THE STEROID ACTIVITY AND MULTIPLICITY OF CRYSTALLINE HORSE LIVER ALCOHOL DEHYDROGENASE[1]

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Recently, it has been reported (McKinley-McKee, et al., 1965) that crystalline horse-liver alcohol dehydrogenase (ADH) exhibits multiple zones upon starch-gel electrophoresis which have been explained in terms of binary and ternary complexes of the enzyme with coenzyme, anions or inhibitors. It has also been noted (Ungar, 1960; Ungar, et al., 1965; Graves, et al., 1965a; Waller, et al., 1965) that ADH catalyzes the NAD-dependent interconversion of certain 3β-hydroxy- and 3-keto-steroids. We wish to report that the steroid activity of ADH is associated with a minor electrophoretically homogeneous zone that still exhibits substantial activity towards classical ADH substrates.

Crystalline ADH from different commercial sources (Worthington and Boehringer) was assayed in the reductive direction with three different substrates, acetaldehyde at 1.1×10^{-3} M, cyclohexanone at 1.1×10^{-4} M and the most active steroid tested, 17β-hydroxy-5β-androstan-3-one (5β DHT) at 1.1×10^{-4} M. Acetaldehyde, a good substrate for ADH, exhibited virtually maximal velocity at this concentration (Table I) while cyclohexanone, a poor substrate ($K_m = 1.7 \times 10^{-2}$) was reduced at a much lower reaction rate. The steroid, although present at close to a saturating concentration ($K_m = 5 \times 10^{-5}$) exhibited an activity even lower than that of cyclohexanone.

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The activity ratio acetaldehyde/cyclohexanone was relatively constant for both enzymes but the steroid activity (5β DHT) was more than threefold higher for the Worthington enzyme which indicated that the activity might not be inherent to ADH itself.

Table I

Specific[1] Activity of Horse Liver Alcohol Dehydrogenase (ADH) from Different Sources

Source[2]	Substrate		
	Acetaldehyde (A) (1.1×10^{-3} M)	Cyclohexanone (C) (1.1×10^{-4} M)	5β DHT[3] (1.1×10^{-4} M)
Worthington	89,000	430	106
Boehringer	110,000	496	32

[1]Expressed as activity units/mg protein (1 unit = optical density change of .001/min). The assay system contained $NADH_2$, 1.75×10^{-4} M, the substrate at the stated concentration and phosphate buffer, .03 M, pH 7.0, total volume 3.0 ml.

[2]ADH was purchased as a crystalline powder from Worthington Biochemical Corp. and from C. F. Boehringer as a crystalline suspension in phosphate buffer containing 10% ethanol. Both preparations were dialyzed against .03 M phosphate buffer before assay. Enzyme protein concentrations were determined by the ratio of optical density at 215 and 225 mμ.

[3]17β-Hydroxy-5β-androstan-3-one. The steroid was dissolved in .01 ml of methanol for assay purposes.

Electrophoresis on Sepraphore 3 at pH 8.5 followed by staining with Ponceau S revealed an identical pattern for each enzyme of five cathodically migrating zones. Protein concentrations were spectrophotometrically estimated by elution of the stained zones and are listed in Table II with the zone migrating fastest towards the cathode numbered as 1. It is apparent that although zone 3 is the major component in both cases, the Worthington enzyme contains relatively greater proportions of zones 1, 4 and 5 while zone 2 appears to be present in each enzyme in essentially equal concentrations.

Table II

Estimation of Relative Protein Concentrations of the Five Zones from Sepraphore 3 Electrophoresis[1] of Worthington and Boehringer ADH

Zone	Enzyme	
	Worthington	Boehringer
1[2]	4.1%	1.0%
2	1.2%	1.3%
3	64.0%	77.0%
4	22.6%	15.1%
5	8.2%	5.6%

[1] Sepraphore strips were pre-soaked in .02 M tris-HCl, pH 8.5 and electrophoresis run for 45 min. in tank buffer .05 M, pH 8.5. The enzyme was applied in .02 M tris-HCl buffer, pH 8.5. The zones were developed with Ponceau S, eluted with 0.1 N sodium hydroxide and protein concentrations estimated by the absorption at 540 mμ.

