximately equal (approx. 50 μ M). Samples (100 μ l) were then pipetted into 5ml conical centrifuge tubes, with one tube for each time-point. The tubes were simultaneously placed in a water bath at the desired temperature, either 60° or 70°C. At pre-determined time-intervals a tube was removed and immediately chilled in an ice bath. Before assay, the tubes were centrifuged to sediment the white precipitate that formed. The supernatant of each sample was assayed for acetaldehyde and 3-oxo-5 β -androst-17 β -ol activities.

Hybridization experiments

Dissociation in urea followed by the reconstitution procedure was carried out as described by Pietruszko & Theorell, (1969).

Ionagar electrophoresis

Ionagar, purchased from Wilson Diagnostics (Glenwood, Ill., U.S.A.), was added to a 1.5% concentration in 5mM-sodium phosphate buffer, pH7.0, and dissolved by heating. About 3.5ml of the hot ionagar solution was pipetted evenly on to clean microscope slides (25mm $\times$ 75mm) and left to cool. Slots were made just before sample application by dipping the edge of a 3–4mm strip of dry Whatman no. 3 filter paper into the gel. Electrophoresis was performed in an apparatus built to the specifications of Feldstein (1968). The electrode gels consisted of 1.5% bacteriological agar (Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.) in 5mM-sodium phosphate buffer, pH7.0, which was also used as the well buffer. Light petroleum (b.p. 30–60°C) or hexane was used in the centre compartment as the coolant. All electrophoretic runs were performed in a cold-room at 100V for 45min.

Activity stains for ionagar and starch gels

NAD⁺ (15mg; Boehringer Mannheim Corp., New York, N.Y., U.S.A.), 10mg of Nitro Blue Tratrastolium (Sigma Chemical Co.) and 1mg of

phenazine methosulphate (Sigma Chemical Co.) were dissolved successively in 25ml of 25mM-Tris/HCl buffer, pH8.6. For ethanol substrate staining, ethanol (Commercial Solvent Corp., Terre Haute, Ind., U.S.A.) was added to give a concentration of 15mM. For steroid substrate staining, 3 β -hydroxy-5 β -androst-17-one (Sigma Chemical Co.) was added in double-distilled dioxan (80 μ l/25ml of staining solution). The final concentration was 50 μ M. When a combined ethanol/steroid stain was required some 3 β -hydroxy-5 β -androst-17-one was dissolved in 95% (v/v) ethanol, and 0.1ml of this solution used.

Results

Electrophoretic separation, nomenclature and subunit composition of horse liver alcohol dehydrogenase isoenzymes

The nomenclature and subunit composition formerly ascribed (Pietruszko & Theorell, 1969) to horse liver alcohol dehydrogenase isoenzymes, electrophoretically separable on starch-gel electrophoresis, is shown in the first three columns of Table 1. By the established criteria (Pietruszko & Theorell, 1969) the SS isoenzyme is the fastest migrating band that is also active with steroid substrates. Since two kinds of 'SS isoenzyme' occur naturally (Pietruszko, 1974) the preparations used here and judged by these criteria will be referred to as the fastest cathodal steroid-active band with source specified as either the A-type or the S-type liver. A-type liver is the liver from which the preparation of the fastest cathodal steroid-active band has a ratio of acetaldehyde or cyclohexanone/3-oxo-5 β -androst-17 β -ol activity of approx. 20:1 and S-type liver is the liver from which the preparation of the fastest cathodal steroid-active band has an acetaldehyde or cyclohexanone/3-oxo-5 β -androst-17 β -ol activity ratio of approx. 1:1 (Pietruszko, 1974).

