INACTIVATION OF ES AND SS ISOZYMES OF HORSE LIVER ALCOHOL DEHYDROGENASE BY 2R, 4S-2, 4-CYCLO-5α-ANDROSTANE-3α, 17B-DIOL, A CYCLOPROPYL STEROID

PAUL DOUGLAS SKIRVING

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by

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ABBREVIATIONS

DHT	Dihydrotestosterone
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
HLADH	Horse Liver Alcohol Dehydrogenase.
Thr	Threonine
Val	Valine
Gly	Glycine
Ile	Isoleucine
Leu	Leucine
Asp	Aspartate
ADPR	Adenine Phosphate Ribose
tlc	Thin Layer Chromatography
hplc	High Performance Liquid Chromatography
DEAE	Diethylaminoethyl
CM-52	Carboxymethyl
THF	Tetrahydrofuran
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
n.m.r.	Nuclear Magnetic Resonance
p.m.r.	Proton Magnetic Resonance
P.p.m.	Parts per million

1. Review of the Literature

1. A. Horse Liver Alcohol Dehydrogenase.

i. Isolation and Purification

One of the first successful attempts to isolate horse liver alcohol dehydrogenase (HLADH) was reported by Bonnischsen and Wassén (1) in 1948. They extracted ground horse liver with water, separated the aqueous extract by centrifugation and precipitated an active dehydrogenase fraction with ammonium sulphate. A further purification was effected by ethanol precipitation from phosphate buffer.

Ten years later, Dalziel (2) separated a more acidic HLADH fraction using an ion exchange column (carboxymethyl cellulose). Up to 4 HLADH fractions, later termed isozymes, could be resolved by starch gel electrophoresis (3). Ethanol was found to be a substrate for HLADH; later, Ungar (4,5) noted the NAD - dependent interconversion of certain 3-keto and 3β-hydroxysteroids. These reactions formed the basis for the classification of HLADH isozmes, as discussed in Section ii.

By 1970, HLADH had been separated into 12 distinct bands by electrophoresis (6). Von Wartburg (7) separated HLADH into its major components by chromatography on carboxymethyl cellulose. Starch gel electrophoresis followed a further diethylaminoethyl (DEAE) cellulose column, and showed homogeneous components.

An important advance in the purification of HLADH was the use of affinity techniques which permitted the development of a single step purification of HLADH from the crude mixture of the enzymes (8). Affinity techniques generally involved an immobilized cofactor (NAD or AMP) or inhibitor (pyrazole) attached to a support (9, 10, 11, 12). Dehydrogenases selectively bind to the cofactor or inhibitor presumably by accepting the ligand into some enzymic pocket and forming a stable binary complex (13). The enzymes are then differentially eluted as a cofactor or inhibitor is applied in the eluent. An interesting development is the use of an immobilized enzyme (HLADH) in the purification of a mixture of cofactors (14).

ii. Structural Features

By 1967 it was known that HLADH was dimeric and comprised of two subunits (15). Steroid activity was associated with only one type of subunit, while ethanol activity was found in both. Theorell (16) named the two types of protein chains E (for ethanol active) and S (for steroid active). The main isozymes were thus named EE, ES and SS. SS is the most basic (pI = 10), ES is intermediate (pI = 9.3) and EE is the least basic (pI = 8.7) (7).

Commercial preparations of HLADH contain EE sometimes with a small amount of ES. Crude horse liver homogenates contain relatively large amounts of EE and ES with considerably less SS (observation by Mr. G. Murphy of our laboratory).

The amino acid sequence of the E subunit was determined by Jornval (17), and is seen in Fig. 1. The sequence for S was not determined de novo but by detecting the peptides in the S subunit that differed on electrophoresis from the E peptides. Each subunit contains 374 amino acids. The E to S exchanges are shown below the E sequence. There are only 6 amino acid differences. Of these known differences, the aspartate to serine change is not entirely certain. It is possible that there has been a deletion rather than a serine substitution (17). Undetected changes which would not cause a difference in the charge of the peptide fragment used in the sequencing may also occur. The amino acid exchanges resulted in the S chain being three units more positively charged than the E. This is in agreement with the measured electrophoretic mobilities of the isozymes containing the S subunit. The molecular weight, originally determined to be 80,000 - 88,000 (18, 19, 20) was calculated to be 79,958 (17).

Minor intermediate fractions are seen on electrophoresis (3, 7, 21). A complete schematic pattern of electrophoretic separation is shown in Fig. 2.

A third type of subunit, named A, has been reported (22). Electrophoretically it behaves like S but it is not a steroid dehydrogenase. The composition of this subunit has not yet been reported. It can be distinguished from S by its much higher activity towards acetaldehyde. Apparently, few horses have the A variant.

Horse liver alcohol dehydrogenase contains four Zn^{++} ions per molecule; two ions per subunit (23). The Zn^{++} is probably coordinated in an octahedral configuration (24). Loss of two of the four Zn^{++} ions resulted in the loss of the catalytic activity of the enzyme (15). The tertiary structure of the enzyme was maintained. It was thus suggested that HLADH contains two different kinds of Zn^{++}. In the subunit one Zn^{++} is required for the catalytic activity and is called the catalytic Zn^{++}; the other may be required for the maintenance of the tertiary structure and is called the noncatalytic Zn^{++}.

The Zn^{++} - free apoenzyme of HLADH binds NAD, NADH and ADP - ribose and forms ternary complexes with the coenzyme and substrate competitive inhibitors (25). Their dissociation constants are similar to those of the native enzyme.

The three dimensional structure of horse liver alcohol dehydrogenase was determined by X-ray crystallography in 1976 (26). Each subunit contains two domains; one which binds the coenzyme and one which contains the two Zn^{++} ions. One Zn^{++} is located near the active site of the enzyme (catalytic Zn^{++}), while the other is held internally (noncatalytic Zn^{++}) see Fig. 3. The crystals of the native enzyme are not isomorphous with those of the coenzyme complex (27), but the tertiary structures are sufficiently similar that discussion of the active site on the basis of these structures is probably valid.

Examination of a model of HLADH with ADP-ribose in position (obtained from Vis-Aid Devices, 857 Mulvey Ave., Winnepeg, Manitoba, Canada) suggested that the corresponding portion of NAD sits in the same location since this analogue competes with the coenzyme at the binding site (28, 29). If a NAD molecule is constructed to the same scale as the model and placed so that the ADP-ribose functions coincide with that of the analogue, the nicotinamide moiety fits nicely into a pocket comprised of the residues Thr-178, Val-203, Val-292, Gly-293 and Ile-318 (30). This fully extended conformation has been shown to be very similar to that of NAD in lactate dehydrogenase (31). In this form the carboxyamide of the nicotimamide can easily hydrogen bond to Thr-178 and the carbon C-4 is 4.5 Å from the catalytic Zn^{++} (32). The A side of the cofactor faces the Zn^{++}, in agreement with the known 4-pro-R stereochemistry of hydride transfer (32).

On examining the active site in the EE isozyme, an amino acid corridor is evident. Brändén (33) calls this the "hydrophobic barrel", see Fig. 4. The catalytic Zn^{++} is located at the end of this tunnel. On the basis of best fit of substrate and the facility of hydride transfer, Brändén has identified potential substrates and predicted their relative Km values. It should, however, be noted that Km is related to "stickiness" only if the "substrate-on" rate is significantly rate-determining.

Fig. 5. presents a different view of the active site of an E-subunit. The steroid substrate, 3β-hydroxy-5β-cholanoic acid, experiences an unfavorable interaction between its C-18 methyl and Leu-116. This interaction presumably prevents the steroid from binding in the assumed productive orientation in the E subunit. The corresponding 3α-hydroxy-5α-steroid has most of its body out of the active site in a space densely occupied by amino acids of the protein chain. Brändén has predicted that an 18-nor steroid (5α or 5β) should be a substrate for the EE isozyme because it lacks the critical C-18 --- Leu-116 repulsion (33).

Because the E and the S subunits differ in only 6 detected amino acids out of 374 in the subunit, it is expected that both will have the same general conformation and indeed the ES isozyme has been crystallized (34). One expects the differences to be confined to the areas of these exchanges.

One of the amino acid exchanges is at amino acid 115 where aspartate is changed to a serine. It is conceivable that this change may result in a loss of the repulsion of Leu-116 with the steroid C-18. The Leu-116 may bend out of the hydrophobic barrel thus allowing steroids access to the active site. A salt bridge exists from Asp-115 to Arg-120, see Fig. 5. This apparently produces this steric "hump" in the active site. The exchange of Asp-115 for serine, as seen in the S subunit, may remove this hump and enable steroid molecules to function as substrates for the enzyme.

iii. Catalytic Properties

Horse liver alcohol dehydrogenase (EC 1.1.1.1.) catalyses the oxidation of a wide variety of alcohols to their corresponding aldehydes and ketones with nicotine adenine dinucleotide (NAD) as cofactor. This conversion is reversible. The 4 - pro-R hydrogen of NADH (denoted H*) is probably transferred as a hydride anion or it's equivalent to the 1 - pro-R position of simple primary alcohol products such as ethanol, see Fig. 6. As a steroid dehydrogenase, oxidation and reduction at the 3-position has been reported only for 3β- alcohols. In Fig. 6 these two reactions are shown in the same orientation.

A unique, compulsory ordered kinetic mechanism, described by Theorell and Chance (35), applies to the primary alcohol dehydrogenase reaction catalysed by HLADH. It is characterized by the following sequence of steps.

1. Coenzymes bind to the free enzyme before the substrate.
2. The ternary complexes, enzymes-NADH-aldehyde and enzyme-NAD-alcohol are in rapid equilibrium.
3. The substrate is less tightly bound to the enzyme than to the coenzyme and therefore the ternary complexes liberate the substrate first.
4. The dissociation of the binary enzyme-coenzyme complex is the last phase of the reaction and is rate limiting, when both substrate and coenzyme are

at high concentration. This mechanism does not apply to substances such as secondary alcohols (36, 37).

It was noted that when methanol or isopropanol are substrates, ternary complex interconversion is the rate limiting step if both substrate and coenzyme are present in saturating (equimolar) conditions (38).

Isotope effects for deuterium (up to kH/kD = 6) and tritium transfer have been observed. The catalytic mechanism suggested by Brändén (39) is seen in Fig. 7. X-ray structure information suggests that the Zn^{++}, the zinc-bound water molecule, the hydroxyl group of Ser-48 and the imidazole group of His-51 may be involved in the binding and polarization of the reactive part of the substrate. This process is mediated by electrophillic catalysis by the active site Zn^{++}. The alcohol may bind to Zn^{++} as the negatively charged alcoholate ion, see Fig. 7a.

Recent structure-activity studies seem to indicate that no charge develops on the substrate during catalysis (40). Therefore, the release of the proton of the alcohol and the hydride transfer occur simultaneously. In this mechanism, Fig. 7b, the hydroxide ion remains bound to Zn^{++}; the Zn^{++} bound hydroxide ion is thought to be the general base catalyst for the oxidation of alcohols (41). Correspondingly, the Zn^{++} bound water molecule would be the acid catalyst for the reduction of aldehydes in the acid - base catalysis mechanism.

iv. Effect of Stimulators and/or Inhibitors on Structure/Activity Relationships of HLADH

The major form of HLADH found in liver is EE. Commercial as well as laboratory preparations of HLADH produced by the method of Bonnischsen and Wassén contain from 50 - 80% EE isozyme (observation of G. Murphy of our laboratory).

Both E and S forms oxidize ethanol. The S form catalyses the oxidation at 1/10 the rate of the E form (42). The S form catalyses the oxidation of the same substrates as the E subunit and in addition oxidizes 3β-hydroxy steroids. Steroids with an A/B cis configuration (5β-hydrogen) are preferred and oxidized at approximately ten times the rate of similar steroids with an A/B trans configuration (5α-hydrogen) (43, 44). The EE enzyme is apparently not inhibited by steroids indicating that binding does not occur (42).

CHEMICAL MODIFICATION OF HLADH

a. Cysteine Residues

HLADH is inhibited by mercurials and other thiol reagents (24). Two active site cysteine residues were identified using iodoacetate (Cys-46 and Cys-174) (45, 46, 47, 48, 49, 50). Cysteine residues have been alkylated with a salicylate molecule substituted with mercury (39). Cys-46 and Cys-174 modification inactivated both the E and the S subunits, however, the enzyme was protected against

inactivation by NAD and NADH. Cys-46 and Cys-74 are located in the amino acid-lined cavity through which the cofactor is extended; prior binding of the cofactor would be expected to prevent entry of the chemical modifier.

b. Lysine Residues

Plapp (51) found that methyl picolinimidate enhanced the activity of HLADH. Most of the lysine ω-amino residues were modified; but it was found tha the activation was the result of only one amino group substitution per molecule. The lysine group was protected from modification by NAD and pyrazole or NADH and isobutyramide (52), and was thus thought to be near the coenzyme binding site. This residue has been identified as Lys-228 (53, 54) which is very close to the adenine moiety of the cofactor; modification probably results in removal of a steric crowding.

Methylation of lysine residues by reaction with formaldehyde and sodium hydroxide was found to activate the enzyme (55, 56).

Imido esters (52) have been used to introduce radioactivity into HLADH without loss of activity.

c. Histidine Residues

Histidine residues have been preferentially destroyed by photooxidation (57, 58). The degree of activity loss varies with different coenzyme analogues and the affected residues have not been identified.

d. Tyrosine Residues

Iodination of tyrosine does not affect enzyme activity indicating that there are apparently no strategic tyrosine residues in the enzyme or that iodination did not occur, or that the space so filled was not critical for the substrate (ethanol) used in the assay (59).

e. Arginine Residues

Enzyme activity is lost when butanedione and phenylglyoxal are used to modify the enzyme arginine residues (60). There may be two arginine residues associated with the loss of enzyme activity, but so far these have not been identified. The enzyme is protected against the arginine modification and the loss of enzyme activity by NADH. It is thought that the arginine residues participate in the binding of the cofactor. Arg-47 which lines the cavity through which the cofactor extends is the most likely residue affected. Modification of this residue may result in the inability of the cofactor to bind to this cleft.

There are two atoms of Zn^{++} present per subunit in the enzyme (23). The Zn^{++} associated with the active site (catalytic Zn^{++}) is that first to be removed during dialysis. If all four zinc ions are removed, the enzyme remains dimeric, hence the second Zn^{++} is not necessary for subunit association. HLADH activity is retained when Zn^{++} was substituted by other divalent cations such as Co^{++} and

Cd^{++} (61, 62, 63). HLADH loses activity and Zn^{++} at pH = 5 and remains in a dimeric state (64, 65). At lower pH the enzyme dissociates into subunits in 7 to 8 M urea (18, 66, 67, 68), 5 to 6 M guanidine HCl (69, 70) and in 5 mM dodecylsulfate (71).

NAD and NADH protect against thermal inactivation (72, 73).

1. Review of the Literature (continued)

1. B. Chemistry of Cyclopropanols

i. Use as Substrate

Simple cycloalcohols have rarely been studied as substrates for HLADH. It is known that cyclohexanol and cycloheptanol are substrates but not cyclopentanol (74, 75, 76). Cyclopropanol is of special interest; if this compound is a substrate for the enzyme, then, following oxidation, cyclopropanone would be produced. Cyclopropanone is a very unstable species: it is not known at room temperature but has been observed below -78°C (77). There is good evidence that cyclopropanones such as 2 are intermediates in the Favorskii Rearrangement, see Fig. 8a., although it has not been isolated and identified. Hydroxyl (OH^-) attack on this cyclopropanone would give the product that indeed is isolated in the reaction (78, 79).

Using this rearrangement as a model, we would expect that if a cyclopropanol were converted to the cyclic ketone then attack by a nucleophile in the active site would cause covalent attachment of the substrate, presumably by a mechanism such as that seen in Fig. 8b.

Recently cyclopropanol has been shown to inactivate methanol dehydrogenase isolated from Methylomonas methanica strain S1 (80). This is presumed to be a mechanism-based or suicide inactivation of the enzyme. When radioactive

cyclopropanol was used, the radioactivity was seen to be associated with the enzyme following oxidation.

In order to attach a steroid covalently to the S subunit, it would first be necessary to incorporate the cyclopropanol functionality into the steroid. Since the steroid active enzyme is a 3β-hydroxy steroid dehydrogenase, the alcohol functional group of the cyclopropanol must be in the 3-position of the steroid. This would restrict the A-ring of the steroid to the type shown as structure 7 of Fig. 8.

A literature search revealed that compound 8 had been synthesized in 1975 by Templeton and Wie (81) from 5α-DHT. Both isomers at the 3-position (3α- and 3β-hydroxy) had been synthesized and separated. It is known that the closely similar 5α-androstane-3β,17β-diol is a substrate for the enzyme (83).

ii. Synthesis of Cyclopropanols/Cyclopropanones

Cyclopropane has been synthesized in high yield by treating 1,3-dibromo compounds with sodium metal in refluxing solvents (Wurtz Reaction) (84). Bicyclobutane has also been prepared in this fashion by the action of sodium metal on 1,3-dibromocyclobutane (85). It is not known if cyclopropanols can be prepared by this method.

