ANTIBODY STUDIES WITH THE MULTIPLE ENZYMES OF HORSE LIVER ALCOHOL DEHYDROGENASE I.[1]

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Recent studies (Pietruszko, et al., 1966, Theorell, et al., 1966) have demonstrated that crystalline horse-liver alcohol dehydrogenase (ADH) consists of five electrophoretically separable components. The fastest cathodically migrating enzyme form (C_1) exhibits activity towards conventional LADH substrates, such as ethanol, acetaldehyde, cyclohexanone and, in addition, catalyzes the interconversion of 3-ketosteroids of the A/B cis series and the corresponding 3β-hydroxy compounds. This steroid activity persists in highly purified preparations of C_1 which are homogenous on gel electrophoresis. However, competitive and alternate product inhibition experiments demonstrate that the reaction of steroids and the reaction of "alcohols"[4] occurs at different binding sites. This and certain steroid considerations have led to the conclusion that C_1 is a form of alcohol dehydrogenase containing both an alcohol site and a steroid site in a single molecule.

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[4]The active site involved in the transformation of ethanol, acetaldehyde, cyclohexanone and other small substrates is referred to as the "alcohol" site.

In contrast the major ADH component (C_3) contains two identical "alcohol" sites and is devoid of steroid activity. The molecular weight (ca. 80,000) of C_1 and of C_3 are indistinguishable within experimental limits. In the present paper, we detail certain antibody studies that further substantiate the dual active site nature of C_1, the close relationship of C_1 and C_3, and the presence of a "steroid" site and an "alcohol" site attached to a single protein molecule (C_1).

Rabbit antiserum, which was prepared[5] against purified and electrophoretically homogenous C_3 as the stimulating antigen, was found to form a precipitate with C_1 as well as C_3. When antibody was added to C_1 and the resulting precipitate removed by centrifugation, both steroid activity and alcohol activity underwent a progressive decrease. Figure 1 illustrates the results of an

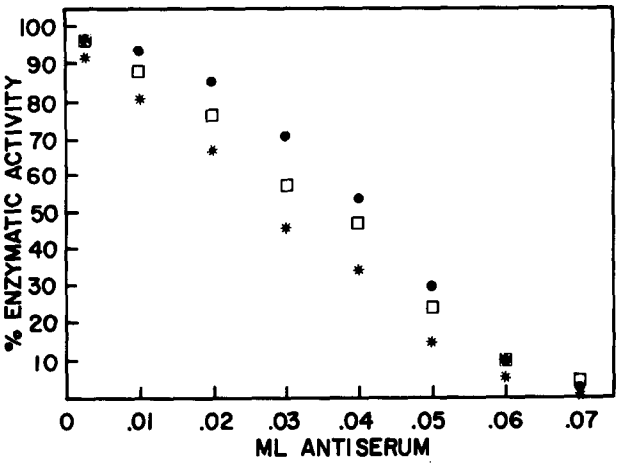

Figure 1: Enzymatic activity of C_1 and C_3 in the presence of increasing amounts of antiserum to C_3. High concentration of enzyme and antiserum; precipitate removed.

● - 5βDHT as substrate, enzyme C_1
* - cyclohexanone as substrate, enzyme C_1
□ - cyclohexanone as substrate, enzyme C_3

[5]We are grateful to Mr. J. Everse and Professor Nathan Kaplan, Brandeis University, for preparation of the antiserum.

experiment carried out with 17β-hydroxy-5β-androstan-3-one (5βDHT) as the representative steroid and cyclohexanone as the substrate for the "alcohol" site. Enzyme C_1 at a concentration of 90μg/7.25 ml was incubated with increasing volumes of undiluted antiserum in the presence of NADH, the antibody-enzyme complex removed and the enzymatic activity remaining in solution determined spectrophotometrically by addition of the appropriate substrate. With the addition of .07 ml of antibody solution, both the steroid and cyclohexanone activity fell to less than 5% of the respective control values. This volume of antiserum appears to be the equivalence point for a 1:1 insoluble complex with the enzyme. The fact that both the steroid and cyclohexanone enzymatic activity are precipitated by antiserum to an enzyme free of steroid activity (i.e., C_3), and at essentially the same equivalence point, strongly argues that the two sites are part of the same enzyme molecule.

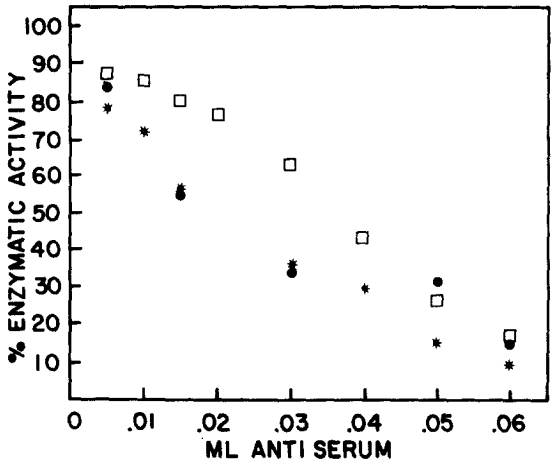

Figure 2: Enzymatic activity of C_1 and C_3 in the presence of increasing amounts of antiserum to C_3. Low concentration of enzyme and antiserum; precipitate removed.