Purification of the fastest cathodal steroid-active band of horse liver alcohol dehydrogenase

Purification procedures from both A-type and S-type livers were identical and involved only two mild steps: (NH₄)₂SO₄ fractionation and a passage through DEAE-cellulose with negative absorption. The preparations (monitored by starch-gel electrophoresis) contained only the fastest migrating band (see Table 1), none of the other known isoenzymes of alcohol dehydrogenase were detectable on gels. Although about 2000-fold purification from the liver homogenate was achieved with regard to protein other than alcohol dehydrogenase the purity was only about 17% as shown by comparison of the results obtained from active-site titration (Winer & Theorell,

Table 1. Separation pattern of horse liver alcohol dehydrogenase isoenzymes on starch-gel electrophoresis: nomenclature and subunit composition

Diagrammatic pattern of electrophoretic separation	Nomenclature as in Pietruszko & Theorell (1969)	Subunit composition as in Pietruszko & Theorell (1969)	Subunit composition of alcohol dehydrogenase isoenzymes in S-type livers	Subunit composition of alcohol dehydrogenase isoenzymes in A-type livers	Nomenclature used here
Anode					
—	EE''	Unknown	Unknown	Unknown	
—	EE'	Unknown	Unknown	Unknown	
—	EE	EE	EE	EE	
—	ES''	Unknown	Unknown	Unknown	
—	ES'	Unknown	Unknown	Unknown	
—	ES	ES	ES	ES+EA	
—	SS''	Unknown	Unknown	Unknown	
—	SS'	Unknown	Unknown	Unknown	
—	SS	SS	SS	SS+AS+AA	Fastest cathodal steroid-active band
Cathode

Fig. 1. Effect of butan-1-ol on the activity of preparations from A-type liver with 3-oxo-5 β -androstan-17 β -ol and acetaldehyde

Enzyme activity (expressed as percentage of the activity without inhibitor) was measured in 0.1M-sodium phosphate buffer, pH7.0, with 170 μ M-NADH at 25°C. The concentrations of 3-oxo-5 β -androstan-17 β -ol (114.6 μ M) (○) and of acetaldehyde (477 μ M) (●) are adjusted to represent approximately five to six times their respective K_m concentrations.

1960) and colorimetric estimation of total protein (Layne, 1957).

Methods used during this investigation (except for total protein estimation) are based on the reactivity of the enzyme and do not depict the inert protein. Pietruszko (1974) showed that up to this stage of

purification the catalytic properties of the preparations of the fastest cathodal steroid-active band depend solely on the livers from which the preparations were obtained. To ensure that components whose existence forms the basis of the polymorphism would not be lost (except for the hybridization experiments mentioned at the end of the Results section) no attempt was made at this stage to purify further the fastest cathodal steroid-active band. In addition, purification often creates electrophoretic artifacts (Johnson & Grossman, 1975), whose appearance might obscure interpretation of the experimental results.

Characterization of the fastest cathodal steroid-active band from A-type and S-type livers

Catalytic-site heterogeneity in the preparations of the fastest cathodal steroid-active band from A-type liver. When the preparations from A-type livers were assayed in a mixture of 3-oxo-5 β -androstan-17 β -ol and cyclohexanone, the rate of the reaction was the sum of the rates obtained when these substrates were used separately. Thus in the conditions described in the Materials and Methods section at a constant concentration of enzyme, the change in E_{340} was 0.188/min for cyclohexanone, 0.098/min for 3-oxo-5 β -androstan-17 β -ol and 0.286/min for the mixture of steroid and cyclohexanone. When the effect of butan-1-ol (an alternate product) on the reduction of 3-oxo-5 β -androstan-17 β -ol and acetaldehyde was investigated, there was no inhibition of 3-oxo-5 β -androstan-17 β -ol activity up to 1.8mm-butan-1-ol whereas activity with acetaldehyde was almost 50% inhibited (see Fig. 1). Both experiments suggest that

Fig. 2. Heat treatment of preparations from A-type and S-type livers at 60°C (a) and 70°C (b)

Cyclohexanone activity of preparations from A-type (●) and S-type (■) and 3-oxo-5β-androstan-17β-ol activity of preparations from A-type (○) and S-type (□) livers is expressed as percentage of initial enzymic activity. Both preparations (50 μM) were subjected to heat-treatment in 50 mM-sodium phosphate buffer, pH 7.5.

in the preparation of the fastest cathodal steroid-active band from A-type liver the catalytic sites are heterogeneous.