The synthesis of secondary cyclopropanols was first described in 1942 (86). Magnesium bromide, ferric chloride, ethyl magnesium bromide and acid were added successively to

epichlorhydrin (9), see Fig. 9. The reaction presumably occurs by the mechanism shown in the same figure. Methyl lithium and lithium aluminum hydride have also been used to cleave cyclopropyl acetates 16 generated from the thermolysis of pyrazole-1-in-3-yl acetates (87), or the Baeyer-Villiger rearrangement of the ketone 13 (88). The Simmons-Smith cyclopropanation of vinyl acetates 15 does occur, but with poor yields (89).

iii. Synthesis of 2,4-cyclosteroids

In 1975 Templeton and Wie (81) published the synthesis of 2R,4S-2,4-cyclo-5α-androstane-3α and 3β,17β-diol-17 acetate 21a and 22b, see Fig. 10. As mentioned in the introduction (part 1. B. i.) the 3β-cyclopropanol was of special interest because of its potential as a substrate for 3β-hydroxy steroid dehydrogenase. Templeton treated 5α-DHT, 18, with excess bromine to form the dibromo ketone 19. This reaction occurred in high yield (>90%). Reduction with sodium borohydride gave a mixture of the 3α- and 3β-hydroxy epimers 20a and 20b. Fractional crystallization was used to separate the alcohols but this generally gave low yields of pure compounds 21a and 21b. The ratio of the two isomers was found to be 3α/3β = 4 (G.C. of the silyl ethers). A 2% (by weight) zinc/copper couple was found to give a low yield of the steroid cyclopropanols 8 from the dibromoalcohols (<10%).

1. Review of Literature (continued)

1. C. Objectives of the Study

The circulating levels of the hormonal steroids in the blood, generally 10^{-5} - 10^{-10} M, are such that tight (high affinity) binding to specifically tailored regions of proteins is of critical importance in steroid hormone action. Steroid hormones exert their effect by modifying the specific receptor proteins so that they, in turn, pass on the hormonal signal to regulate processes within the cell. The high affinity of steroid precursors for the biosynthetic enzymes and for the steroid degradative enzymes is, of course, important in controlling the level of those hormones in the blood.

The purpose of the proposed study is to attach a steroid by a covalent bond to the active site of an enzyme as a first step in obtaining a better understanding of the interaction between steroids and the active sites of protein molecules. Our understanding of the amino acid residues actually involved in forming the (presumably) hydrophobic pocket suitable for interaction with steroids is still in its infancy. One can expect that amino acids such a tryptophan, phenylalanine and tyrosine, being planar and, in part, hydrophobic are likely to be involved, but this has not yet been established. At this stage of our understanding, it is important to study the interaction of

steroids with pure, well defined proteins about which much is already known. The hydroxysteroid dehydrogenases are a group of such proteins.

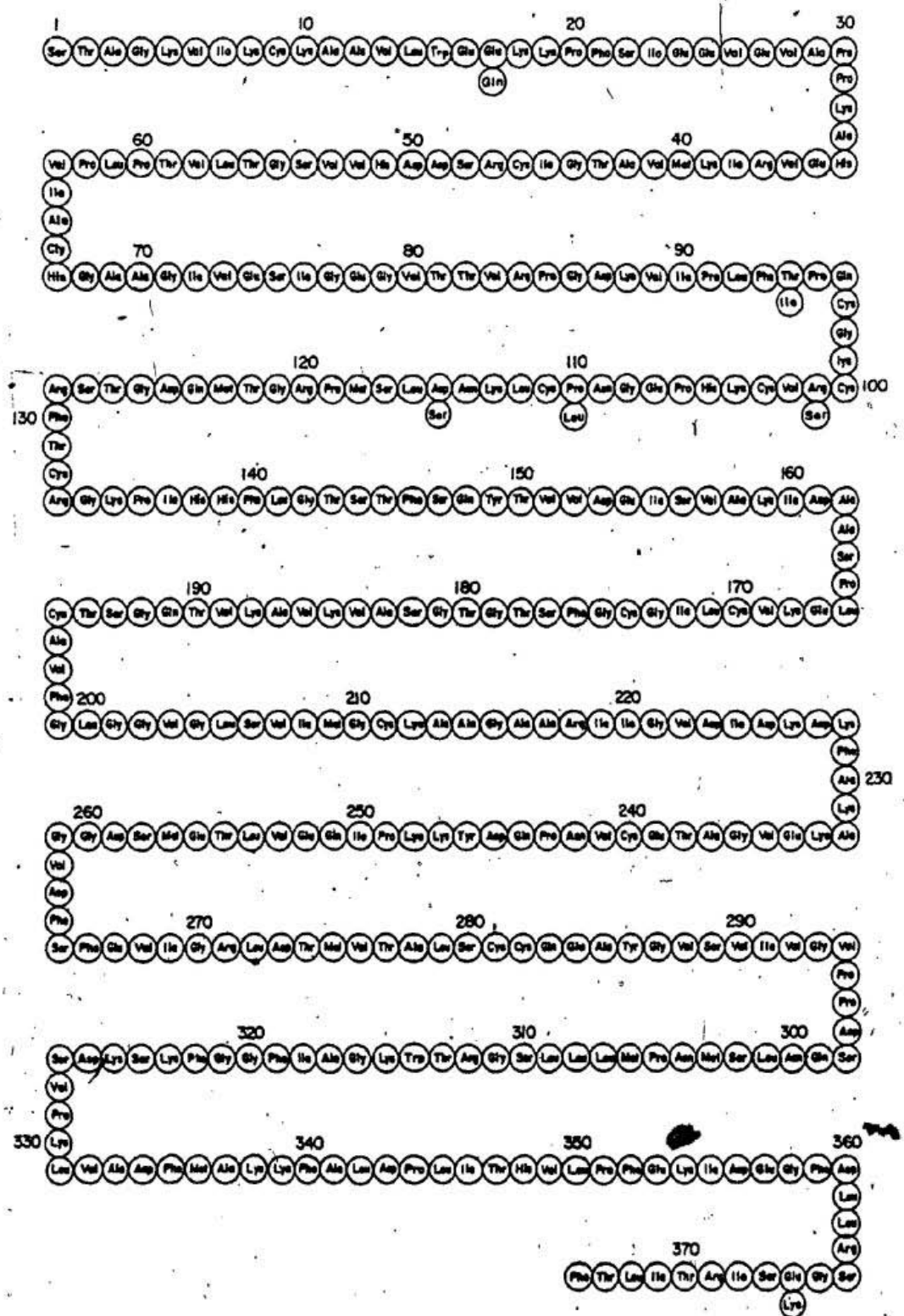
Horse liver alcohol dehydrogenase, in particular, presents some interesting features. The normal substrate for this enzyme appears to be ethanol, which is converted to acetaldehyde. There are apparently two major isozymes of the dehydrogenase; the E and the S. Since the enzyme is dimeric, the active enzyme apears to be either EE, ES or SS. Only ES and SS have activity in the reversible reduction of steroid 3-ketones to the corresponding 3β-alcohol (44). Of particular interest is the fact that the E and the S isozymes differ in only six known amino acid residues out of 374 (17). There are thus two closely similar amino acid sequences in these two isozymes, only one of which is capable of binding and transforming steroid ketones.

Having chosen the enzyme, the choice of the reactive steroid was made. Covalent attachment of steroid to enzyme could be effected by the use either of a reactive substrate or by the use of an unreactive substrate specifically designed to be transformed by the enzyme to a highly reactive (electrophilic) product. The later class of inhibitors are termed "suicide" or "mechanism based" inhibitors (90). A heretofore unexplored type of suicide inhibitor involves the enzymic formation of a highly electrophilic cyclopropanone group. Since a survey of the literature revealed that the appropriate 3-hydroxy-2,4-

cyclosteroids had been synthesized (81), this group was chosen for study.

This thesis describes the purification and characterization of the enzymes and the 2,4-cyclosteroids, the discovery of a new method of chemical synthesis of cyclopropane rings and the inactivation of the enzyme by one of the steroidal cyclopropanols, together with preliminary evidence that covalent linkage has occurred.

Fig. 1. Amino Acid Sequence of the E Subunit of Horse Liver Alcohol Dehydrogenase (S Subunit Exchanges Included). Jörnval et al (17).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Pro
Lys
Ala
60
50
40
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Ile
Ala
Gly
70
80
90
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Ile
Cys
Gly
Lys
120
110
100
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150
160
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Ala
Ser
Pro
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180
170
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Ala
Val
Phe
200
210
220
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Phe
Arg
Lys
230
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250
240
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Val
Asp
Phe
270
280
290
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Pro
Pro
Asp
320
310
300
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Pro
330
Lys
340
350
360
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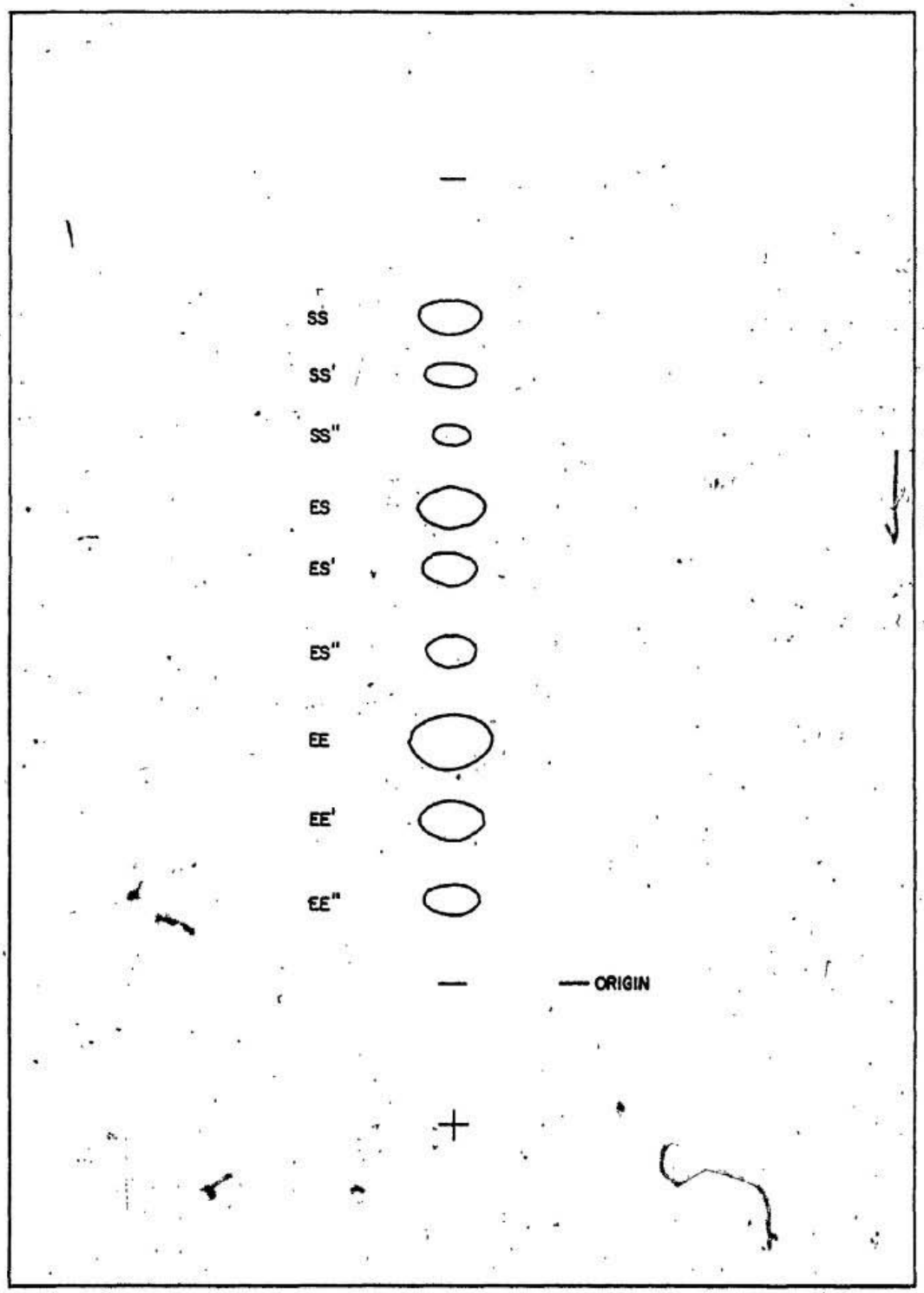
Leu
Leu
Arg
370
Phe Thr Leu Ile Thr Arg Ile Ser Glu Gly Ser
Lys

Fig. 2. Schematic Pattern of Electrophoretic Separation of the Components of HLADH. Pietruszko (21). (Starch Gel; Buffer, Tris.HCl, pH = 8.5)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SS'
SS"
ES
ES'
ES"
EE
EE'
EE"
ORIGIN

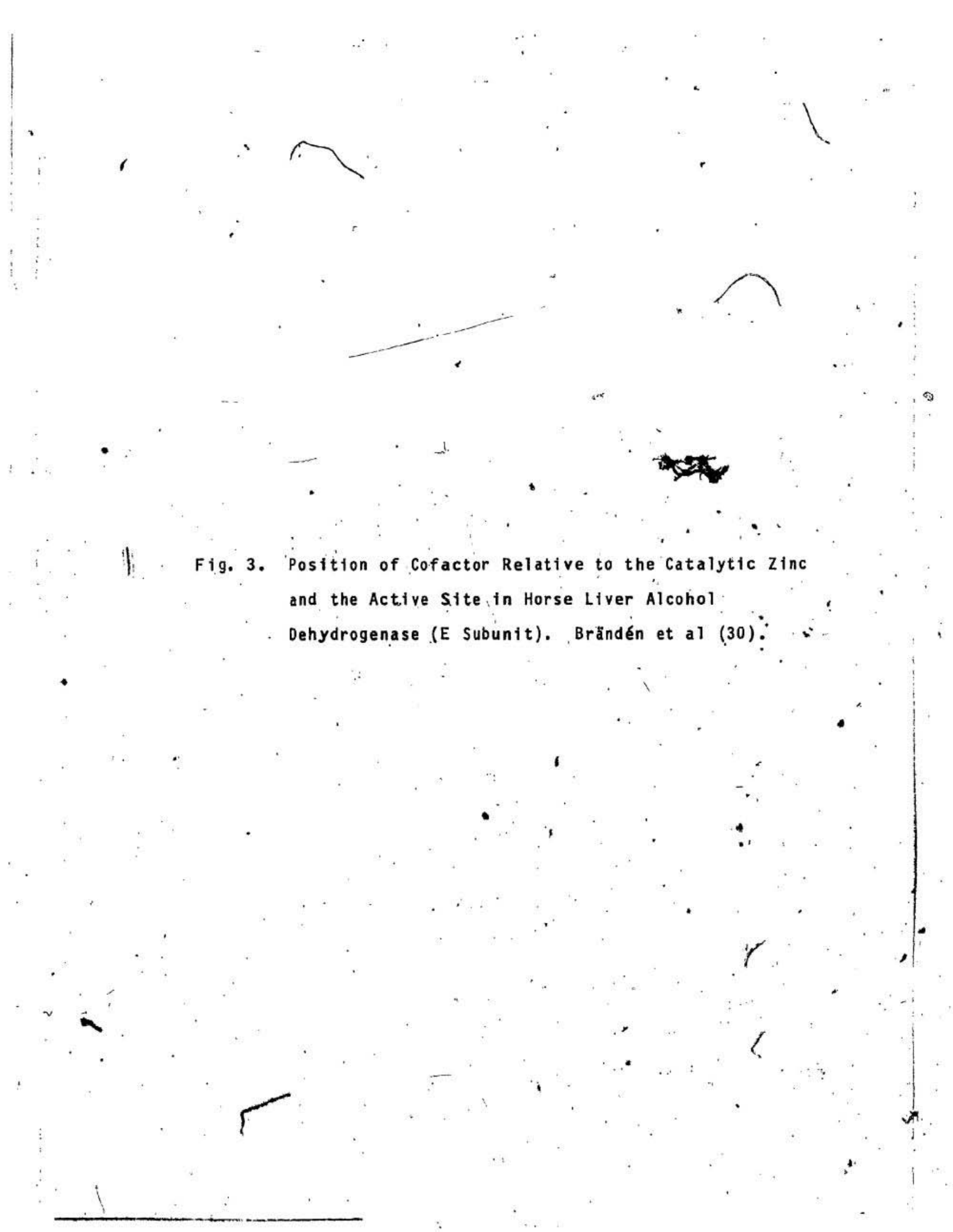


Fig. 3. Position of Cofactor Relative to the Catalytic Zinc and the Active Site in Horse Liver Alcohol Dehydrogenase (E Subunit). Brändén et al (30).