[2] Zone 1 was the fastest cathodically migrating zone and 5, the slowest.

Starch-gel electrophoresis of either enzyme preparation at pH 8.5 in tris-HCl buffer revealed, in accord with the Sepraphore results, five cathodically migrating zones detectable by protein staining with nigrosin. All five zones exhibited catalytic activity and could be readily visualized by the oxidation of ethanol in the presence of NAD, nitro-blue tetrazolium and phenazine methosulfate (Fig. 1). When a steroidal alcohol (3β-hydroxy-5β-androstan-17-one) was substituted for ethanol, oxidation could be detected, on occasions, in the most cathodal zone (zone 1) of the Worthington enzyme but the blank reaction of all zones was usually strong enough to obscure this selective oxidation. Each zone, when re-inserted without extraction or when extracted and subjected to re-electrophoresis, appeared as a single component.

In a preparative electrophoretic run with the Worthington enzyme, the zones were individually extracted and assayed for activity with cyclohexanone, acetaldehyde and 5β DHT (Table III). The recovery of cyclohexanone and acetaldehyde activity was 81% and the distribution of both activities roughly

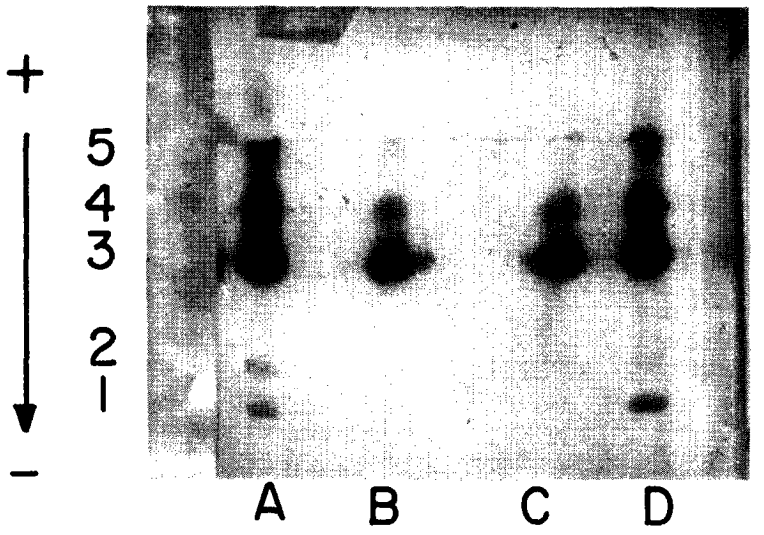

Figure 1. Starch-gel electrophoresis of Boehringer and Worthington ADH before and after CMC purification. (A) Boehringer-crude (B) Alcohol zones (eluate pH 6.6, I = .05) from CMC (C) Alcohol zones (eluate pH 6.6, I = .05) from CMC (D) Worthington-crude.

paralleled the protein distribution (Table II) of the individual zones. The results of a similar electrophoresis of the Boehringer enzyme are also shown for comparison in Table III to illustrate the activity distribution between zones 3, 4 and 5 and again were in rough agreement with the protein distributions of Table II. However, the acetaldehyde activity of zone 3 relative to 4 and 5 was definitely higher than anticipated on the basis of protein concentration (zones 1 and 2 of the Boehringer enzyme cannot be compared in this case since their activity was almost completely lost on extraction, a common occurrence with these zones when the protein concentration was very low).

The recovery of steroid activity in the Worthington electrophoresis was 64% and was seen to be concentrated primarily in zone 1. The steroid activity of the major alcohol dehydrogenase zones 3, 4 and 5 was so extremely low relative to acetaldehyde (1/16,000 - 1/35,000) that it may be stated with

surety that the three major zones of ADH do not contain significant steroid activity. The steroid activity of zone 2 may be real or could be due to carry over from zone 1.