Catalytic-site homogeneity in the preparations of the fastest cathodal steroid-active band from S-type livers. With the preparations from S-type liver the rate of NADH oxidation with 3-oxo-5β-androstan-17β-ol and cyclohexanone mixture was the same as that with 3-oxo-5β-androstan-17β-ol alone (the K_m for the steroid is 30 μM and that for cyclohexanone is 10 mM). Moreover, acetaldehyde and 3-oxo-5β-androstan-17β-ol activities were inhibited to a similar extent by the steroid alcohol, 3β-hydroxy-5β-androstan-17-one (50 mM), used as an alternate product.

Effect of storage and heat-treatment on catalytic properties of the fastest cathodal steroid-active band from A-type and S-type livers. When preparations from either liver type were stored in solution in 0.05 M-phosphate buffer, pH 7.5, steroid activity gradually decreased and in 1 month the enzymes were almost completely inactive towards steroids. With preparations from the S-type livers, the loss of steroid activity on storage was paralleled by the loss of cyclohexanone activity. The ratio of cyclohexanone/3-oxo-5β-androstan-17β-ol activity was maintained at 1.4 at all times suggesting that both activities were associated with the same catalytic site. However, with preparations from A-type livers the steroid activity loss occurred at a higher rate than the cyclohexanone activity loss; in one sample, after 4 weeks

of storage, 80% of the initial cyclohexanone activity was retained whereas the 3-oxo-5β-androstan-17β-ol activity decreased to 2% of the original value. This suggested that these activities were associated with different catalytic sites.

The results obtained by subjecting both preparations to heat-treatment were similar to those obtained from storage. With the preparations of the fastest cathodal steroid-active band from S-type livers the loss of 3-oxo-5β-androstan-17β-ol and cyclohexanone activities occurred at similar rates at both 60° and 70°C (see Figs. 2a and 2b); the ratio of activities remained constant. With the preparations from A-type livers the loss of 3-oxo-5β-androstan-17β-ol and cyclohexanone activities occurred at different rates during heat-treatment (Fig. 2b); loss of steroid activity occurred faster than the loss of cyclohexanone activity. Further, the stability to heat-treatment of the preparations from A-type and S-type livers was different. Both activities of preparations from A-type livers remained stable to heat-treatment for 20 min at 60°C whereas both activities of the preparations from S-type livers decreased to about 30% of the control (Fig. 2a).

Electrophoretic demonstration of differences between the preparations of the fastest cathodal steroid-active band from A-type and S-type liver. Even though preparations from A-type and S-type livers were indistinguishable by electrophoresis on starch gel, where both migrated as a single fastest cathodal band

(see Table 1), differences could be demonstrated by electrophoresis on ionagar. At pH 7.0 on ionagar the preparations from A-type liver had demonstrably different electrophoretic mobility from the preparations from S-type livers (Plate 1a). In addition, only a single activity band was detected in the preparations from S-type livers, whereas two activity bands were detected in the preparations from A-type liver.

Differences in substrate specificity of the two alcohol dehydrogenase components present in the preparations from A-type livers. All alcohol dehydrogenase components of preparations from either liver type could be easily located on ionagar gels by activity staining with ethanol as substrate. When steroid alcohol (3 β -hydroxy-5 β -androstan-17-one) was substituted for ethanol, the single component of preparations from S-type livers was detectable as readily as with ethanol. However, only one of the components (the faster migrating) of the preparations from A-type livers could be detected with 3 β -hydroxy-5 β -androstan-17-one irrespective of the concentration of the steroid or the duration of staining.