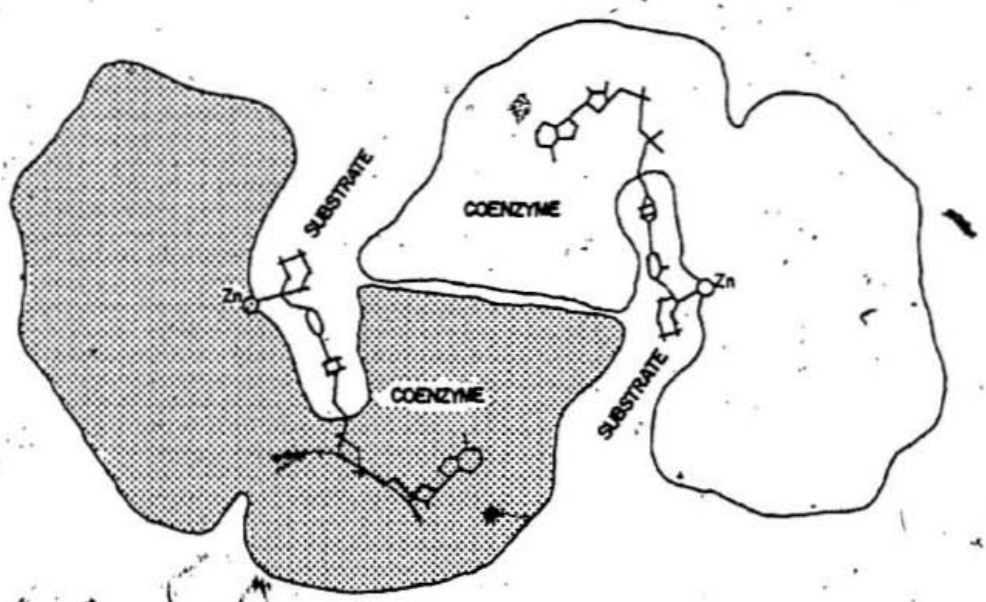
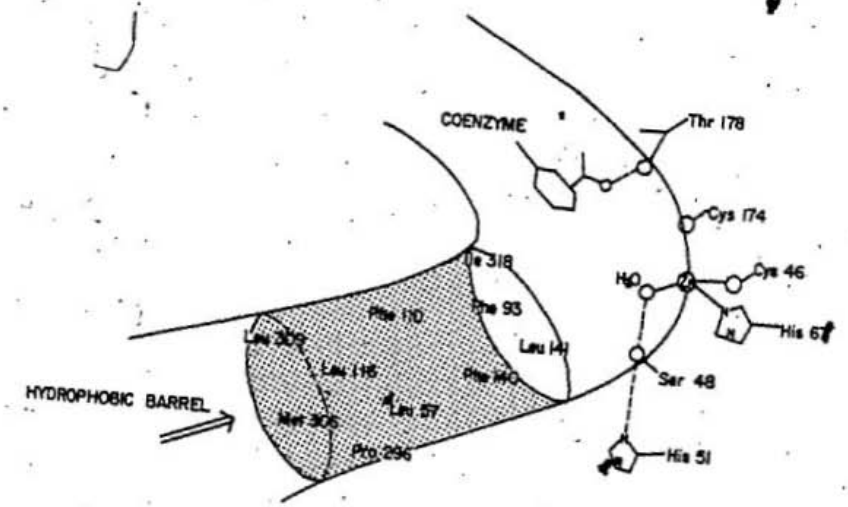

SUBSTRATE
COENZYME
Zn
COENZYME
SUBSTRATE
Zn

Fig. 4. Hydrophobic Barrel in Active Site in the E Subunit of Horse Liver Alcohol Dehydrogenase. Brändén et al (30).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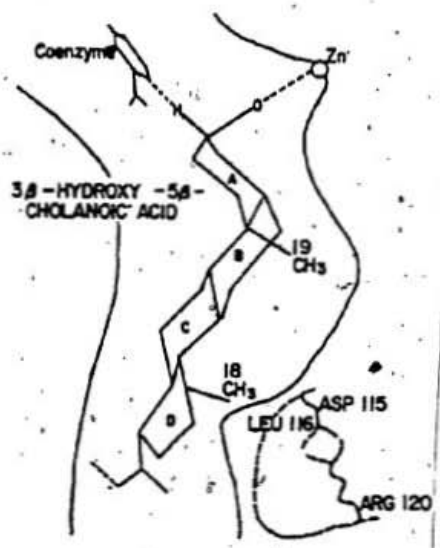
His 67
Leu 309
Leu 44
Leu 116
Phe 140
Ser 48
HYDROPHOBIC BARREL
Leu 57
Met 305
His 51
Pro 296

Fig. 5. Steroids Placed in the Active Site of the E Subunit of Horse Liver Alcohol Dehydrogenase. Brändén et al (30).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N
O
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B
C
D
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LEU 116
ARG 120

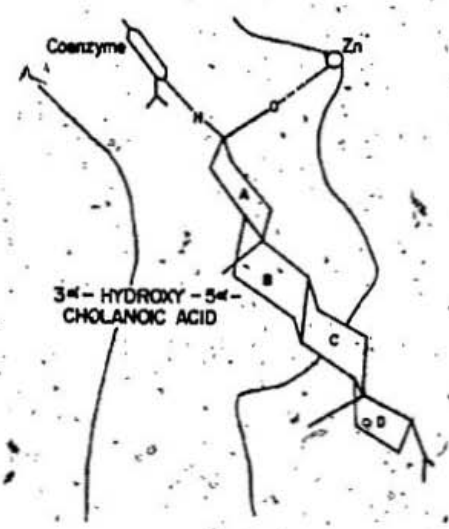
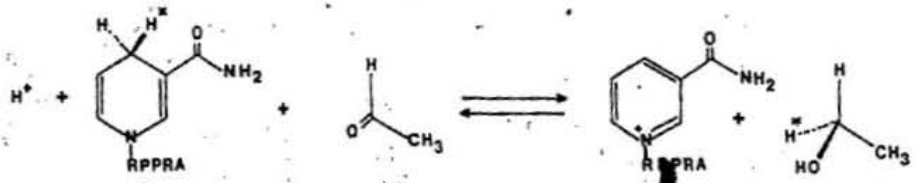

Coenzyme
Zn
N
O
3α-HYDROXY-5α-CHOLANOIC ACID
A
B
C
D

Fig. 6. Stereochemistry of the Reversible Reduction of Ethanol and 5α-DHT by Horse Liver Alcohol Dehydrogenase.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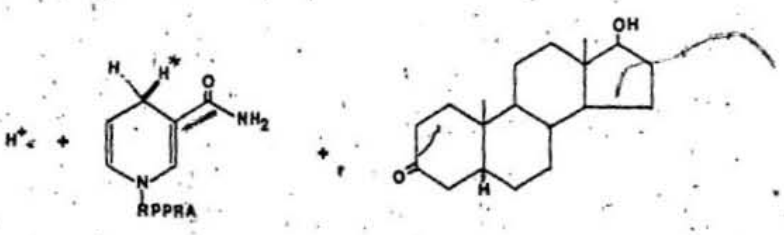
RPPRA
CH3
HO


H+
NH2
RPPRA
OH
O

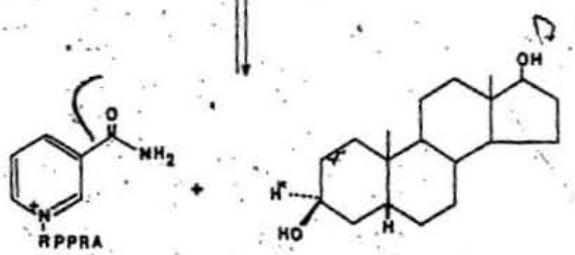
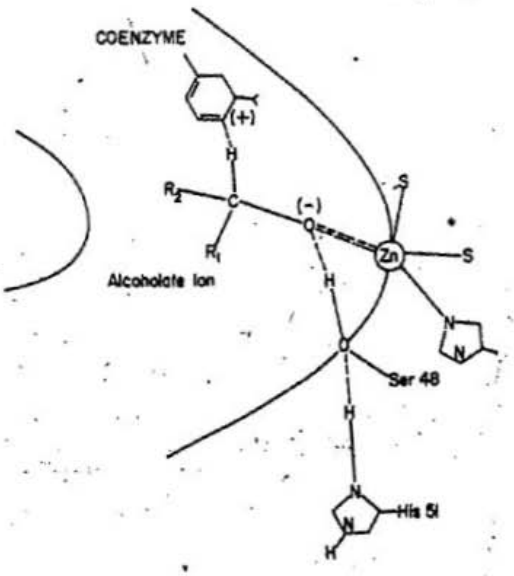

NH2
RPPRA
OH
HO

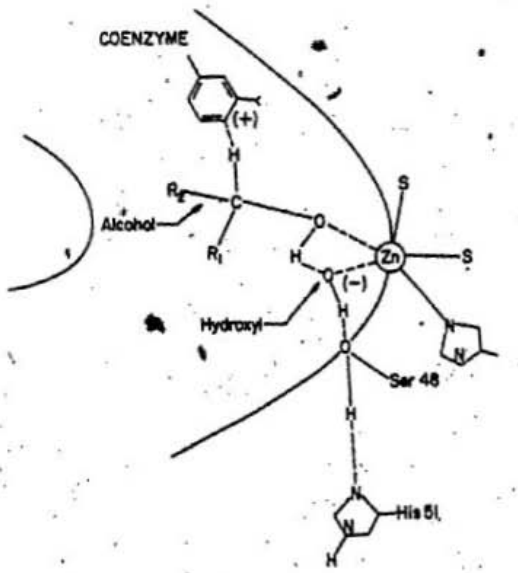
Fig. 7. Diagram of the Assumed Productive HLADH - NAD^+ - Alcohol Complex Suggested by:

a) Brändén et al (39).

b) Dworschack and Plapp (40).


COENZYME
(+)
H
R2
C
(-)
O
R1
Alcoholate ion
S
Zn
S
H
O
Ser 48
N
N
H
N
N
H
His 51

a

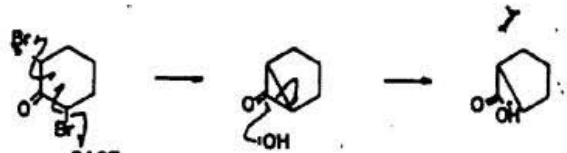

COENZYME
(+)
H
R2
C
O
Alcohol
R1
H
O
(-)
H
Hydroxyl
S
Zn
S
O
Ser 48
N
N
H
N
N
H
His 51

b

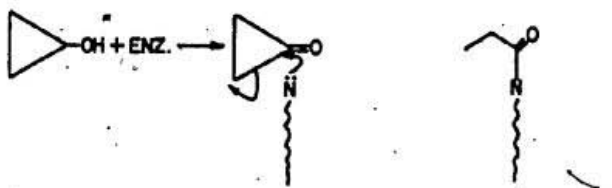
Fig. 8. a) Favorskii Rearrangement.

b) Inactivation of Methanol Dehydrogenase by Cyclopropanol.

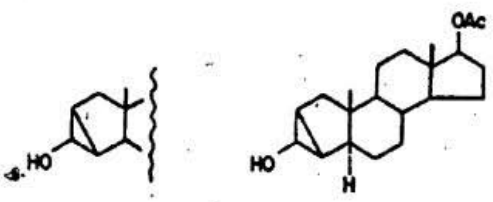
c) A Ring of Potential 3β-hydroxy Steroid Dehydrogenase Inactivator.



1 2 3 a

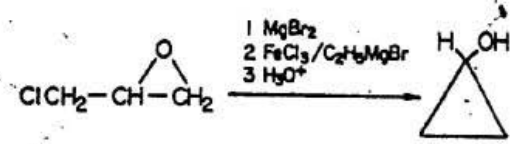


4 5 6 b

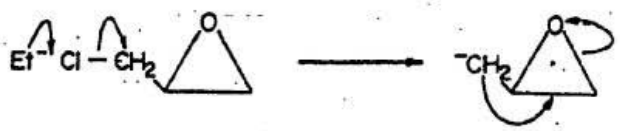


7 8 c

Fig. 9. Methods for Producing Cyclopropanols.


1 MgBr2
2 FeCl3/C2H5MgBr
3 H3O+
ClCH2–CH–CH2
H
OH

9 10


Et
Cl
CH2
CH2

11 12

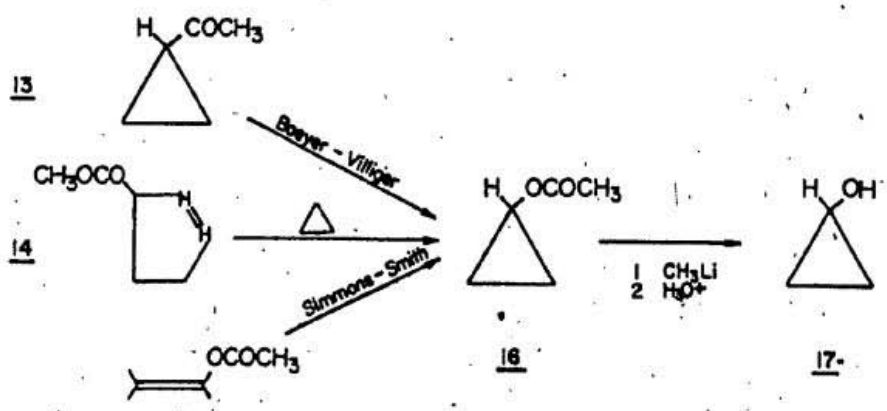
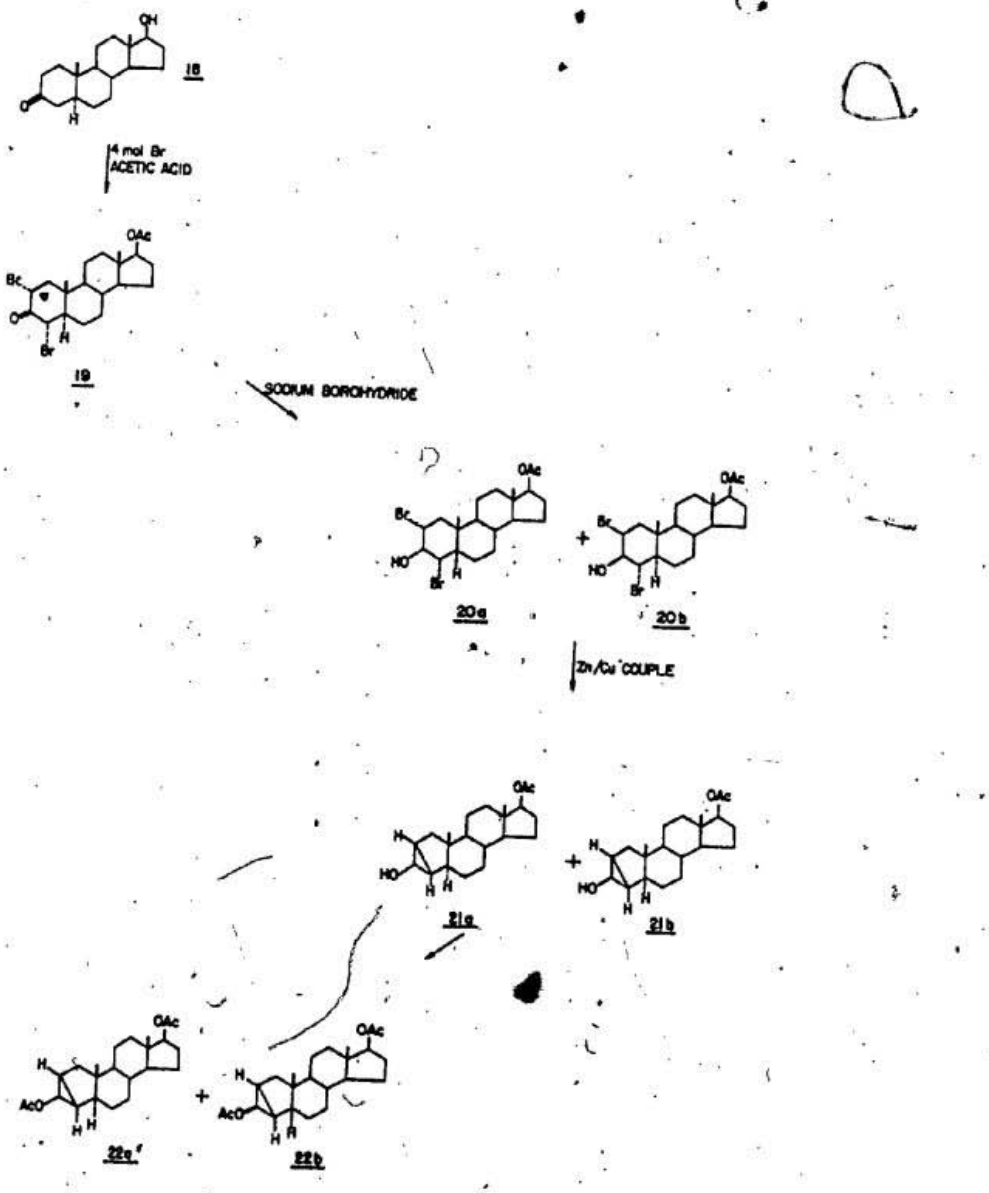

H
COCH3
13
Boeyer - Villiger
CH3OCO
H
H
14
Simmons - Smith
OCOCH3
H
OCOCH3
1 CH3Li
2 H3O+
H
OH
16
17
15

Fig. 10. Templeton Procedure for Producing 2,4-steroid Cyclopropanols (81.).


18
4 mol Br
ACETIC ACID
19
SODIUM BOROHYDRIDE
20a
20b
Zn/Cu COUPLE
21a
21b
22a
22b

2. Materials and Methodology

2. A. Isolation of Horse Liver Alcohol Dehydrogenase

i. Sources of Materials

Horse liver used in this procedure was supplied by "Boucherie Raymond", 4025 Est Rue Jean Talon, Montreal, Quebec, Canada. The liver had been frozen immediately after slaughter. DEAE cellulose was purchased from Pharmacia Fine Chemicals (Sephacel). CM-52 was obtained from Whatman Chemicals. Dialysis was performed using Spectrophor #2 tubing (12,000 to 14,000 m.w. cutoff). The dialysis tubing was prepared by heating in water containing a trace of EDTA and sodium bicarbonate.

ii. Enzyme Assays

Protein assays were performed by the method of Lowry (91). Enzyme activity assays were performed by the method of Pietruszko (92).

a. Acetaldehyde Assay

An aliquot (100 μL) of a stock solution of acetaldehyde (0.20 mL of freshly distilled acetaldehyde in 100 mL of water) was added to a 3.0 mL cuvette containing phosphate buffer (2.9 mL, 0.1 M, pH = 7.0) with NADH (0.18 mM). The final acetaldehyde concentration was 0.12 mM. The reaction was started by the addition of the enzyme and the rate of change of absorption (340 nm) was monitored on a Gilford UV

Spectrophotometer Model 6050. Solutions containing all assay constituents but without enzyme ("no-enzyme blank") and another without substrate ("no-substrate blank") were run simultaneously to assure that the observed change in absorption was due to reduction of acetaldehyde by the dehydrogenase enzyme. A cuvette containing pure water was used to check for instrumental variations.

b. 5β-DHT Assay

An aliquot (10 μL) of a solution of 5β-DHT (Steraloids, 10.0 mg in 1.0 mL freshly distilled t-butanol) was added to a 3.0 mL cuvette containing phosphate buffer (2.9 mL, 0.1 M, pH = 7.0), with NADH (0.18 mM). The final 5β-DHT concentration was 0.11 mM. The reaction was started by the addition of enzyme and monitored at 340 nm as in the acetaldehyde assay, "no-enzyme", "no-substrate" and water blanks were used. Note especially that the "no-substrate" blank still contained the steroid solvent, t-butanol (see page 97).