Table III

Distribution of Total Enzymatic Activity after Starch-gel Electrophoresis[1] of Worthington[2] (W) and Boehringer[3] (B) ADH

Enzyme Source	Zone	Substrate[4]			Activity Ratio	
		A	C	5β DHT	A/C	A/5β DHT
W	1[5]	15,600[6]	93	392	168	40
	2	8,700	55	71	158	122
	3	352,000	1,740	21	202	16,700
	4	105,000	470	3	223	35,000
	5	36,000	159	2	226	18,000
B	3	73,900				
	4	8,200				
	5	3,100				

[1] Starch buffer, .025 M tris-HCl, pH 8.5; electrode buffer, .3 M; 25 milliamp, 150 V, 17 hr.

[2] Applied in .025 M tris-HCl, pH 8.5, 7.2 mg enzyme containing 640,000 units A(acetaldehyde) activity, 3,100 units C(cyclohexanone) activity and 765 units 5β DHT (17β-hydroxy-5β-androstan-3-one) activity.

[3] Applied 97,400 units A activity.

[4] The assay and substrate concentrations were as detailed in Table I. Individual electrophoretic zones were extracted with .03 M phosphate buffer pH 7.0 containing tetra-sodium EDTA at a concentration of 300 mg/ℓ. The addition of EDTA was found to increase the activity recovery without inhibiting the enzymes at the final concentrations utilized in the assays.

[5] Zone numbers are defined as in Table II.

[6] Activity is expressed as the total number of activity units extracted from the particular electrophoretic zone.

Confirmation of these findings and the removal of steroid activity from zones 3, 4 and 5 was readily demonstrated by column chromatography of each enzyme preparation on carboxymethyl cellulose. At pH 6.6, phosphate buffer (I = .05) eluted, without significant separation, the major alcohol zones 3, 4 and 5 while at pH 7.0 (I = .1), steroid zone 1 containing traces of 2

and 3 was eluted (Fig. 2). Utilizing the same solvent system, re-chromatography of the steroid zone from the Worthington enzyme gave electrophoretically pure zone 1 (Fig. 2), while re-chromatography of Boehringer zones 3, 4 and 5 (which had not separated from each other) gave an alcohol zone virtually free of steroid activity. Table IV summarizes the specific activity of central fractions.

Table IV

Specific[1] Activity of Central Fractions from CMC Chromatographs of Worthington and Boehringer ADH

Enzyme Source	Substrate[1] A	C	5β DHT
W			
Before chromat.	89,000	430	106
1st Chromat-alcohol zone[2]	107,000	531	18
1st Chromat-steroid zone[3]	19,300	108	282
Rechromat-steroid zone	32,000	173	407
B			
Before chromat.	110,000	496	32
1st Chromat-alcohol zone	113,000	515	18
1st Chromat-steroid zone	8,200	46	96
Rechromat-alcohol zone	133,000	515	10

[1]Substrate concentrations and the definition of activity units are given in Table I.

[2]Alcohol zone refers to the pH 6.6, I = .05 eluate which contained electrophoretic zones 3, 4 and 5. The enzyme was applied to the column in phosphate buffer, pH 6.4, I = .05.

[3]Steroid zone refers to the pH 7.0, I = .1 eluate which contained primarily electrophoretic zone 1 and a trace of 2 and 3 after the first chromatograph and only zone 1 after the second chromatograph.

It is of great interest that although zone 1 contained the steroid activity it also exhibited high activity towards acetaldehyde (A) and cyclohexanone (C) with an A/C activity ratio not greatly different from zones 3, 4 and 5; therefore, it must be classed as an alcohol dehydrogenase[3].

[3]Professor Hugo Theorell has kindly informed us that his laboratory has confirmed the localization of steroid activity in an electrophoretically homogeneous zone distinct from the major ADH zones but still retaining significant activity towards ethanol.