When storage and heat-treatment were monitored by ionagar electrophoresis it could be readily demonstrated that the loss of steroid activity of the preparations from A-type livers was associated with the loss of the faster-migrating component (Plate 1b). Only the slower-migrating component, inactive with 3-oxo-5 β -androstan-17 β -ol and 3 β -hydroxy-5 β -androstan-17-one as substrates, remained in the preparation after 15 min of heat-treatment at 70°C or after prolonged storage. Since the loss of the faster-migrating component is associated with complete

loss of steroid activity, the steroid activity of the preparations from A-type liver is confined to this component (see Plates 1a and 1b).

The K_m value and turnover number at maximum velocity with ethanol as substrate for the slower-migrating heat-stable component was determined. At pH 9.5 in 0.062 M-glycine buffer, at 500 μ M-NAD⁺, the K_m for ethanol was found to be 0.6 mM and the turnover number at maximum velocity was $81 \times \text{active site}^{-1} \times \text{min}^{-1}$.

Similarities of catalytic properties with steroid substrates of the preparations from A-type and S-type livers. Despite the fact that the steroid-active components of the preparations from A-type and S-type livers differed from each other in electrophoretic mobility and stability to heat-treatment, their catalytic properties with steroid substrates were almost identical. Thus K_m values for the steroid alcohol, 3 β -hydroxy-5 β -androstan-17-one, were the same for both preparations (Table 2) as were the K_m values for the steroid ketone, 3-oxo-5 β -androstan-17 β -ol. The K_i values with lithocholic (3 α -hydroxy-5 β -cholanoic) acid, an inhibitor of the steroid site of the ES isoenzyme of horse liver alcohol dehydrogenase (Theorell *et al.*, 1966), were also similar for both preparations when determined kinetically with 3 β -hydroxy-5 β -androstan-17-one as substrate (Table 2). When isobutyramide, a classical inhibitor of horse liver alcohol dehydrogenase, was used with 3-oxo-5 β -androstan-17 β -ol as substrate the K_i values determined for both preparations were also numerically similar (the difference shown in Table 2 is within experimental error of the procedure).

Table 2. Kinetic constants with 3 β -hydroxy-5 β -androstan-17-one and 3-oxo-5 β -androstan-17 β -ol and inhibition constants with lithocholic acid and isobutyramide for the preparations of the fastest cathodal steroid-active band

Alcohol (0.6–13 μ M) oxidation was determined in 0.062 M-glycine buffer, pH 9.5, at 500 μ M-NAD⁺; ketone (10–114 μ M) reduction was followed in 0.1 M-phosphate buffer, pH 7.0, at 170 μ M-NADH. All measurements were made in a Beckman DB-GT spectrophotometer at 25°C.

Variable substrate	Constant substrate	Inhibitor	Preparation from A-type liver			Preparation from S-type liver		
			K_m variable substrate (μ M)	V (turnover no. $\times$ active site ⁻¹ $\times$ min ⁻¹)	K_i (μ M)	K_m variable substrate (μ M)	V (turnover no. $\times$ active site ⁻¹ $\times$ min ⁻¹)	K_i (μ M)
3 β -Hydroxy-5 β -androstan-17-one	NAD	—	3.0	53*		3.0	69	
3 β -Hydroxy-5 β -androstan-17-one	NAD	Lithocholic acid			3.0			5.0
3-Oxo-5 β -androstan-17 β -ol	NADH	—	20	67*		30	150	
3-Oxo-5 β -androstan-17 β -ol	NADH	Isobutyramide			4200			8800

* Highest turnover number obtained with fresh preparations.