Starch gel electrophoresis was performed by the method of Pietruszko (92) (pH = 8.5 in tris-HCl buffer). Polyacrylamide gel electrophoresis was performed by the method of Taber and Sherman (93).

iii. Isolation of the Isozymes of Horse Liver Alcohol Dehydrogenase, see Fig. 11.

The preparation of ES and SS isozymes was generally performed using a 500-1000 g sample of horse liver. This

was removed from the whole frozen (-80°C) liver and thawed in buffer (Tris-HCl, 20 mM, pH = 9.0). A typical preparation is given.

A sample of frozen horse liver (607 g) was ground in a meat grinder and further homogenized in a Sorval-Omni Mix. Buffer (Tris.HCl, 20 mM, pH = 9.0) was added to give a volume of 810 ml. The homogenate was centrifuged at 15,000 X g, 1 1/2 h, 4.0°C (Beckman J-21 B centrifuge). The supernatant (300 ml) was collected and centrifuged at 100,000 X g, 1 1/2 h, 4.0°C (Beckman L2-65 Ultracentrifuge).

The supernatant (225 ml) was collected. 130 ml was placed on a DEAE cellulose column, see Fig. 12, and eluted with buffer (Tris.HCl, 20 mM, pH = 9.0). Fractions 56 - 76 appeared cloudy. Fractions 56 - 88 had significant acetaldehyde and 5β-DHT dehydrogenase activity. Fractions 56 - 76, containing SS and ES isozymes (starch and polyacrylamide gel electrophoresis) were combined separately from fraction 77 - 88, which contained ES and EE isozymes. The 56 - 76 pool was centrifuged (15,000 X g, 0.5 h, 4.0°C, Beckman J-21 B centrifuge). Both pools were then dialized separately (2 X ten fold volume of buffer, sodium phosphate, 0.01 M, pH = 7.0, 8 h, 4.0°C).

The 77 - 88 pool was chromatographed on CM-52 cellulose (first CM-52 Cellulose Column, see Fig. 13). A linear gradient from 0.01 to 0.1 M sodium phosphate buffer, pH = 7.0 was applied. The 56 - 76 pool was chromatographed under the same conditions (second CM-52 Cellulose Column, see Fig.

14). Starch gel electrophoresis showed one fraction of pure ES isozyme from each pool (fraction 15 from the first CM-52 column and fraction 39 from the second CM-52 cellulose column, see Fig. 15). The SS isozyme was not easily detected on starch gel. Polyacrylamide gel electrophoresis was therefore used to identify this isozyme. One pure sample was seen (sample # 43, second CM-52 column) see Fig. 16. Fraction 14 (first CM-52 column) contained a contaminant that migrated between EE and SS - possibly ES'. Fraction 40 (second CM-52 column) contained a small amount of EE resulting in a steroid/acetaldehyde activity ratio lower than in fraction 39.

2. B. Synthesis of Steroids (see Fig. 17)

i. Sources of Chemicals and Instrumentation

All melting points were taken on a Reichert-Kofler apparatus and are corrected. Rotary evaporation was performed on a Buchi-Brinkman Rotavapor-R. N.m.r. spectra were recorded using a Brukker WP 80. All steroids were dissolved in deuteriochloroform with tetramethylsilane as an internal standard. Mass spectra were recorded on a Varian MAT 311-A instrument. Tlc was performed on silica-GF obtained from Analtech Inc. with ether/toluene (2:1) eluent. A Waters 244 Liquid Chromatograph equipped with a R401 differential refractometer and a model 450 variable wavelength detector was used for hplc separations. Steroids were separated on either a Whatman Partisil 10/25 Column or a Waters Bondapak C18 (30 cm) column. 5α-DHT was supplied as a gift by Dr. W. Edwards, Syntex Inc. Palo Alto, California, U.S.A. Authentic samples of 2,4-cyclo-5α-androstane -3α and 3β,17β-diol-17 monoacetates were kindly provided by Dr J. Templeton, Dept. of Pharmacology, University of Manitoba.

ii. Synthesis of 2α,4α-dibromo-5α-androstan-3-one-17β -acetate 19.

Bromine (8.40 mL, 163.1 mmol) in acetic acid (200 ML) was added dropwise with stirring to 5α-DHT 18 (20.0 g, 68.9 mmol) in acetic acid (1.5 L) at room temperature. The

solution was stirred for 24 h, then poured into water (2 L). The precipitate that formed was filtered, washed with water (3 X 400 mL) and dried in a vacuum dessicator overnight to give 2α,4α-dibromo-5α-androstan-3-one-17β-acetate 19 (32.2 g, 96%). The crude product was recrystallized several times from methylene chloride/methanol to give product whose melting point range was 187.0 -191.5°C, lit. (81) M.p. = 202 - 204°C. P.m.r. ($CHCl_3$) 4.76 (1, d of d, J = 6 and 13 Hz, 2β -proton), 1.16 (3, s, 19-methyl) and 0.80 (3, s, 18-methyl) p.p.m.. Molecular weight (mass spec) = 490 ($C_{21}H_{32}$ ^{79}Br ^{81}Br O_3 most abundant species).

iii. Synthesis of 2α,4α-dibromo-5α-androstane-3α and 3β ,17β-diol 17-acetate (20a and 20b).

Sodium borohydride (500 mg, 13.2 mmol) was stirred in ethanol (10 mL) for 40 min. After centrifugation (1600 rpm) to remove approximately 5 mg of hazy precipitate, the clear solution was added dropwise to 2α,4α-dibromo-5α-androstan-3-one-17β-acetate (7.5 g, 15.3 mmol) in ethanol (1.5 L) with stirring at room temperature. After 30 min, the reaction mixture was evaporated. Water (200 ml) was added and the crude product was extracted with ethyl acetate (3 X 200 mL), filtered through cotton wool and evaporated. This resulted in a quantitative yield of a mixture of the 3α- and 3β-epimers (20a and 20b). The ratio of the isomers by hplc ($CHCl_3$/Hexane, 1:1 on a silica column, Partisil 10/25) was 3α /3β = 3.85. M.p. (3α-isomer = 205 -206°C, lit (81) m.p. =

206 - 207°C. M.p. (3β isomer) = 248.5 - 251°C, lit. (81) m.p. 253 - 255°C. P.m.r. (3α-isomer) ($CHCl_3$) 4.59 (1, t, J = 8.0 Hz, 17α-proton), 4.36 (1, m, 2β-proton), 4.27 (1, t, J = 2.5 Hz, 3β-proton), 2.03 (3, s, 17β-acetoxy methyl), 0.89 (3, s, 19-methyl) and 0.77 (3, s, 18-methyl) p.p.m.. P.m.r. (3β-isomer) ($CDCl_3$) 4.59 (1, t, J = 8.0 Hz, 17α-proton), 3.84 (1, t, J = 10.0 Hz, 3α-proton), 2.03 (3, s, 17β-acetoxy methyl), 0.89 (3, s, 19-methyl) and 0.77 (3, s, 18-methyl) p.p.m. Molecular weight (mass spec) = 492.

iv. Synthesis of Zinc/Copper Couple

Zinc dust (14.13 g, 0.2 mmol) was ground with cupric acetate monohydrate (0.86 g, 0.004 mmol) in a mortar and pestle. The mixture was added with stirring to boiling glacial acetic acid (150 mL). The slurry was stirred for 20 min after which cold glacial acetic acid was added (50 mL, 0°C) and the mixture was cooled to room temperature. The slurry was filtered through a medium sintered glass funnel. Ether was added to the couple so that during filtration it always remained wet. The ether - moist couple was used immediately.

v. Synthesis of 2R,4S-2,4-cyclo-5α-androstane-3α and 3β,17β-diol, 17-acetate (21a and 21b). Method of Templeton and Wie (81)

2α,4α-dibromo-5α-androstane-3α and 3β,17β-diol, 17-acetate (3α/3β = 3.85, 1.0 g, 2.02 mmol) in ethanol (300 mL)

was treated with zinc/copper couple (1.0 g, ether-moist couple). The slurry was stirred at 50°C for 1 1/2 h. The reaction mixture was centrifuged (1600 rpm) and the supernatant poured off and collected. Solvent was removed by rotary evaporation and the residue extracted with ethyl acetate (50 mL). The organic layer was washed with water (3 x 50 mL), filtered through cotton wool and evaporated. The crude product was found to contain about 20% yield of a mixture of 3α- and 3β-epimers of 2R,4S-2,4-cyclo-5α-androstane-3α and 3β,17β-diol, 17-acetate in the same ratio (3α/3β) as in the starting material, (hplc, normal phase, chloroform/hexame,4/1). (tlc, ether/toluene, 2/1, R.f. (3α-) = 0.81, R.f. (3β-) = 0.72. M.p. (3α-isomer) = 145 - 146°C, lit. (81) m.p. = 143 - 146°C. M.p. (3β-isomer)= 130 - 133.5°C, lit. (81), m.p. 130 - 132°C. P.m.r. (3α-isomer) ($CDCl_3$) 4.59 (1, t, J = 8.0 Hz, 17α-proton), 3.84 (1, d of d, J = 10 and 2.5 Hz, 3β-proton), 2.03 (3, s, 17-acetoxy methyl), 0.94 (3, s, 19-methyl) and 0.77 (3, s, 18-methyl) p.p.m. P.m.r, (3β-isomer) ($CDCl_3$) 4.55 (1, t, J = 8.0 Hz, 17α-proton), 3.13 (1, unresolved t, 3α-proton), 2.03 (3, s, 17-acetoxy methyl), 0.85 (3, s, 19-methyl) and 0.77 (3, s, 18-methyl) p.p.m.. Molecular weight (mass spec) = 332 for both isomers, the spectra were indistinguishable. Both had a characteristic fragmentation: loss of 31 a m u from the molecular ion and from M-15.

vi. Synthesis of 2R,4S-2,4-cyclo-5α-androstane-3α and 3β,17β-diol, (23a and 23b).

a. From the monoacetates.

To 2R,4S-2,4-cyclo-5α-androstane-3α or 3β,17β-diol, 17-acetate (gift of Dr. J. Templeton, 100 mg, 0.30 mmol) in dry ether (10 mL) was added 1.0 mL of a concentrated ether solution of lithium aluminum hydride. After 15 min, water (15 mL) was cautiously added with stirring until no more evolution of gas was seen. The ether layer was removed and washed with water (3 X 10 mL), filtered through cotton wool and evaporated to give a quantitative yield of crude diol. If the 3-epimers were not separated before removal of the 17-acetate, then these could easily by separated on hplc (normal phase, chloroform/hexane = 4, tlc, R.f. (3α-) = 0.52, R.f. (3β-) = 0.48, (ether/toluene = 2).

b. From 2α,4α-dibromo-5α-androstane-3αor 3β, 17β -diol, 17-acetate, (20a and 20b).

Liquid ammonia (100 mL) was delivered from an inverted cylinder into an Erlenmeyer flask (Clear - Fit) surrounded by vermiculite and protected from contact with the air by a loose fitting Clear - Fit stopper. Lithium wire (30 X 3 mm) was cut, washed (2 X 30 mL hexane) and added to the liquid ammonia; the solution was stirred magnetically using a glass - jacketed magnet. The intense blue color that immediately formed indicated the presence of excess solvated electrons. 2α,4α-dibromo-5α-androstane-3αand 3β, 17β-diol, 17-acetate (200 mg, 0.41 mmol) was dissolved in dioxane (20 mL, freshly

distilled from sodium metal) and added to the liquid ammonia solution. More lithium was added as necessary to maintain the blue colour. After 30 min, no starting material remained (hplc, normal phase, chloroform/hexane = 4). The product was a mixture of 2R,4S-2,4-cyclo-5α-androstane-3α and 3β,17β -diols (tlc, silica GF, ether/toluene = 2, hplc, normal phase, chloroform/hexane = 4). In this experiment, mixtures of the 2,4-dibromo 3α and 3β -epimers 20a and 20b were used. The resulting cyclopropanol epimers at the 3-position (23a, 23b) were of the same ratio as that of the starting material.

Solid ammonium chloride was added until the blue color disappeared. The flask was removed from the insulating vermiculite, allowing the ammonia to boil off. When most of the ammonia have been removed, the residue was extracted with ethyl acetate (50 mL) and washed with water (4 X 50 mL). The ethyl acetate was filtered through cotton wool into a round bottom flask and the solvent removed by rotary evaporation. This gave 2R,4S-2,4-cyclo-5α-androstane-3$\alpha$and 3β,17β-diol (23a and 23b) (0.107 g, 90%). Tlc, R.f. (3α -isomer) = 0.52, R.f. (3β-isomer) = 0.48, (silica GF, ether/toluene = 2). P.m.r. (3α -isomer) ($CDCl_3$) 3.77 (1, t, J = 6.0 Hz, 17α-proton), 3.62 (1, t, J = 8.0 Hz, 3β -proton), 0.94 (3, s, 19-methyl) and 0.73 (3, s, 18-methyl) p.p.m.. P.m.r. (3β -isomer) ($CDCl_3$), 3.62 (1, t, J = 8.0 Hz, 17α -proton), 3.11 (1, t, J = 4.0 Hz, 3α -proton), 0.85 (3, s, 19-methyl) and 0.73 (3, s, 18-methyl) p.p.m.. M.p. (3α-

isomer) = 73 - 75°C, m.p. (3β-isomer) = 104 - 105.5°C.

Molecular weight (mass spec) (3α- and 3β -isomers) = 290.

2. C. Enzyme Inactivation

In a typical experiment the enzyme was dissolved in buffer (pyrophosphate. 0.05 M, pH = 9.0); the final enzyme concentration was 0.01 - 0.05 mM. NAD was then added to give a final concentration of 6 - 11 mM. This solution was then divided into two equal volumes (experimental and control) and placed in a water bath maintained at 25.0°C. The actual inactivation experiment was started by the addition of steroid (dissolved in t-butanol, freshly distilled). The same volume of t-butanol was added to the control. Aliquots (usually 100 μL) were removed periodically and assayed for acetaldehyde and 5β- DHT dehydrogenase activity. The assay solution was carefully monitored for precipitation of either the steroid or the enzyme. Steroid concentrations greater than 0.261 mM were not used due to solubility problems.