● - 5βDHT as substrate, enzyme C_1
* - cyclohexanone as substrate, enzyme C_1
□ - cyclohexanone as substrate, enzyme C_3

Figure 2 illustrates an experiment identical to that described above, except that the enzyme and the antiserum were diluted 1:10 prior to mixing. It may be noted from the initial portion of the curves that for a given antiserum ratio there was a greater relative decrease of enzymatic activity in the more dilute reaction than in the concentrated one, although the equivalence point was essentially similar in both experiments. The curves for 5βDHT and cyclohexanone (Figure 2) were found to be virtually superimposable while in the more concentrated solution (Figure 1) the reduction of 5βDHT activity was less than that of cyclohexanone when enzyme was present in excess.

In Figure 3, the enzymatic activity is shown when the antigen-antibody complex which had been formed in dilute solution was not removed. Under these conditions the decrease in enzymatic activity was less for a given volume of antiserum and established that the antigen-antibody precipitate retains some enzymatic activity

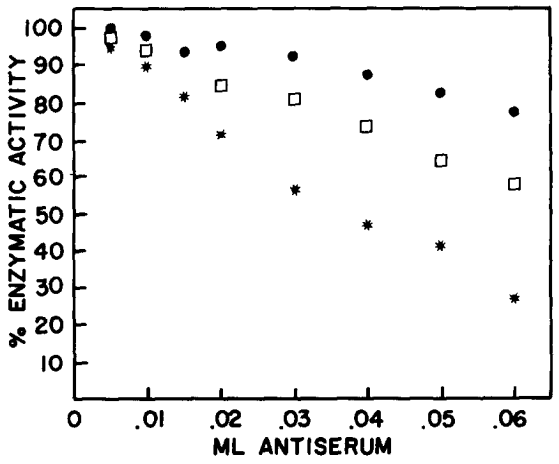

Figure 3: Enzymatic activity of C_1 and C_3 in the presence of increasing amounts of antiserum to C_3. Low concentration of enzyme and antiserum; assay carried out in the presence of precipitate.

● - 5βDHT as substrate, enzyme C_1
* - cyclohexanone as substrate, enzyme C_1
□ - cyclohexanone as substrate, enzyme C_3

which was readily confirmed by visual staining of the isolated precipitate. The steroid activity remained particularly high with 77% retained in the presence of .06 ml of antiserum. These results indicate that the initial interaction of C_1 and antibody occurs in such a manner that the enzymatic activity of the "alcohol" site is altered while the steroid site remains relatively unaltered. Therefore, in the presumed 1:1 complex, steroid activity will be significantly reduced only when the precipitate is removed.

In Figures 1, 2 and 3, the effect of increasing antibody concentration on the enzymatic activity of the homologous antigen, enzyme C_3, is included for comparison. Cyclohexanone was the substrate and the concentrations of C_3 and C_1 were kept identical in each experiment. It may be noted that in all three experiments the percentage decrease in enzymatic activity was less for C_3 than for C_1 until the equivalence point was reached and the precipitate was removed. The difference in behavior may be seen most readily in Figure 3, which indicates that for a given volume of antiserum, the decrease in C_3 activity was approximately one half of the C_1 decrease. Bearing in mind that C_3 contains two apparently identical "alcohol" sites, this data indicates that the first equivalent of antibody interferes with the catalytic activity of only one of the two sites.

The relationship of the multiple forms of alcohol dehydrogenase will be discussed in a forthcoming paper (Pietruszko, et al., 1969) where evidence will be presented that C_3 contains only identical subunits, while C_1 is a hybrid enzyme containing a subunit identical with that from C_3 and a new subunit which forms the steroid site.

Procedure:

Antiserum Preparation[5] - ADH (C_3) at a concentration of 250µg/ml in .02M tris-HCl pH 7.5, was diluted with an equal volume of Freund's adjuvant and four weekly injections of 0.5 ml each administered into the toe pad of a rabbit. Seven days after the last injection a booster dose of 250µg of enzyme in 1 ml of buffer was administered intravenously and the rabbit bled five days later. The blood was allowed to stand for 24 hr. at room temperature, the serum was then poured off and spun for 10 min. to remove residual blood cells. The clear serum was active against C_3 at a dilution of 1:18,000 in the micro-complement fixation test of Wasserman and Levine (1960) and gave a single Ouchterlony diffusion band with C_3 but none with C_1. Aliquots were frozen and thawed immediately before use.

Interaction of Antiserum with ADH-C_1 and -C_3

(A) Concentrated Solutions - The enzyme (C_1 or C_3 [90µg]) in .1 ml of buffer was added to a series of ice-cooled tubes containing graded concentrations of antiserum (0-.07 ml) and NADH (1 mg) in .03M phosphate buffer pH 7.0. The final volume was 7.25 ml in each case and the protein concentration was kept constant at 9.8 mg in each tube by the prior addition of the requisite amount of bovine serum albumin. The mixtures were allowed to stand for 66 hr. at 4° and then centrifuged for 70 min. at 34,000 rpm. An aliquot of 2.9 ml of clear solution from each tube was transferred to a cuvette for determination of enzyme activity. The enzymatic reactions at 27° were initiated by the addition of cyclohexanone (169µg) or 5βDHT (100µg) contained in .01 ml of methanol and followed on a Beckman DUR Spectrophotometer with a Gilford 2000 attachment by the disappearance of the 340mµ NADH maximum. The control tube lacking antiserum showed virtually no decline of enzyme activity during the period of the experiment.

(B) Dilute Solutions - The procedure was identical with A, except that only 9µg of enzyme was added and the antiserum solution was diluted 1:10 prior to addition. With these lower concentrations it was also possible to carry out enzymatic assay in the presence of the antibody-enzyme complex.

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[5] Ibid.