EXPLANATION OF PLATE I

Ionagar electrophoresis of the preparations of the fastest cathodal on starch gel steroid-active band from (a) A-type and S-type liver and (b) preparations from A-type liver after heat-treatment

Gels were comprised of 1.5% ionagar in 5 mm-sodium phosphate buffer, pH 7.0, which was also used as the well buffer. Electrophoresis was performed in a cold-room at 100V for 45 min. Duration of heat treatment at 70°C in (b) is indicated. Sample application slots are marked by the arrow. Gels were developed by activity staining, a mixture of ethanol and 3 β -hydroxy-5 β -androstan-17-one being used as substrate.

EXPLANATION OF PLATE 2

Electrophoretic demonstration of the presence of the S-type alcohol dehydrogenase component in the preparations from A-type livers

Sample application slots are marked by the arrow. The middle slot (2) shows A-type liver component with electrophoretic mobility similar to that of S-type liver component (bottom slot, 3) and faster than the two major components of preparations from A-type liver (top slot, 1). Experimental conditions were as in Plate 1.

EXPLANATION OF PLATE 3

Hybridization of a mixture of the SS and AA isoenzymes

Application slots (marked by the arrow) are numbered: 1, preparation from A-type liver containing AA and AS isoenzymes, used as control; 2, SS isoenzyme after dissociation and reconstitution; 3 and 4 SS isoenzyme controls; 5, mixture of AA isoenzyme with the SS isoenzyme after dissociation and reconstitution; 6, AA isoenzyme control. Experimental conditions were as in Plate 1.

Fig. 3. Cyclohexanone/3-oxo-5 β -androstan-17 β -ol activity ratios in fractions of the fastest cathodal steroid-active band from A-type and S-type livers eluted from DEAE-cellulose (DE11) column

Cyclohexanone/3-oxo-5 β -androstan-17 β -ol activity ratios in preparations from A-type (●) and S-type (■) liver.

The maximum velocities (Table 2) are based on active-site number determined by spectrofluorimetric titration (Winer & Theorell, 1960) which does not distinguish between steroid-active and steroid-inactive sites of alcohol dehydrogenase. Because the steroid-active component of preparations from A-type livers is less stable than the steroid-inactive component, the values with steroids are variable and depend on the duration of storage. The values quoted here are the highest obtained during this investigation when freshly prepared enzymes were used.

Evidence for the presence of a component characteristic of the preparations for S-type livers in preparations from A-type livers. Although preparations of the fastest migrating steroid-active band from A-type livers passed through DEAE-cellulose columns without apparent retardation, detailed analysis of the eluate has demonstrated that partial fractionation occurred. The initial fractions had a low cyclohexanone/3-oxo-5 β -androstan-17 β -ol activity ratio (approx. 1:1) resembling that of preparations from S-type livers; this ratio increased gradually (Fig. 3) to the value characteristic of the preparations from A-type livers (approx. 20:1). When the initial fractions were carefully analysed by ionagar electrophoresis (after 10–20-fold concentration by vacuum dialysis) a third component was detected. This component (present only in trace amounts) was

Fig. 4. A scheme for correlating electrophoretic mobility on ionagar with the nomenclature of isoenzymes present in preparations from A-type and S-type livers

Nomenclature of isoenzymes is shown in the Figure.

electrophoretically identical with the single component of the preparations from S-type livers (Plate 2).

Isoenzyme composition of preparations from S-type and A-type livers and their nomenclature

To simplify the terminology used to describe the isoenzymic forms present in the two preparations, a diagrammatic representation of the isoenzymes demonstrated by ionagar electrophoresis in the preparations from A-type and S-type liver is shown in Fig. 4. In the preparations from A-type livers AA and AS isoenzymes are present as major components (Fig. 4) and SS isoenzyme present as a trace component. Alcohol dehydrogenase in the preparations from S-type livers consists only of SS isoenzyme. Attempts to demonstrate AA or AS isoenzymes in preparations of the fastest cathodal steroid-active (starch gel) band from S-type livers were unsuccessful.