Fig. 11. Enzyme Purification Procedure

MAIN LINE FRACTION

DISCARD FRACTIONS

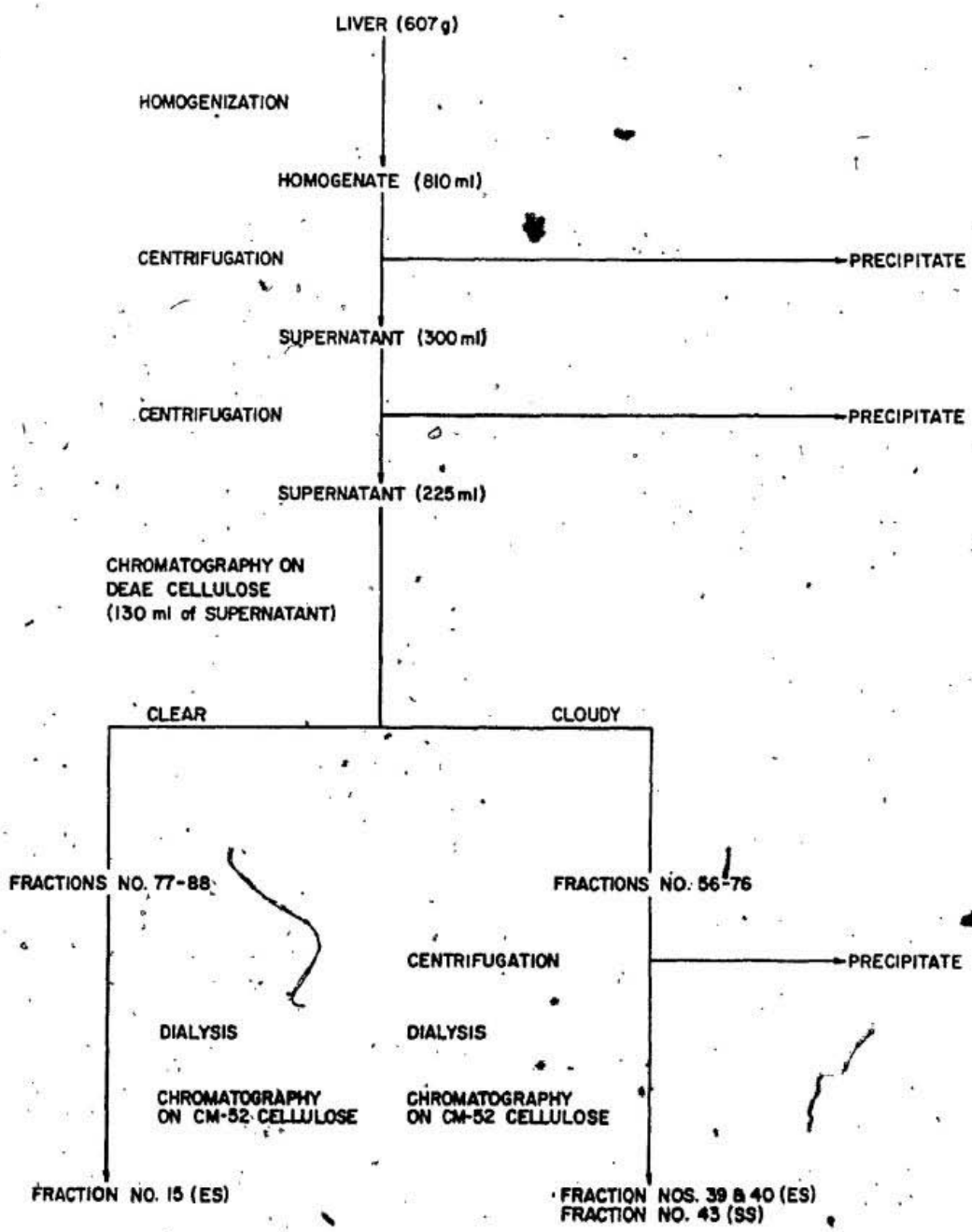


Fig. 12. Diethylaminoethyl (DEAE) Cellulose Column.
Protein (mg) -△
Acetaldehyde Activity (μmol/min.mg) -○
5β-DHT Dehydrogenase Activity (μmol/min.mg) -□
(Buffer, Tris.HCl, 20 mM, pH = 9.0)

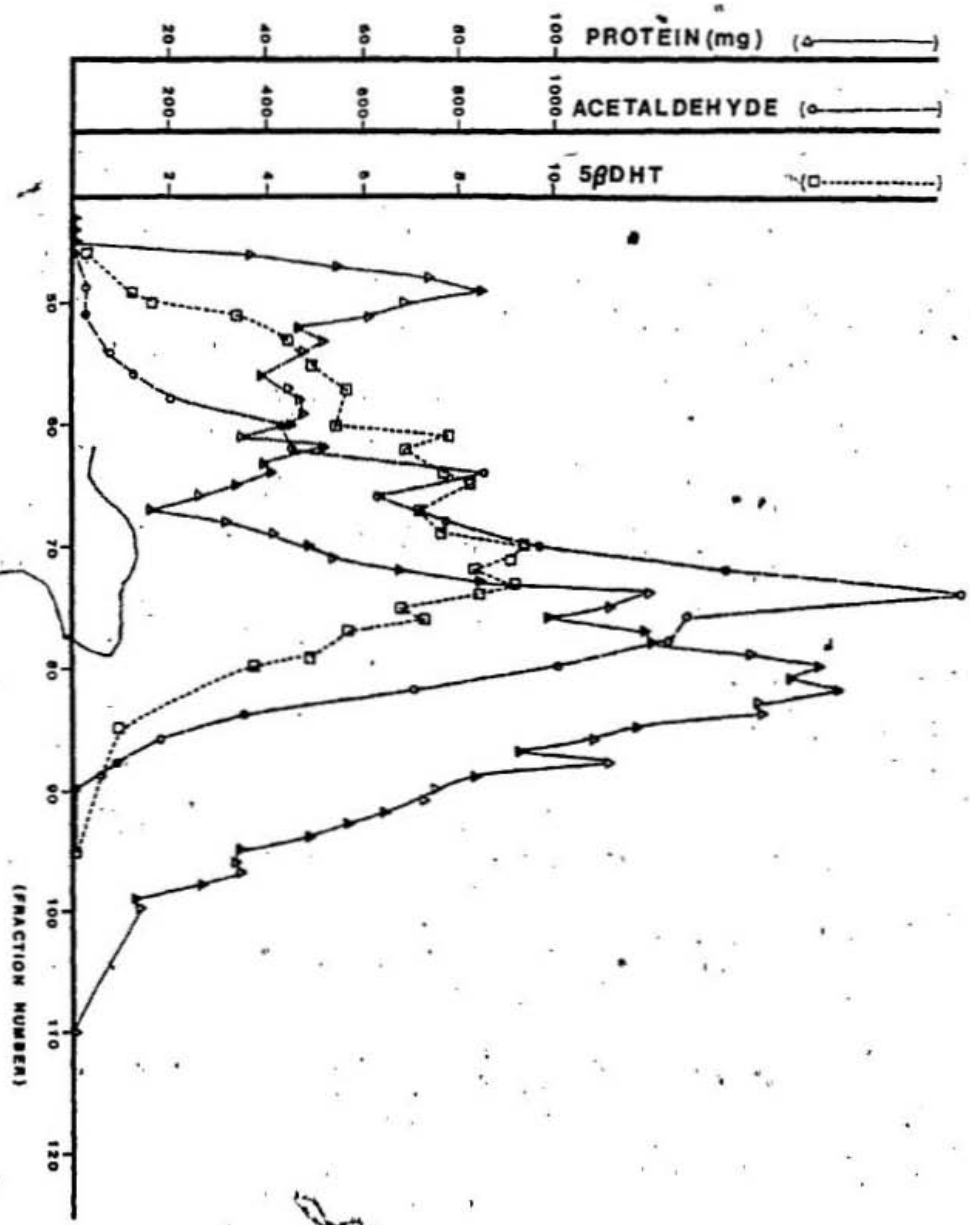

PROTEIN (mg)
ACETALDEHYDE
5βDHT
(FRACTION NUMBER)

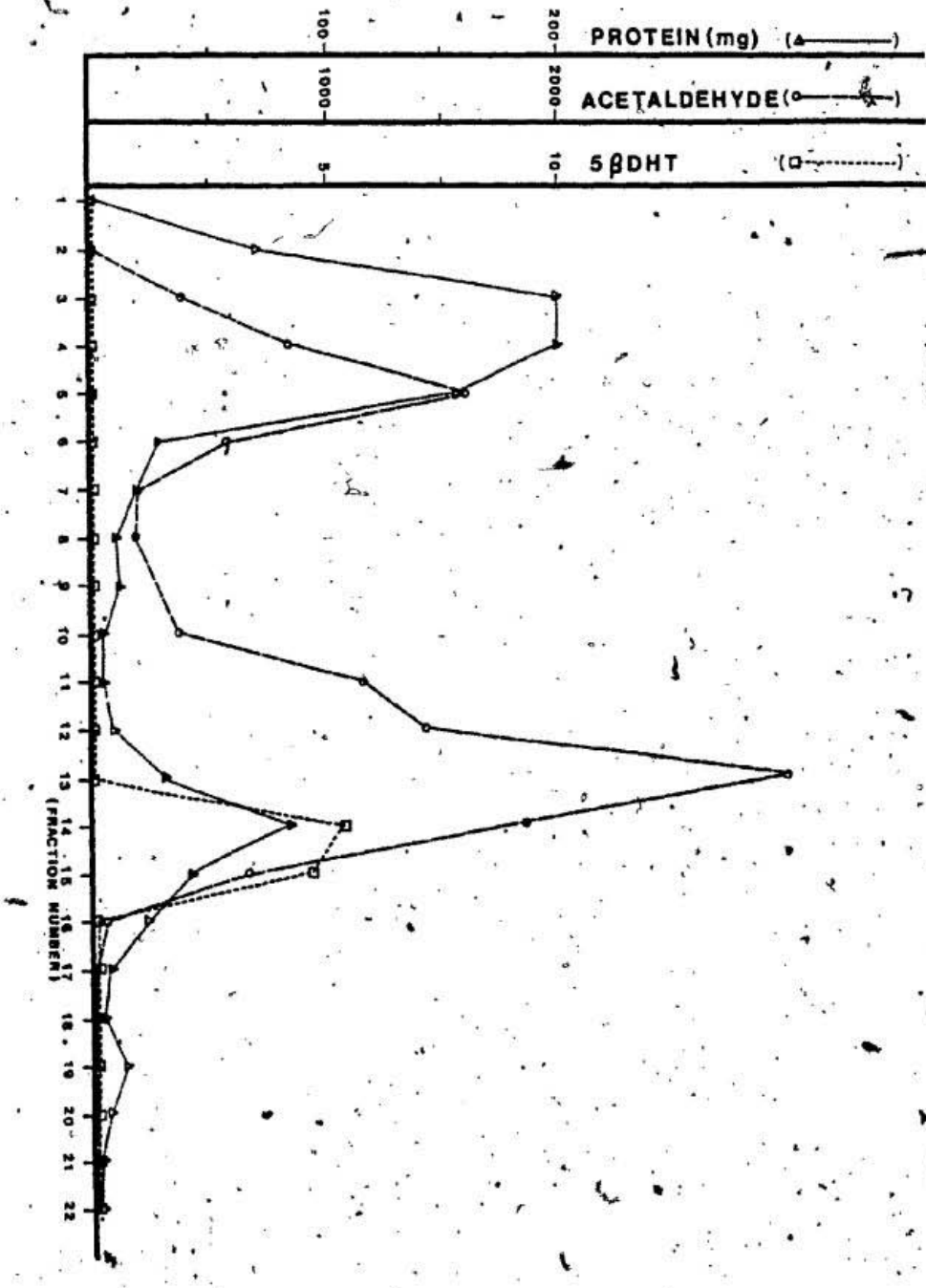
Fig. 13. First Carboxymethyl Cellulose Column.

Protein (mg) -Δ

Acetaldehyde Activity (μmol/min.mg) -○

5β-D[illegible] Dehydrogenase Activity (μmol/min.mg) -□.

(Buffer, Sodium Phosphate, 0.01 - 0.1 M Linear Gradient, pH = 7.0)


PROTEIN (mg)
ACETALDEHYDE
5βDHT
100
200
1000
2000
5
10
(FRACTION NUMBER)

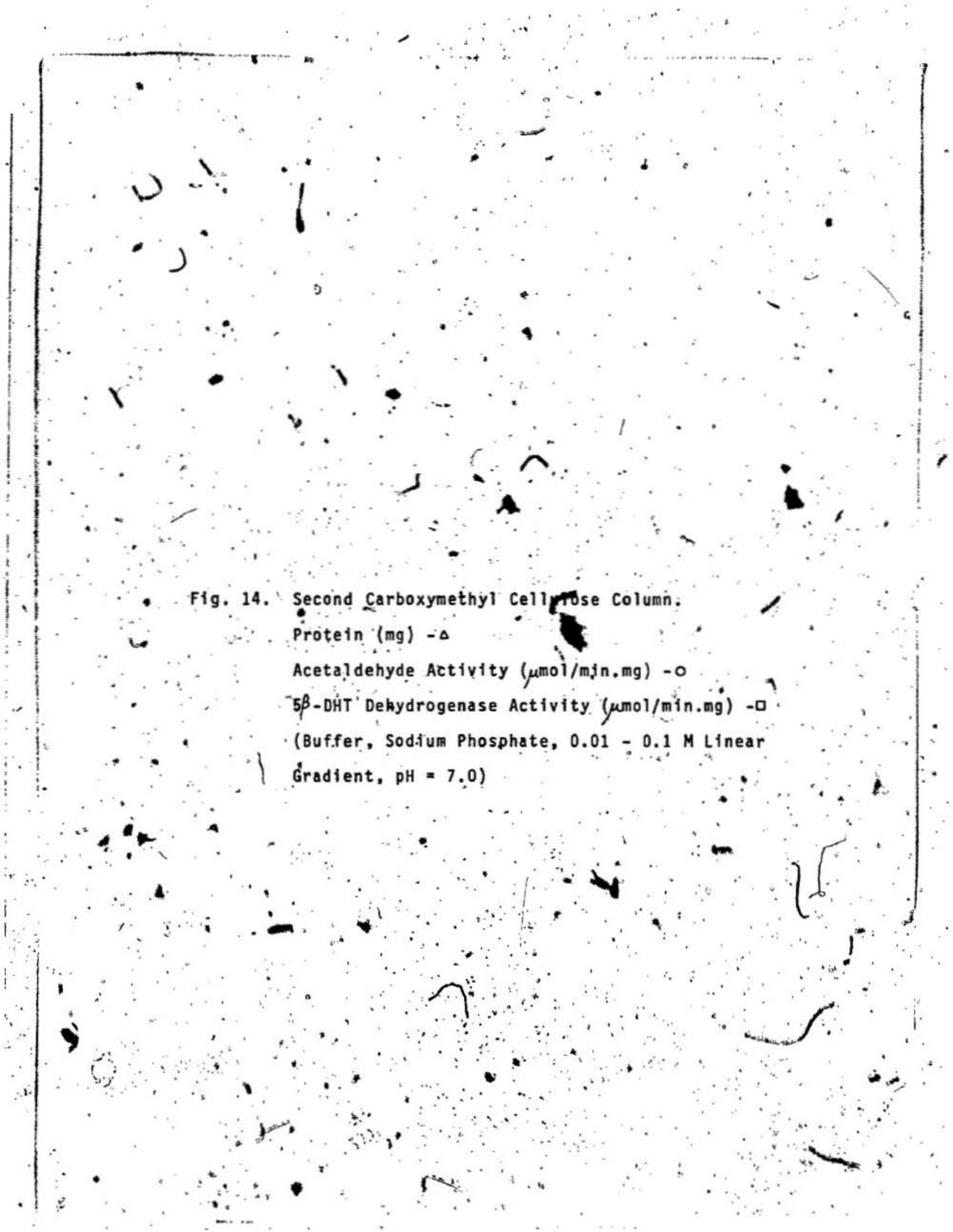


Fig. 14. Second Carboxymethyl Cellulose Column.
Protein (mg) -△
Acetaldehyde Activity (μmol/min.mg) -○
5β-DHT Dehydrogenase Activity (μmol/min.mg) -□
(Buffer, Sodium Phosphate, 0.01 - 0.1 M Linear Gradient, pH = 7.0)

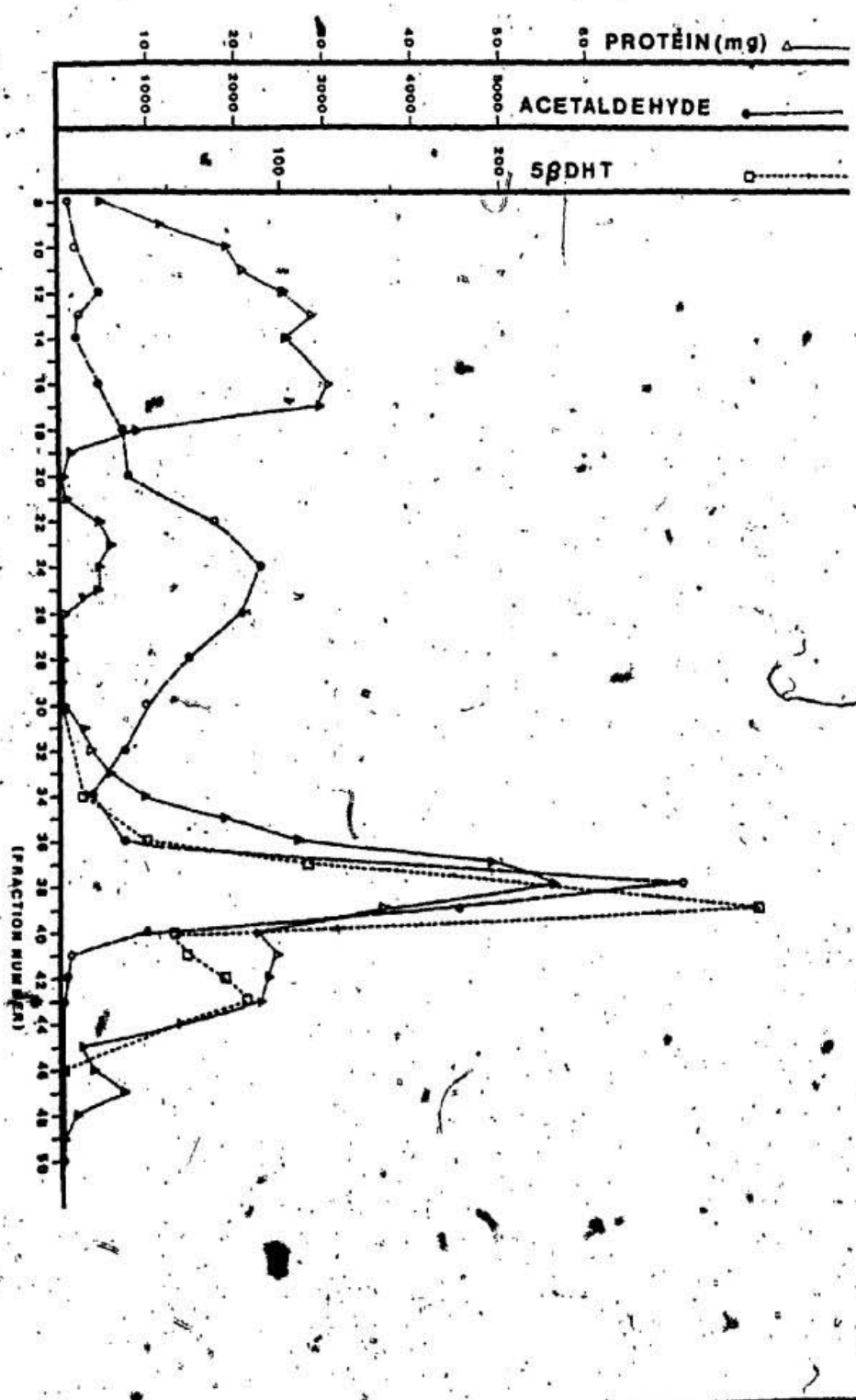
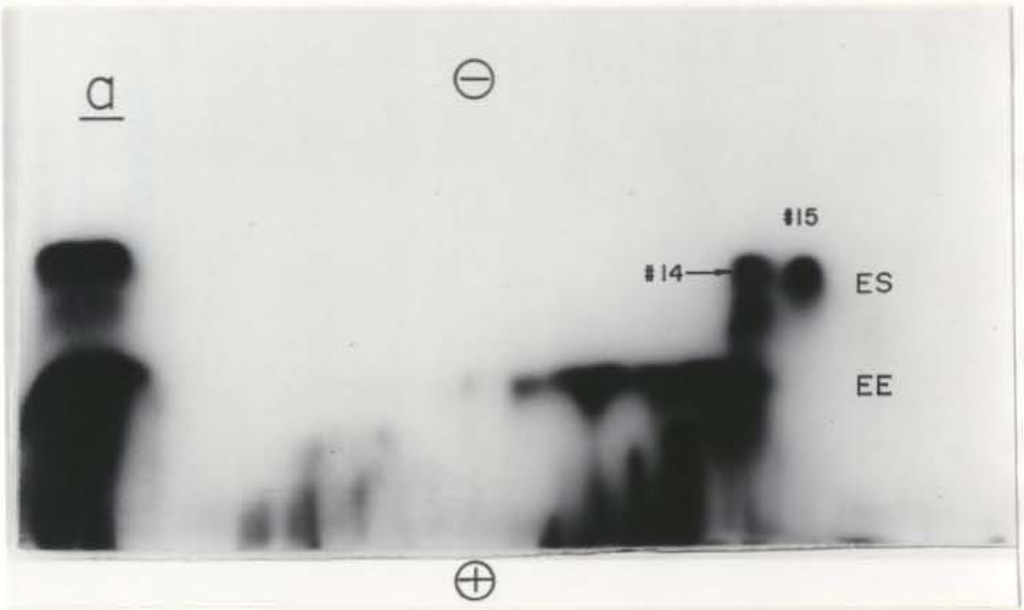