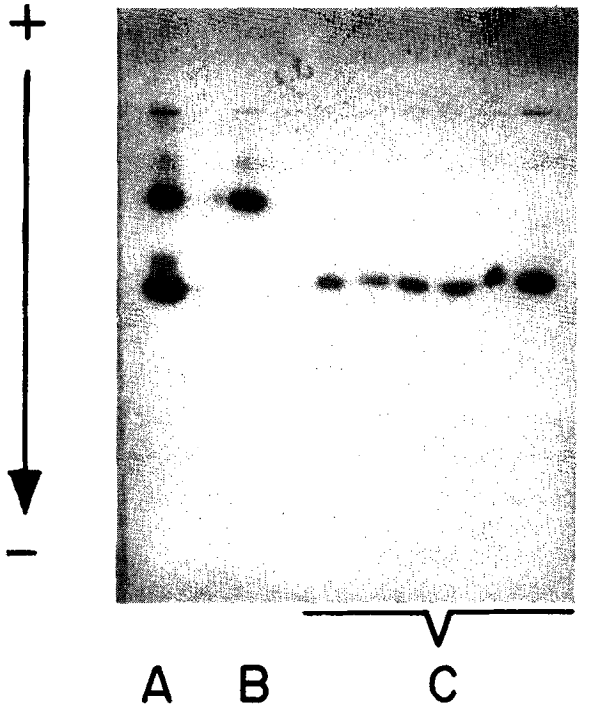

Figure 2. Starch-gel electrophoresis of zones from CMC chromatograph of Worthington ADH. (A) - 1st chromat. - Eluate pH 7.0, I = .1 (B) Rechromat. of A- Eluate pH 6.6, I = .05 (C) Rechromat. of A- Individual fractions pH 6.8-7.0, I = .05 - .1.

While the steroid activity could be due to an undetected enzyme, all attempts to dissociate steroid activity from acetaldehyde-cyclohexanone activity have been unsuccessful. Spontaneous inactivation by storage in dilute solution has led to parallel decreases in activity, and electrophoresis under varying pH conditions has failed to reveal more than a single zone. However, inhibition data indicates that if the same enzyme is responsible for both activities the steroid and acetaldehyde (or the steroid and cyclohexanone) cannot be reacting at the same catalytic site. With the twice-chromatographed steroid zone from the Worthington enzyme K_m for 5β DHT was found to be 5×10^{-5} and V_{max} only 1/55 of the maximal velocity for cyclohexanone or acetaldehyde. Yet, at a concentration of 1.1×10^{-4} M, the steroid failed

to inhibit in any manner the reduction of equal concentrations of cyclohexanone ($K_m = 1.5 \times 10^{-2}$) or of acetaldehyde. Further, n-butanol at a concentration of 10^{-2} M exhibited virtually complete alternate product inhibition of the reduction of cyclohexanone and acetaldehyde but did not decrease the reduction rate of 5β DHT by more than 10%. The overlapping but independent activities may be explained on the basis of two independent sites on the same enzyme or by contamination of ADH by a small amount of steroid specific enzyme. However, specificity studies indicate marked similarities in the behavior of zone 1 towards steroid substrates, and zones 3, 4 and 5 towards certain bicyclic derivatives which may formally be considered as steroid analogs. Most striking is the finding that with zone 1, d-19-nor-17β-hydroxy-5α-androstan-3-one underwent reduction to the 3β-alcohol while the enantiomeric ℓ-compound was reduced to the 3α-alcohol. The enantiomeric 10-methyl-trans-2-decalones underwent reduction with zones 3, 4 and 5 in an identical steric manner (Graves, et al., 1965b) which leads us to believe that all of these enzymes are interrelated and bear certain common structural features that dictate steric specificity.

The fact that the individual zones were not altered by electrophoresis or chromatography clearly argues against multiplicity as an effect of enzyme-inhibitor or enzyme-coenzyme complexes. The precise relationship between the five different alcohol dehydrogenases can only remain speculative at the present time. Since all five enzymes were found to be present in crude extracts of horse liver, the possibility can be excluded that some of the zones are artifacts of the purification procedure. The differences between the Worthington and Boehringer enzymes in the relative concentrations of the individual zones may be explained on the basis of animal variation or by differences in the purification procedure. Specific anti-body studies are in progress to determine the relationship of the five enzymes.

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