Hybridization experiments with AA and SS isoenzymes

AA isoenzyme was purified by heat-treatment and subjected to dissociation in 8M-urea containing 0.1M-2-mercaptoethanol followed by a reconstitution procedure (Pietruszko & Theorell, 1969). The recovery of catalytic activity was 25%. After concentration by vacuum dialysis and electrophoresis on ionagar the reconstituted isoenzyme migrated as one band with the mobility of the untreated control, suggesting that it consisted of identical subunits. SS isoenzyme was purified from S-type liver and subjected to the same procedure. Only one band with the electrophoretic mobility of the untreated control was detected showing that SS isoenzyme also consisted of identical subunits, but distinct from those of the AA isoenzyme. However, when a mixture of AA and SS isoenzymes was subjected to hybridization, three bands were observed on electrophoresis (Plate 3). The fastest and the slowest migrating bands were the same as the starting materials; the band with inter-

mediate mobility corresponded electrophoretically to AS isoenzyme.

Discussion

As judged by the criteria used here, the preparations from S-type livers consist of a single alcohol dehydrogenase isoenzyme which migrates as a single band in several electrophoretic systems and forms only itself, and no other isoenzymes on hybridization (Plate 3). Several lines of evidence indicate that the same catalytic sites are concerned with interconversion of classical substrates and of steroids. When the preparation from the S-type liver is subjected to heat-treatment (Figs. 2a and 2b), acetaldehyde, cyclohexanone and 3-oxo-5 β -androstane-17 β -ol activities are lost at similar rates. Although loss of total enzyme activity occurs on prolonged storage, the cyclohexanone/3-oxo-5 β -androstane-17 β -ol activity ratio remains constant. Activities with 3-oxo-5 β -androstane-17 β -ol and cyclohexanone are not additive and inhibition of both activities by 3 β -hydroxy-5 β -androstane-17-one (an alternate product) can be readily demonstrated. The ratio of cyclohexanone/3-oxo-5 β -androstane-17 β -ol activities is the same as that determined for the SS dimer (Pietruszko & Theorell, 1969). The preparations from S-type livers consist therefore of the SS isoenzyme described (Pietruszko & Theorell, 1969); a homodimer consisting of identical S subunits (Plate 3 and Table 1).

The preparations of the fastest cathodal steroid-active band from A-type livers consists of isoenzymes electrophoretically and physicochemically distinct from SS isoenzyme (Plate 1a and Fig. 4). AA and AS isoenzymes are the major components of preparations from A-type liver (Fig. 4); SS isoenzyme occurs in these preparations only in trace amounts. The AA isoenzyme is stable to heat-treatment and is readily freed of any AS isoenzyme by this procedure. Purified AA isoenzyme is inactive with 3-oxo-5 β -androstane-17 β -ol and 3 β -hydroxy-5 β -androstane-17-one as substrates, resembling EE isoenzyme (Pietruszko & Theorell, 1969), rather than SS isoenzyme, in this respect. Its K_m value with ethanol as substrate (0.6 mM at pH 9.5) is of the order reported (2.0 mM) for the classical horse liver alcohol dehydrogenase (Sund & Theorell, 1963), consisting mostly of EE isoenzyme, but an order of magnitude lower than that of the SS isoenzyme (11 mM). Thus the AA isoenzyme resembles the EE isoenzyme in substrate specificity and the Michaelis constant with ethanol, but it is readily distinguishable from the EE isoenzyme by its electrophoretic mobility on starch gel where it migrates like the SS isoenzyme. The AA isoenzyme occurs only in some horse livers, unlike the SS isoenzyme which appears to be more generally distributed. The AA isoenzyme is more resistant than the SS isoenzyme to denaturation by storage and

heat-treatment. Dissociation and reconstitution of purified AA isoenzyme yields only the AA isoenzyme, indicating that it consists of identical sub-units (see the Results section). Thus AA isoenzyme is a new alcohol dehydrogenase homodimer and subunit A, distinct from either the E or S subunits, is a new, previously unidentified subunit of horse liver alcohol dehydrogenase.