PROTEIN(mg)
ACETALDEHYDE
5βDHT
(FRACTION NUMBER)

Fig. 15. Starch Gel Electrophoresis of Fractions from Carboxymethyl Cellulose Protein Columns.

a. Fractions from First Column.
(Fraction #15 Labelled)

b. Fractions from Second Column.
(Fraction #39 Labelled)

(15% Starch Gel, Electrophoresis was performed at 125 v. for 16 h. at 4.0°C with Tris.HCl Buffer, 0.03 mM, pH = 8.6, Well Buffer, Tris. HCl, 0.3 M, pH = 8.6)


a
⊖
#15
#14
ES
EE
⊕

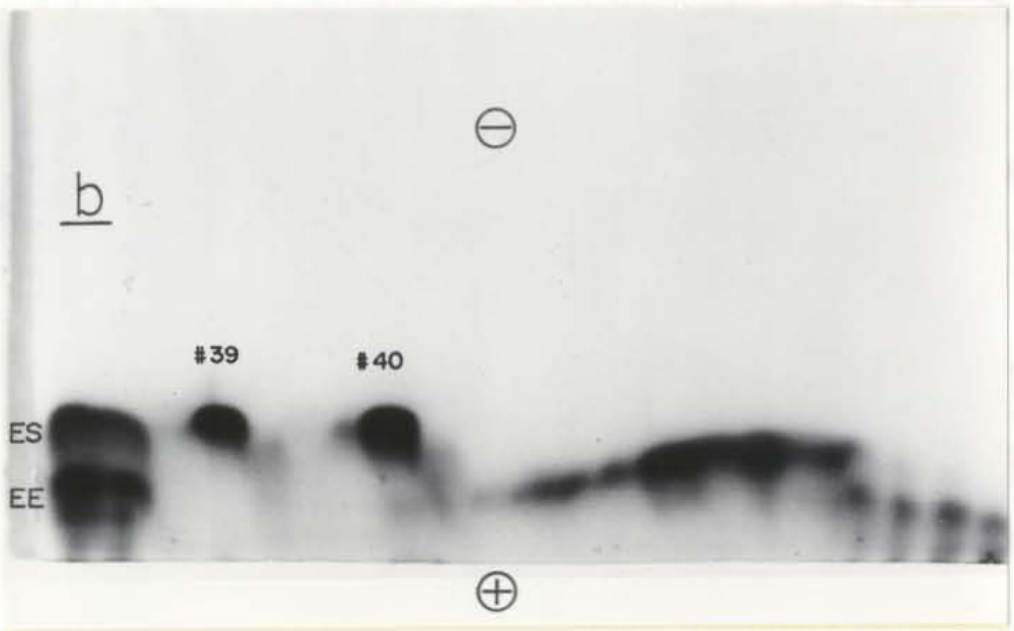

b
⊖
#39
#40
ES
EE
⊕

Fig. 16. Polyacrylamide Gel Electrophoresis of Fractions from Second Carboxymethyl Cellulose Protein Column.
(Electrophoresis was performed at 125 v. for 16 h. at 4.0°C with Glycine.KOH Buffer, 0.63 M, pH = 7.3, Well Buffer, Glycine.KOH, 0.18 M and Lutidine, 0.3 M, pH = 8.3)

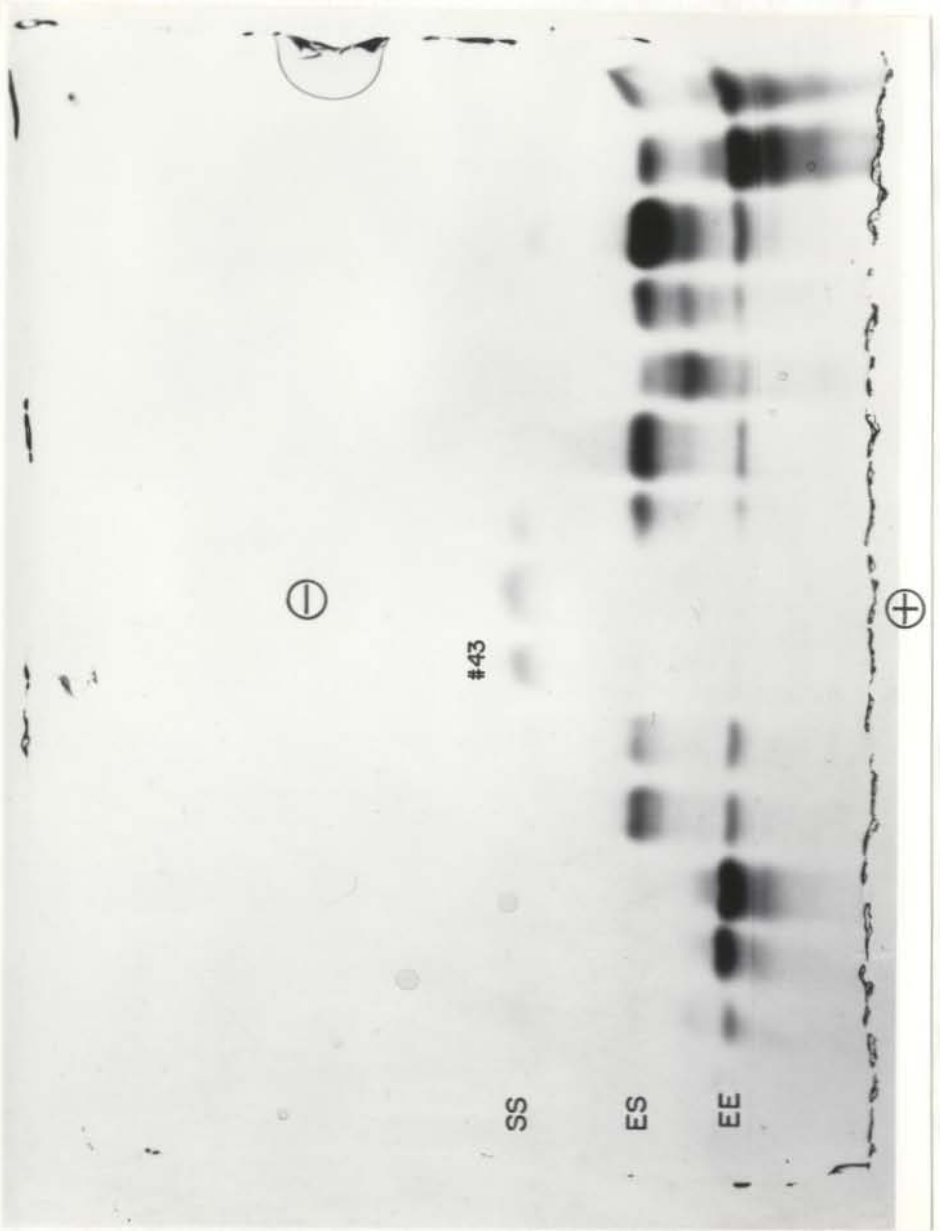
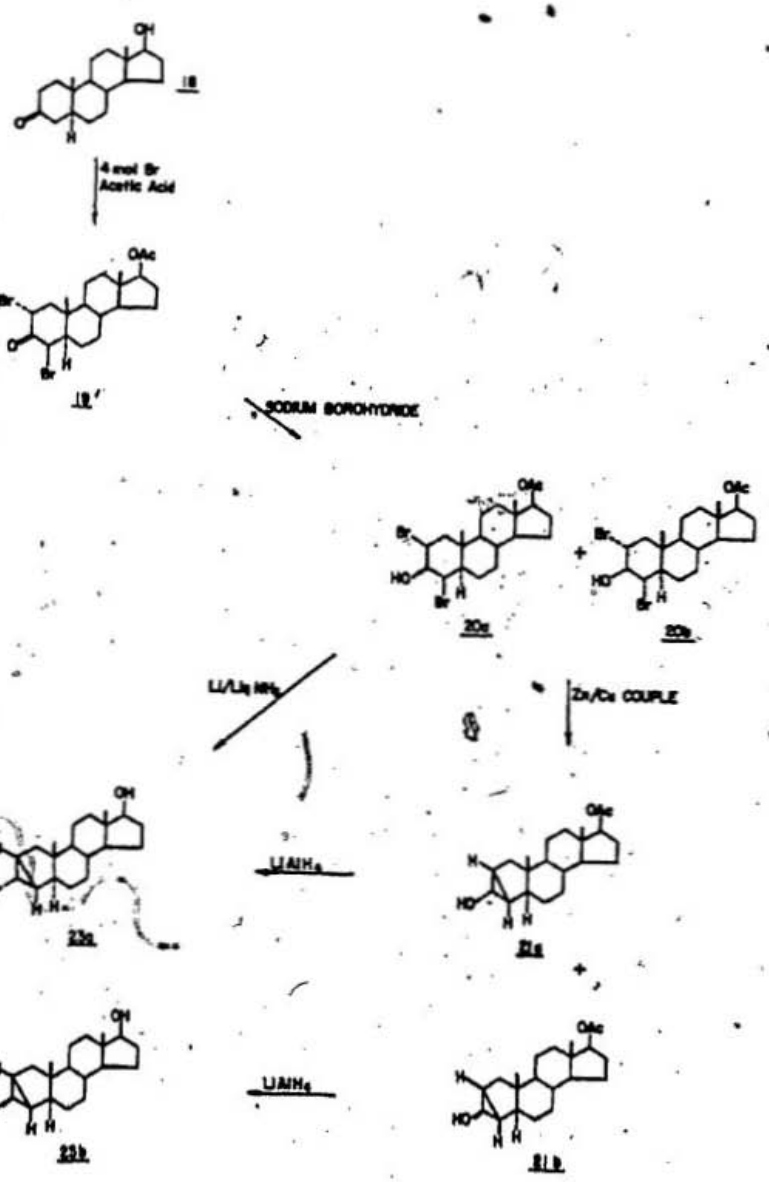

⊖
#43
SS
ES
EE
⊕

Fig. 17. Pathway for Producing 2R,4S-2,4-cyclo-5α-androstane -3α and 3β,17β-diol from 5α-DHT.


4 mol Br
Acetic Acid
SODIUM BOROHYDRIDE
Li/Liq NH3
Zn/Cu COUPLE
LiAlH4
LiAlH4

3. Results and Discussion

3. A. Enzyme Purification

Horse liver samples had been frozen immediately after slaughter and stored at -80°C until used. Periodic sampling of the frozen livers showed that there was no change in the isozyme pattern during storage (2 years). Fresh liver was also obtained and exhibited the same isozyme pattern as that of the frozen material.

A schematic diagram of the steps of the enzyme purification is shown in Fig. 11, Section 2. A. The procedure is based on the unusually high isoelectric point of the S-containing isozymes. Enzyme purification results are shown in Table 1.

Approximately 40% of the protein applied to the DEAE column was recovered. A large quantity of solid material remained precipitated at the head of the column; also negatively charged protein remained associated with the column packing material as the positively charged isozymes of HLADH were eluted. Use of a prior short column (DEAE) significantly reduced the quantity of protein that was applied to the large DEAE colulmn.

The specific acetaldehyde activity of the pool of protein from the DEAE column was 5.72 μmol/min.mg (see Table 1); 5β-DHT activity was 0.036 μmol/min. mg. Activity could

not be measured on the crude material because its intense color reduced light transmission sufficiently to prevent its assay.

Protein yields from the CM-52 columns were of the order of 90%. The ES sample from the first CM-52 column (fraction #15) had a specific acetaldehyde activity of 16.52 μmol/min.mg and 5β-DHT activity of 0.113 μmol/min.mg. That for the second CM-52 column ES sample (fraction #39) was 125.8 μmol/min.mg and 0.87 μmol/min.mg respectively. The ratio of the 5β-DHT/acetaldehyde activity was the same for ES samples from the two separate CM-52 columns (= 0.007). Specific acetaldehyde activity of the SS sample from the second CM-52 column (fraction #43) was 4.38 μmol/min. mg, and specific 5β-DHT activity was 0.70 μmol/min.mg. The ratio of 5β-DHT/acetaldehyde activity is 0.16. Starch and polyacrylamide gels of these fractions can be seen in Figs. 15, 16.

Several protein samples were examined on hplc. Most hplc columns are derivatized silica beads. The TSK 3000 SW (Altex Industries) is one such column that has an isopropylglycerol function attached to the silanol groups. Separation of proteins is primarily a function of molecular size and shape. Empirically, there appears to be a relationship between the logarithm of molecular weight and elution volume within the specified molecular weight range of the column (10,000 - 200,000).

The three major isozymes of HLADH have essentially the same molecular weight and should therefore coelute from the TSK column at high ionic strength. Using 0.05 M sodium phosphate buffer it is possible, however, to achieve a small but significant separation of the three isozymes based on charge, see Fig. 18 and Fig. 19. The order of the isozyme elution is EE, ES, and lastly SS. Whether the greater positive charge or a size effect is responsible for this difference is not yet known. This difference can be used for identification of the isozmes but only after calibration of the column with known samples.

As seen in Fig. 18a, a commercially available sample (Sigma Chemicals) contained about 15% of an impurity, possibly inactive SS (sample showed no steroid activity). Another commercially available sample (Sigma Chemicals - different lot number) is shown in Fig. 19a. This sample contained approximately 50% of a protein eluting after SS. The order of elution of the isozymes is EE first, then ES and SS last, Fig. 18.

A sample of ES was stored at 4°C for two months. While retaining its acetalehyde and steroid dehydrogenase activity, polyacrylamide gel electrophoresis showed that ES had been converted to ES' which runs on polyacrylamide between EE and ES (see Fig. 2, Section 1. A.). Hplc of this species showed a homogeneous peak that anomalously eluted after SS, Fig. 19b.

3. B. Synthesis of Steroid Inactivator

The reaction sequence for producing 2R,4S-2,4-cyclo-5α-androstane-3α and 3β,17β-diol form 5α-DHT is shown in Fig. 17 (Section 2. B.) Bromination of 5α-DHT occurs quickly and in high yield (> 90%). Bromine is first added at the 2α-position and secondly at the 2β-position. The solution is left overnight and rearranges to give the most sterically stable dibromo species with the two bromines in the 2 and 4 equatorial positions. The product may be isolated by precipitation when the glacial acetic acid solution is poured into water or by methylene chloride extraction. In glacial acetic acid solution an acetate group is introduced at the 17-position. The bromination has also been performed in ethyl ether thus retaining the 17-alcohol function.

The precipitate was washed thoroughly to remove all traces of acetic acid and then dried in a vacuum desiccator over $CaCl_2$. The crude product was recrystallized from methylene chloride/methanol to give long needle-shaped crystals. There is a slow decomposition of the bromo compound and the product should be stored at low temperature in the dark.

The sterically hindered dibromo ketone 17-acetate (19) is considerably less polar on tlc than the starting material (R.f. (5α-DHT) = 0.27; R.f. (19) = 0.72; chloroform). Sodium borohydride reduction of this ketone proceeded rapidly to give a mixture of the 3α- and 3β- hydroxy isomers

20a and 20b. The axial alcohol (3∝-) is the major product due to equatorial crowding by the bromines (hplc; reverse phase-octadecylsilane, chloroform eluent). N.m.r. spectra of the compounds 20a and 20b showed the presence of a triplet at 4.27 p.p.m. (J = 2.5 Hz) and 3.84 p.p.m. (J = 10.0 Hz) respectively. This is consistent with axial-equatorial coupling with an equatorial proton at C-3 (3∝-isomer) and diaxial coupling of an axial proton at C-3 (3β-isomer) respectively.