The AS isoenzyme is another major component of the preparations of the fastest cathodal steroid-active band from A-type livers. Its stability and electrophoretic mobility is intermediate between those of AA and SS isoenzymes. Although the AS isoenzyme has not yet been purified, it has been demonstrated that on heat-treatment the loss of steroid activity from preparations from A-type liver is associated with the loss of this isoenzyme (Plate 1b). After electrophoresis on ionagar, the AS isoenzyme is demonstrably steroid-active by staining with steroidal alcohol.

The steroid activity of the preparations from either liver type is of similar magnitude (see Table 2). Whereas in preparations from S-type livers the steroid activity is associated with the SS isoenzyme the steroid activity of preparations from A-type livers is associated with the AS isoenzyme. Yet Michaelis constants and inhibition constants determined with steroids as variable substrates are the same for preparations from A-type and S-type livers (Table 2). These results indicate that the catalytic sites concerned with steroid interconversion in preparations from both liver types are identical.

Similarity of catalytic properties with steroidal substrates, occurrence of SS isoenzyme in preparations from A-type liver (Figs. 3 and 4, Plate 2), and the fact that electrophoretic-migration and heat-stability characteristics (Plate 1a) of the AS isoenzyme are intermediate between those of SS and AA isoenzymes, suggest that the AS isoenzyme is a hybrid between the subunits composing the SS and AA isoenzymes. In fact, hybridization of AA and SS isoenzymes forms a component with electrophoretic-migration characteristics of AS isoenzyme (Plate 3). These considerations lead to the conclusion that preparations from A-type liver consist of three isoenzymes with the tentative subunit composition AA, AS and SS, listed in order of increasing electrophoretic mobility in the cathodal direction (see Fig. 4). Subunit S was described by Pietruszko & Theorell (1969), whereas subunit A is a new unidentified polypeptide. The AA and AS isoenzymes are the major components of the preparations from A-type liver and are new previously unidentified isoenzymes of horse liver alcohol dehydrogenase.

The preparations from S-type liver consist of a single alcohol dehydrogenase component composed of two S subunits. Activity with steroids (3-oxo-5 β -androstane-17 β -ol and 3 β -hydroxy-5 β -andro-

stan-17-one) is associated with the S subunit, which constitutes the single steroid-active alcohol dehydrogenase component of the preparations from S-type livers and occurs in A-type livers as the AS heterodimer. Both AA and AS isoenzymes are active with ethanol and acetaldehyde, the K_m for ethanol being 11 mM for the 'S site' and 0.6 mM for the 'A site'.

Pietruszko (1974) suggested that the fastest cathodal steroid-active band identified by starch-gel electrophoresis in the preparations from A-type liver is a polymorphic form of horse liver alcohol dehydrogenase. From our current data, it appears that the AA isoenzyme (or more precisely the A polypeptide subunit), is the polymorphic form of the enzyme. Although the present paper and a previous paper (Pietruszko, 1974) are the first reports of polymorphism of horse liver alcohol dehydrogenase, polymorphism of alcohol dehydrogenase from human liver ('atypical' enzyme) has been reported by von Wartburg *et al.* (1965). From the results of genetic and amino acid-sequence studies (Smith *et al.*, 1971; Berger *et al.*, 1974) it was concluded that in individuals possessing 'atypical' enzyme the most cathodal alcohol dehydrogenase band on starch gel occurred as a mixture of B'B', B'B and BB, a composition analogous to that of the fastest cathodal steroid-active band in preparations from A-type horse livers.