The 2∝,4∝-dibromo-5∝-androstan-3∝ and 3β,17β-diol 17 acetates (20a and 20b) could not be separated on tlc; the unexpectedly low polarity of the 3β-epimer is presumably due to steric crowding of the 3β-alcohol group by the bromine atoms. Hplc on reverse phase octadecylsilane was used to determine the ratio of the epimers produced in the sodium borohydride reduction. This method was of little use in preparing stocks of the resolved products because the solvent system (methanol/acetonitrile) permitted the separation of only minute quantities of steroids (limited by the solubility of the steroids in the mobile phase). Hplc on silica gave poorer resolution but allowed injection of larger quantities of material. By the separate collection of ascending and descending portions of the peaks (shaving) the epimers were separated, see Fig. 20.

The main peak in Fig. 20a consisted of a mixture of the 3∝- and 3β-epimers (20a and 20b). By collecting the eluent from the hplc column outflow in two fractions (A - ascending

and part of the descending peak; B - tailing portion of the descending peak. Reinjection of portion A is shown in Fig. 20c. Reinjection of portion B is shown in Fig. 20b. Note the resolution of two peaks, corresponding to 3α- and 3β- isomers, not possible in a single injection on our column.

The Waters 244 Liquid Chromatograph has a recycle function that permits the column eluent to be reinjected continuously into the column. A mixture of isomers was recycled so that after 3 passes through the column a tailing peak had been resolved, see Fig. 21. Again, these are the 3α- and 3β- isomers, 20a and 20b.

The 3α- and 3β- eipmers of the 2R, 4S-cyclo series could easly be separated on tlc and hplc; metal/ammonia reductive cyclization was therefore routinely performed on the crude product from the borohydride reduction of the 3-ketone.

Reaction of the dibromo 3α- and 3β-epimeric alcohols with zinc/copper by the method of Templeton et al. (81), consistently gave low yields of the cyclized products in our hands (see Section 2. B. v.). This presumably was due to a less reactive couple than that used by Templeton although there also, the unsaturated compounds were found to be the major products. Results were equally poor with zinc/copper couples activated by hydrochloric acid. These results prompted the search for an alternate method of cyclizing the A ring. Birch reduction (by Li or Yb in liquid ammonia) was

found to give a high yield (>80%) of the cyclized product. This apparently represents a new synthesis of cyclopropanols.

The cyclopropanol steroids are unstable in solution and quickly ring open to give aldehydes in the presence of acid or base (81). Lithium hydroxide is present during the workup after the Birch reduction so a rapid extraction is essential for high yield. The steroids should be stored at low temperature in the dark. In the crystalline form they are apparently quite stable.

The C-18 and C-19 methyl peaks of the bromoketone-17-acetate (19) appear at 0.80 and 1.16 p.p.m. respectively. Sodium borohydride reduction shifts the C-19 to 0.89 p.p.m., while the C-18 methyl is shifted only slightly to 0.77 p.p.m. The two stereoisomers 20a and 20b have both angular methyl absorptions at the same field positions (3α- and 3β-C-18 = 0.77 p.p.m.; C-19 = 0.89) and are indistinguishible based on these data (see Table 2.). The spectra differ, however in the resonances due to the 3-proton, the 3α-proton = 3.84, 3β-proton = 4.27 p.p.m.

Cyclization of the C-2, C-4 position in the A ring has little effect on the C-18 angular methyl field position in either isomer, but the C-19 methyl is shifted upfield in the 3β- isomer to 0.85 p.p.m. and downfield in the 3α-isomer to 0.94 p.p.m. Removal of the 17-acetate affects only the C-18 methyl, which in each isomer is shifted upfield to 0.73 p.p.m..

On the basis of the Templeton assignment of stereochemistry the 3α-proton of the 3β-alcohol is an unresolved triplet at 3.13 p.p.m. while the 3β-proton of the 3α-alcohol is a doublet of doublets at 3.84 p.p.m. On inspection of models (Fig. 22) the difference in coupling constants could not be related to the difference in dihedral angle between the 3-hydrogens and the vicinal proton. As is noted in Jackman and Steinhall (94) p. 286, a cyclopropane ring being planar, the substituents lie at dihedral angles of 0^{0} or 120^{0} which correspond, using the Karplus relation to coupling constants of 7-12Hz and 4-10Hz respectively. This is too close for discrimination of stereochemistry to be reliable. The evidence for assignment of structure given by Templeton does, however, appear to be well based on the very large difference in reactivity of the derived 3-toluene-p-sulfonates.

The mass spectra of the 3α- and 3β-isomers 23a and 23b are indistinguishable. This is true for both the 17-alcohol and 17-acetate derivatives. Both have well defined molecular ion peaks and the 17-acetate shows loss of acetic acid as its most facile elimination.

Analysis of the metastable ions of the DADI spectra (Direct Analysis of Daughter Ions) showed no difference in the stability of the ions produced in the fragmentation of either the 3α- and 3β- isomers (23a and 23b). There is a characteristic loss of fragment CH_2OH due to the three

membered ring opening; the abundance of this fragment is independent of the stereochemistry of the 3-hydroxyl group of its parent compound.

3. C. Enzyme Inactivation Results

A water miscible solvent was required to add the steroid to the aqueous buffer containing the enzyme. Both primary and secondary alcohols are substrates for the enzyme and thus unsuitable as solvents. Dioxane has been commonly used for steroid dehydrogenase assays (93), but in our experiments was found significantly to inactivate the enzyme. A survey of various potential solvents was made, see Table 3.

Aliquots (10 µl) of each solvent was added to a 3.0 ml buffer solution (sodium phosphate, 0.1 M, pH = 7.0) of commercially obtained HLADH enzyme (Sigma Chemicals). The specific activity of the enzyme was measured both before and after addition of the test solvent. Pyridine, dimethylformamide and dimethylsulfoxide are potent inhibitors of HLADH (10 µl of each solvent completely inhibited the enzyme). Tetrahydrofuran removed ~70% of the enzyme activity. Dioxane, Triton X100, formamide and acetonitrile were intermediate inhibitors resulting in 20-40% inhibition. Brij-35 contained a substrate for the enzyme and was therefore unsuitable as a solvent.

t-Butanol is a tertiary alcohol. It is not a substrate for the enzyme since it contains no hydrogens attached to the carbon bearing oxygen (required for substrate activity, see Fig. 6, Section 1. A). It was found to be a good solvent for the steroids, and it did not inhibit the enzyme in the

concentrations used in the assay. Larger volumes (> 20μl) resulted in a significant inhibition. This is the first report of this tertiary alcohol being used as a vehicle for substrate in steroid dehydrogenase assays. It is the preferred solvent for assay of horse liver alcohol dehydrogenase.

A summary of the results of the inactivation experiments involving the cyclopropyl steroids is shown in Table 4. No inactivation was observed when 2R,4S-2,4-cyclo-5α-androstane-3β,17β-diol (A) was incubated with either ES or SS isozyme. The specific activity of the solution containing steroid and cofactor remained unchanged from that of the control containing cofactor but no steroid. Although the enzyme is reported to be a 3β-hydroxy steroid dehydrogenase, the corresponding 3α-isomer was also examained. The results are also shown in Table 4. (3α-hydroxy isomer = B). Various concentrations of A were examained for incubation with ES and SS - ([steroid] / [active site] = 2-7), but no inactivation was observed.

Fig. 23 shows the acetaldehyde activity of a sample of ES enzyme incubated with (B). The "o" symbol represents the enzyme solution containing cofactor (NADH), t-butanol (10μl) but no steroid inactivator ("no steroid" blank or "no steroid" control). The "Δ" symbol shows that incubation with the same constituents but in addition the steroid inactivator (B) ("experimental" ([steroid] / [enzyme] = 5)).

Fig. 24 shows the corresponding 5β-DHT dehydrogenase activity of the "no steroid" control (□) and the

"experimental" (○). Note that over the duration of the experiment (2000 min, 33 h.) 100% of the steroid dehydrogenase activity of the enzyme is lost; compared with 14% of the control activity. 40.6% of the acetaldehyde activity was lost compared with ~4% of the acetaldehyde activity of the control. The ES isozyme is a "dimer" of E and S subunits. Both are capable of reducing acetaldehyde to ethanol but only the S subunit is reactive toward steroids.

In Fig. 27 is shown the results of incubating (B) with SS isozyme. "○" is the acetaldehyde assay result of the "no steroid" control and "△" that of the "experimental" ([steroid] / [enzyme] = 6.8; [steroid] / [active site] = 3.4). "□" and "○" represent 5β-DHT assay results of the 'no steroid control' and 'experimental' respectively. After 2600 min (43 h.) no steroid dehydrogenase activity remained in the experimental compared with ~73% in the control. Approximately 60% of the acetaldehyde activity had been lost compared with only 17% for the control.

Fig. 29 shows an experiment involving SS isozyme where in addition to using a equimolar quantity of steroid (B) ([steroid] / [active site] = 1), a less than one equivalent quantity ([steroid] / [active site] < 1) was also incubated. "□" is the 5β-DHT dehydrogenase activity of the control. "◊" represents the unsaturated experimental ([steroid] / [enzyme] = 1.47; [steroid] / [active site] = 0.71). "○" is the saturated experimental ([steroid] / [enzyme] = 2.1;

[steroid] / [active site] = 1). At the termination of the incubation (1500 min, 25 h.) the solution with equal quantities of steroid and S-subunit ([steroid] / [active site] = 1) had lost all steroid dehydrogenase activity. That with [steroid] / [active site] = 0.71 had lost approximately 57% of its total steroid dehydrogenase activity. This suggests a highly specific, efficient interaction between the 3α-cyclopropyl steroid and the active site of the S subunit. No steroid could be extracted from the remaining enzyme solution (methylene chloride extraction) in these experiments (less than equimolar quantity), whereas, when excess steroid was used, inactivator could be recovered in the organic solvent. This suggests that the steroid may be covalently bound to the protein. The inactivated enzyme behaved similarly to the active enzyme with respect to retention volume on hplc.

A suicide inactivation mechanism requires the enzymic transformation of a benign precursor to a reactive species. The proposed oxidation of the cyclopropanol moiety of the steroid to a cyclopropanone would occur only with associated reduction of cofactor ($NAD^+ + H \rightarrow NADH$). Experiments were performed using both ES and SS isozymes to investigate the potential requirement of cofactor for inactivation. The results are shown in Figs. 30 to 33.

Fig. 30 shows the acetaldehyde assay results of "□" (no steroid, cofactor (NAD) included), "○" (steroid, cofactor (NAD)), "○" (no steroid, no NAD) and "△"

(steroid, no NAD). The enzyme solutions containing no cofactor gradually lost activity. The "no-steroid" control (□) lost ~30% of its acetaldehyde activity over 2000 min (33 h.). The enzyme incubated with steroid and cofactor had only 37% of its initial activity.

The corresponding 5β-DHT activity is shown in Fig. 31. There is virtually no change in the steroid dehydrogenase activity of the control (□). Again, the enzyme solution containing no cofactor gradually lost activity. That containing steroid and cofactor quickly lost steroid dehydrogenase activity; there was no 5β-DHT activity remaining in the enzyme at the end of the experiment.

Fig. 32 illustrates the results for a similar experiment performed with SS isozyme. Again, activity is lost without NAD present. The enzyme incubated with steroid and cofactor retains about 34% of the orignal acetaldehyde activity, the control (no steroid, cofactor included) retains ~70% of the initial activity after 2000 min (33 h.).

In Fig. 33 is shown the corresponding 5β-DHT activities. In keeping with the experiment using ES isozyme, when steroid and cofactor are present, all steroid dehydrogenase activity is quickly lost. Incubations without NAD gradually lose activity. That with no steroid but including NAD retained most of the steroid dehydrogenase activity (~94%) over the duration of the experiment.

Obviously the cofactor, NAD, has a stabilizing effect on the enzyme, as has been noted or the EE isozyme - ADPR.

complex (71, 95). The steroid, 2R,4S-2,4-cyclo-5α-androstane-3α,17β-diol, apparently does not stabilize the enzyme since activity loss is similar to that without the steroid being present (○ , △). In the absence of cofactor the enzyme gradually loses activity regardless of the presence or absence of steroid inactivator. When the steriod and the cofactor are present, approximately 40% of the acetaldehyde activity and all the 5β-DHT dehydrogenase activity is lost. This is in agreement with the proposed suicide mechanism.

The transformation product of the 3α-cyclopropyl steroid is presumably covalently attached to the protein. The amino acid residue to which it may be attached is unknown. Synthesis of radioactive 2R,4S-2,4-cyclo-5α-androstane-3α,17β-diol is required and a demonstrated association of radioactivity with eluting protein on chromatography. Digestion of the enzyme, should further yield a peptide fragment with associated radioactivity. Since the amino acid sequence of the S (steroid active) subunit is essentially known, identification of the residue may be possible.

Because of the complete loss of steroid dehydrogenase activity in the steroid active enzyme, apparently with a steroid to active site ratio of 1:1, and because of the highly reactive cyclopropanone moiety generated by oxidation of the cyclopropanol it is speculated that covalent attachment occurs in or near the active site of the

enzyme. Had the steroid product escaped into solution and only later attacked the enzyme, less than stoichiometric inactivation would be expected. Also there is apparently no non-enzymic reaction involving the steroid since the steroid is recoverable from the incubation solution when no enzyme is present. Acetaldehyde still functions as a substrate, even after complete loss of steroid dehydrogenase activity, suggesting that the active site is not completely obstructed. The cyclopropanone function will most likely react with nucleophilic agents near the active site. Examination of the three dimensional model of HLADH prompted the suggestion of Lsy-113, Lys-315 and Met-118 as possible reactive residues.

Of great interest is the fact that only the 3α-hydroxy-2,4-cyclo isomer is recognized by the enzyme while the 3β-isomer is not. The three membered ring introduced into the A ring of the 5α-DHT greatly changes its geometry. The hydroxyl group of the 3α-hydroxy isomer is located under the A ring in an unlikely location for catalysis (see Fig. 22). It would be likely that the 3β-hydroxy isomer is a substrate for some 3α-hydroxy steroid dehydrogenases.

In Fig. 34 is shown Dreiding models of 3β-hydroxy-5β-DHT and the 3α-hydroxy-2,4-cyclo inactivator. When the B, C and D rings are superimposed the hydroxyl group of the A ring of both steroids are almost coincident. This may help to explain the 3β-hydroxy steroid dehydrogenase recognition of the 3α-hydroxysteroid as a substrate. Because, however,

it is the 3-hydrogen that is transferred by the enzyme, the 3α-hydroxysteroid may enter the active site upside down compared to the entry of 5β-DHT.

Log plots of the inactivation of ES and SS isozymes are presented in Fig. 25, 26 and 28. These indicate that inactivation of the enzyme is not first order; indeed, Figs. 25 and 28 indicate that a biphasic type of mechanism may occur. The nature of this biphasic mechanism was not investigated.

The 3β-hydroxysteroid dehydrogenases are crucial to the biosynthesis of all hormonal steroids. This discovery of a specific inhibitor may prove useful in the study of hormonal mechanisms in experimental animals, and perhaps even be useful in therapy.

Table 1. Enzyme Purification Results.

A change of 1.000 OD units at 340 nm in 3.0 ml is equivalent to transfer of 477.6 nmol of NAD(H), (ε 6200 at λmax 340 nm in H_2O, 1.000 OD units = 159.2 μmol NADH). 1 unit of activity is defined as reducing 1.0 μmol of NAD per minute at 25.0°C. Specific activity is defined as units per mg of protein. % yield is calculated from the total specific activity (5β-DHT) after chromatography on CM-52 cellulose. Purification is calculated from the increase in specific activity (5β-DHT) of the sample after chromatography on CM-52 cellulose. Specific activity of the pool of protein from the DEAE column reported on page 65 is calculated from the total activity of all fractions eluted from the column.