Subunit A hybridizes with the S subunit to form the AS isoenzyme. The AA, AS and SS isoenzymes are electrophoretically superimposable on starch gels. Our preliminary evidence suggests that subunit A also hybridizes with the E subunit to form the AE isoenzyme, electrophoretically superimposable with the ES isoenzyme. Starch gel gives better resolution of the total mixture of the horse liver alcohol dehydrogenase isoenzymes than other electrophoretic systems tested, giving 9–12 electrophoretically separable bands. When electrophoretic separation patterns of isoenzymes present in A-type and S-type horse livers are compared by starch-gel electrophoresis, no difference can be detected. The patterns look identical even though they obviously represent different isoenzymes. In the extracts of A-type horse livers the isoenzyme band of alcohol dehydrogenase (see Table 1) previously thought to represent the SS isoenzyme, represents electrophoretically superimposable AA, AS and SS isoenzymes, and the ES band (see Table 1) represents the ES and EA isoenzymes, which are also electrophoretically superimposable. The subunit composition of horse liver alcohol dehydrogenase as formerly presented in relation to the starch-gel separation pattern of the isoenzymes (Pietruszko & Theorell, 1969) and shown in column 3 of Table 1 applies only to S-type horse livers where the three main isoenzyme bands have the subunit composition of EE, ES and SS.

The existence of the A polypeptide subunit means that the complexity of the isoenzyme system of horse liver alcohol dehydrogenase is greater than had been thought and suggests that other polymorphic forms may exist. Our data and a report of Gurr *et al.* (1972) of a component separable on column chromatography but superimposable on electrophoresis with EE isoenzyme suggest a need for re-examination of the homogeneity of the EE isoenzyme, especially in experiments where active-site interaction is studied. The isoenzyme bands, which have been considered as conformational isomers of EE, ES and SS isoenzymes, may also be more complex.

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References

- Berger, D., Berger, M. & von Wartburg, J. P. (1974) *Eur. J. Biochem.* **50**, 215–225
- Bonnichsen, R. K. & Wassén, A. M. (1948) *Arch. Biochem. Biophys.* **18**, 361–363
- Feldman, R. I. & Weiner, H. (1972) *J. Biol. Chem.* **247**, 260–266
- Feldstein, A. (1968) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), vol. 2, 2nd edn., pp. 195–208, John Wiley and Sons, New York
- Gurr, P. A., Bronskill, P. M., Hanes, C. S. & Tze-Fei Wong, J. (1972) *Can. J. Biochem.* **50**, 1376–1384
- Johnson, R. W. & Grossman, A. (1975) in *Isozymes Molecular Structure* (Clement, L., ed.), pp. 419–432, Markert, Academic Press, New York, San Francisco and London
- Layne, E. (1957) *Methods Enzymol.* **3**, 448–450
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Luttorf, U. M. & von Wartburg, J. P. (1969) *FEBS Lett.* **5**, 202–206
- Luttorf, U. M., Schurch, P. M. & von Wartburg, J. P. (1970) *Eur. J. Biochem.* **17**, 497–508
- Pietruszko, R. (1974) *Biochem. Biophys. Res. Commun.* **60**, 687–694
- Pietruszko, R. & Theorell, H. (1969) *Arch. Biochem. Biophys.* **131**, 288–298
- Pietruszko, R., Clark, A. F., Graves, J. & Ringold, H. J. (1966) *Biochem. Biophys. Res. Commun.* **23**, 526–533
- Smith, M., Hopkinson, D. A. & Harris, H. (1971) *Ann. Hum. Genet. (London)* **34**, 251–271
- Sund, H. & Theorell, H. (1963) *Enzymes* **7**, 25–83
- Theorell, H. & Yonetani, T. (1963) *Biochem. J.* **338**, 537–553
- Theorell, H., Taniguchi, S., Åkeson, Å. & Skursky, L. (1966) *Biochem. Biophys. Res. Commun.* **24**, 603–610
- von Wartburg, J. P., Papenberg, J. & Aebi, H. (1965) *Can. J. Biochem. Physiol.* **43**, 889–898
- Winer, A. D. & Theorell, H. (1960) *Acta Chem. Scand.* **14**, 1729–1742