PROCEDURE	FRACTION NO.	VOLUME ml	UNITS ml	TOTAL UNITS	PROTEIN mg/ml	UNITS mg/prot.	YIELD %	PURIFICATION
DEAE	78-88	66.1	ACETALDEHYDE 150.7	ACETALDEHYDE 9,948	9.02	ACETALDEHYDE 16.71	—	—
			5β DHT 0.47	5β DHT 31.6		5β DHT 0.053		
	56-76	112.0	ACETALDEHYDE 105.2	ACETALDEHYDE 11,784	9.44	ACETALDEHYDE 11.15	—	—
			5β DHT 1.51	5β DHT 168.6		5β DHT 0.160		
1st CM-52 (ES)	15	19.12	ACETALDEHYDE 35.5	ACETALDEHYDE 678.8	2.13	ACETALDEHYDE 16.52	14.7	2.13
			5β DHT 0.242	5β DHT 4.63		5β DHT 0.113		
2nd CM-52 (ES)	39	10.12	ACETALDEHYDE 448	ACETALDEHYDE 4,533.6	3.56	ACETALDEHYDE 125.8	18.7	5.44
			5β DHT 3.12	5β DHT 31.6		5β DHT 0.87		
	40	10.12	ACETALDEHYDE 96.9	ACETALDEHYDE 980.6	2.22	ACETALDEHYDE 43.7	3.07	1.44
			5β DHT 0.51	5β DHT 5.18		5β DHT 0.23		
2nd CM-52 (SS)	43	10.97	ACETALDEHYDE 5.53	ACETALDEHYDE 60.6	1.26	ACETALDEHYDE 4.38	5.72	4.38
			5β DHT 0.88	5β DHT 9.63		5β DHT 0.70		

Fig. 18. HPLC of HLADH Isozymes.
(TSK 3000 SW Protein Column, 0.05 M Phosphate Buffer, pH = 7.0)
a. Commercially available Sample EE (Sigma Chemicals)
b. ES (Fraction #15)
c. SS (Fraction #43)

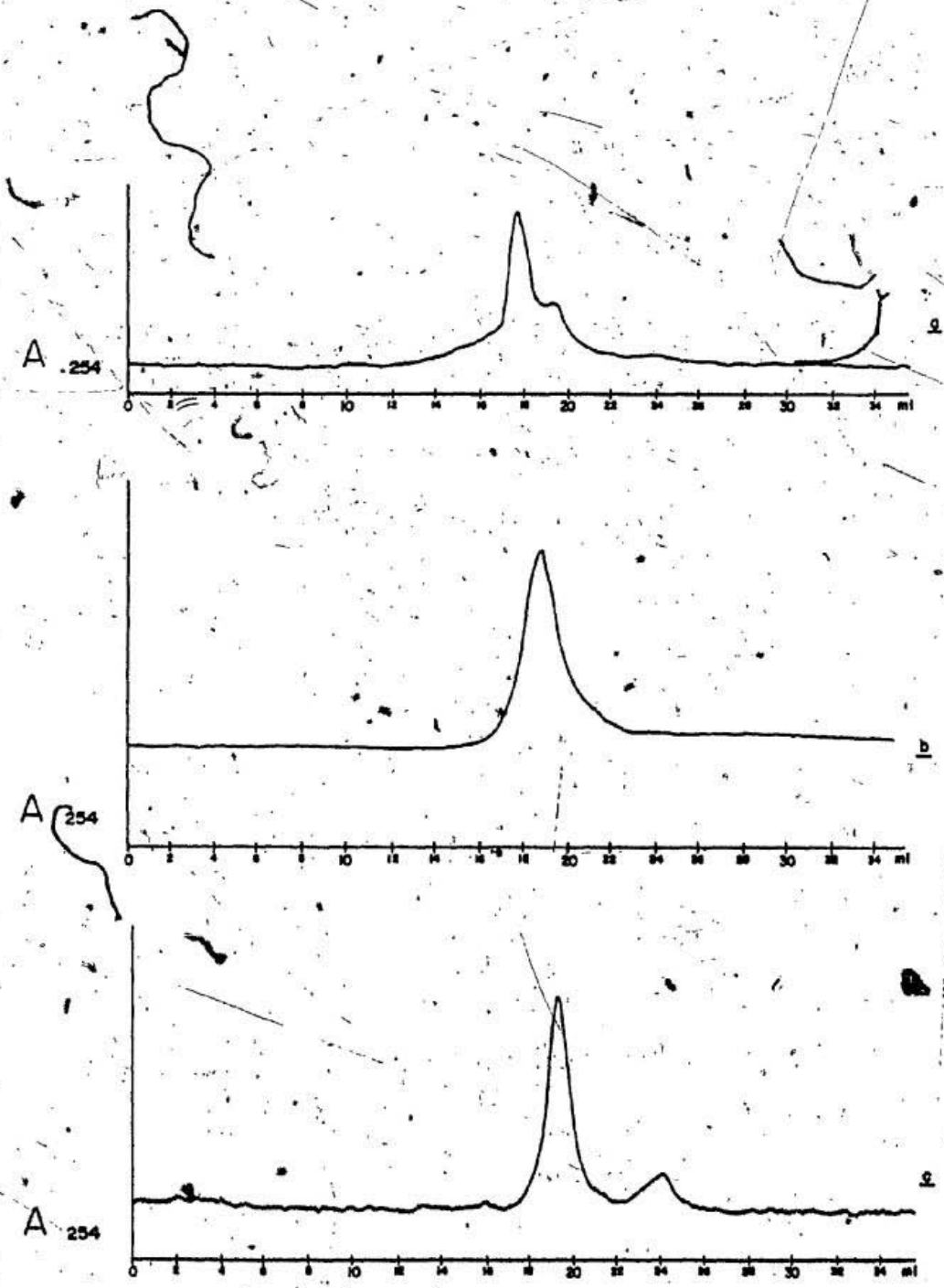
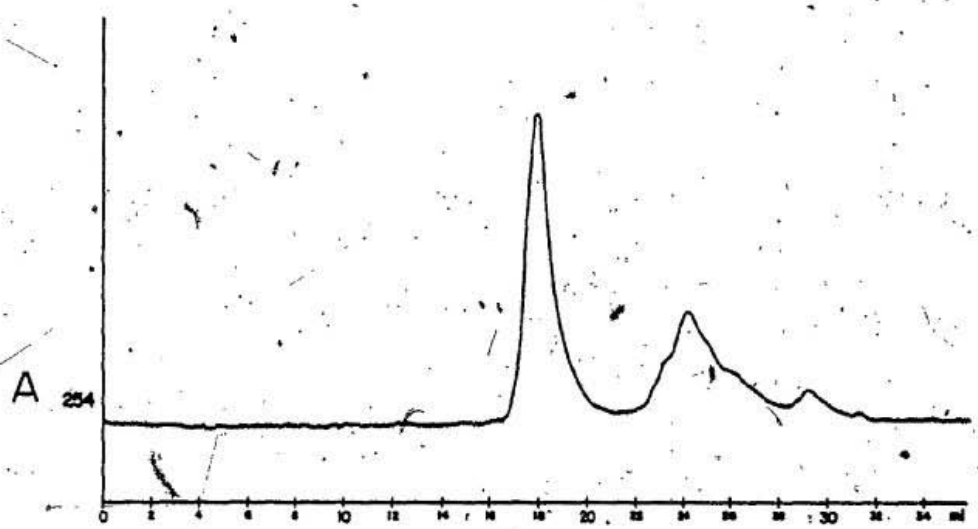

A 254
a
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 ml
A 254
b
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 ml
A 254
c
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 ml

Fig. 19. HPLC of Isozymes Continued.

a. Commercially Available Sample EE (Sigma Chemicals)

b. ES'


A
254
a

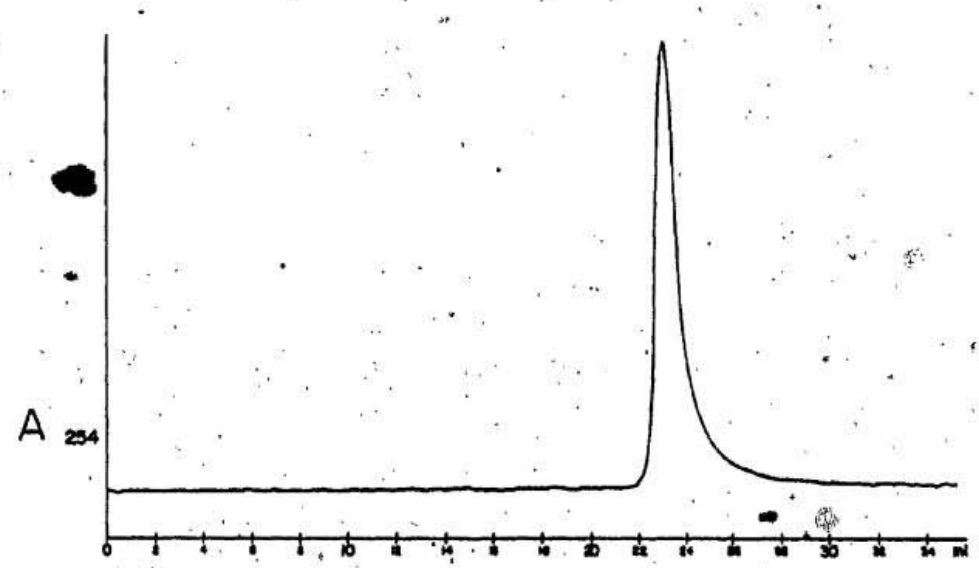

A
254
b

Fig. 20. Illustration of Peak "Shaving" in the Separation of 2α,4α-dibromo-5α-androstane-3α and 3β,17β-diol 17-acetate.
(Whatman Partisil 10/25 Silica Column, chloroform/hexane (1:1) Eluent)


A STOP
B START
B STOP
A START
a
A
B
b
A
c
A 254
2
4
6
8
10
12 ml

Fig. 21. Example of the use of the Recycle in the Separation of 2α,4α-dibromo-5α-androstane-3α and 3β,17β-diol, 17-acetate.
(Whatman Partisil 10/25 Silica Column, chloroform/hexane (1:1) Eluent)

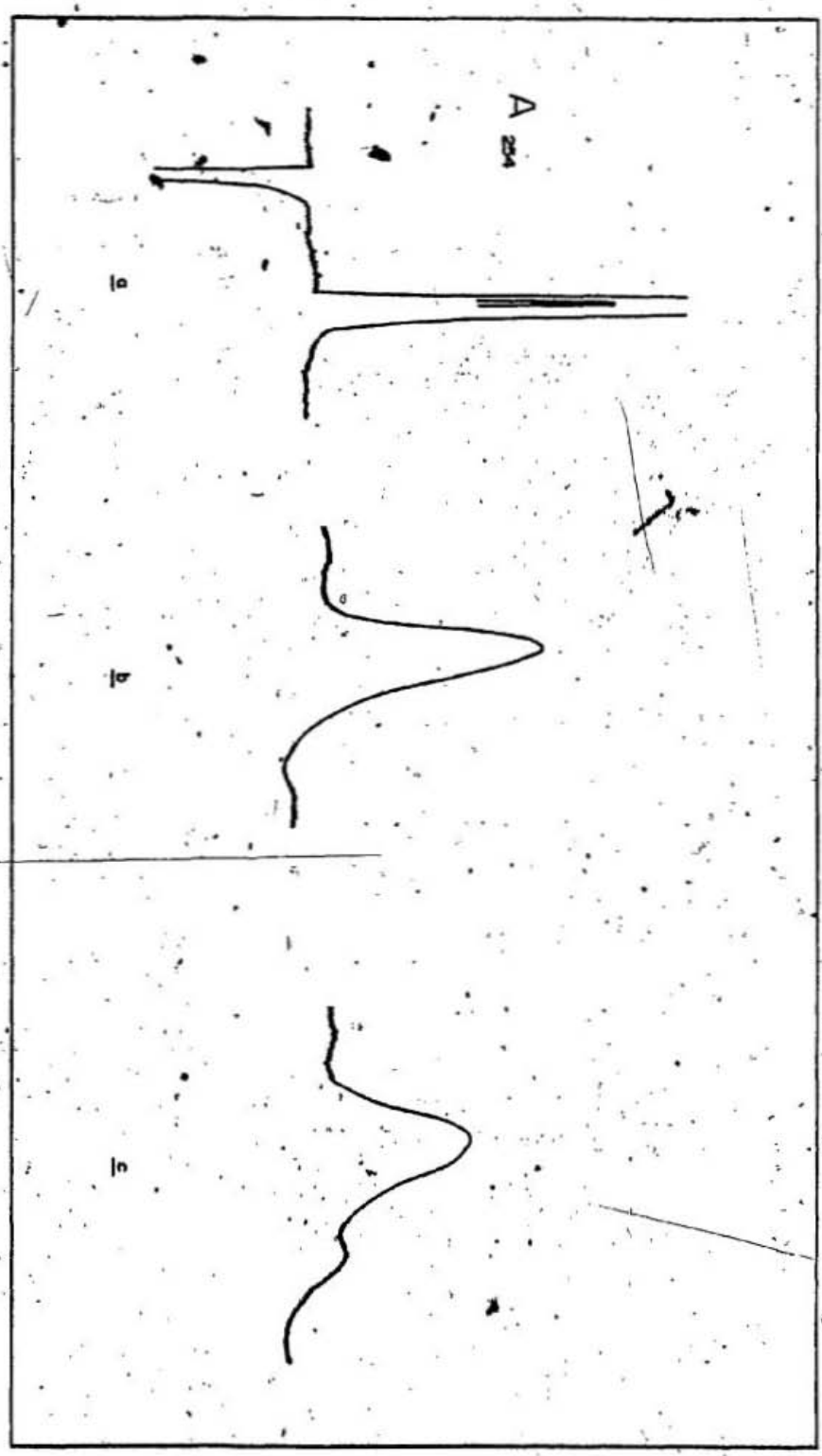

A
Ia
Ib
Ic

Table 2. δ-Values of C-18 and C-19 Methyl Groups of Selected Steroids from the Synthesis of 2R,4S-2,4-cyclo-5α-androstane-3$\alpha$and 3β,17β-diol from 5α-DHT.

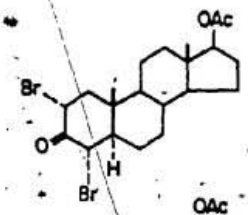
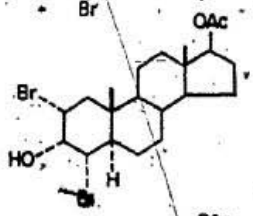
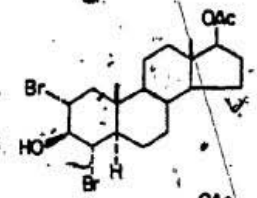
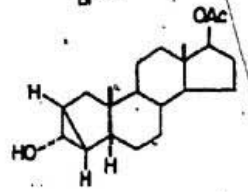
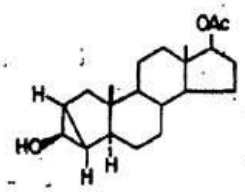
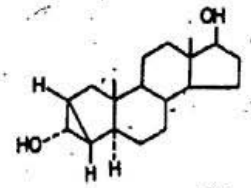
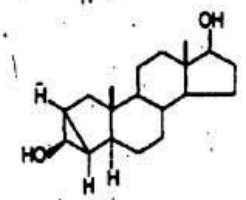
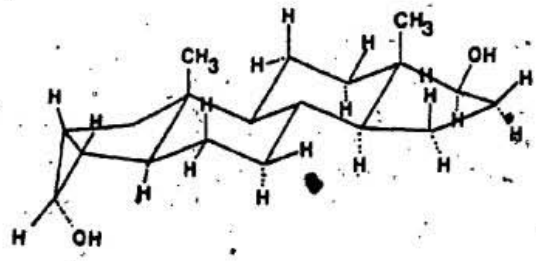
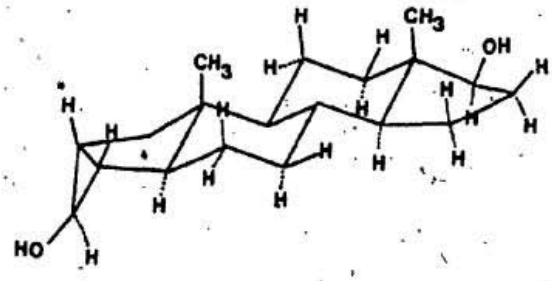
	C19 (p.p.m.)	C18 (p.p.m.)
	1.16	0.80
	0.89	0.77
	0.89	0.77
	0.94	0.77
	0.85	0.77
	0.94	0.73
	0.85	0.73

Fig. 22. Stereochemistry of 3-hydroxyl in 2R,4S-2,4-cyclo-5α-androstane-3α,17β-diol (a) and 2R,4S-2,4-cyclo-5α-androstane-3β,17β-diol (b).



a



b

Table 3. Survey of Inhibition of HLADH by Various Water Miscible Solvents.

Aliquots (10μl) of each solvent were added to a 3.0 ml buffer solution (sodium phosphate, 0.1 M, pH = 7.0) of commercially obtained HLADH enzyme (Sigma Chemicals). The specific activity was measured both before and after addition of the test solvent.

NO.	SOLVENT	INHIBITION	COMMENTS
1.	T-BUTANOL	0%	PREFERRED SOLVENT; NOT SUBSTRATE FOR EITHER ISOZYME
2.	DIOXANE	20%	ALSO FORMED PEROXIDES
3.	TRITON X 100	31%	
4.	FORMAMIDE	39%	
5.	ACETONITRILE	42%	
6.	TETRAHYDROFURAN	69%	
7.	PYRIDINE	100%	
8.	DIMETHYLFORMAMIDE	100%	
9.	DIMETHYLSULFOXIDE	100%	
10.	BRIJ-35	–	CONTAINED A SUBSTRATE FOR THE ENZYME

Table 4. Summary of Inactivation Experiments.

EXPERIMENT NO.	STEROID	ENZYME	STEROID (mM)	ENZYME (mM)	NAD (mM)	FIGURE NO.	% ACTIVITY AT ENDPOINT	
							CH_3CHO	5β-DHT.
1	A	ES	0.040	0.022	8.08	—	NO INACTIVATION	
2	A	SS	0.040	0.022	8.49	—	NO INACTIVATION	
3	B	SS	0.058	0.028	6.95	29	—	0
			0.041	0.028	6.95	29	—	33
4	B	ES	0.261	0.047	8.10	23,24	60	0
5	B	SS	0.191	0.028	6.95	27	40	0

Fig. 23. Inactivation of ES Isozyme.

o - Acetaldehyde assay ("no steroid" control)

Δ - Acetaldehyde assay (experimental)

(Buffer, pyrophosphate, 0.05 M, pH = 9.0; Enzyme, 0.047 mM; NAD, 8.10 mM; Steroid, 0.261 mM; Incubation Temperature, 25.0°C)

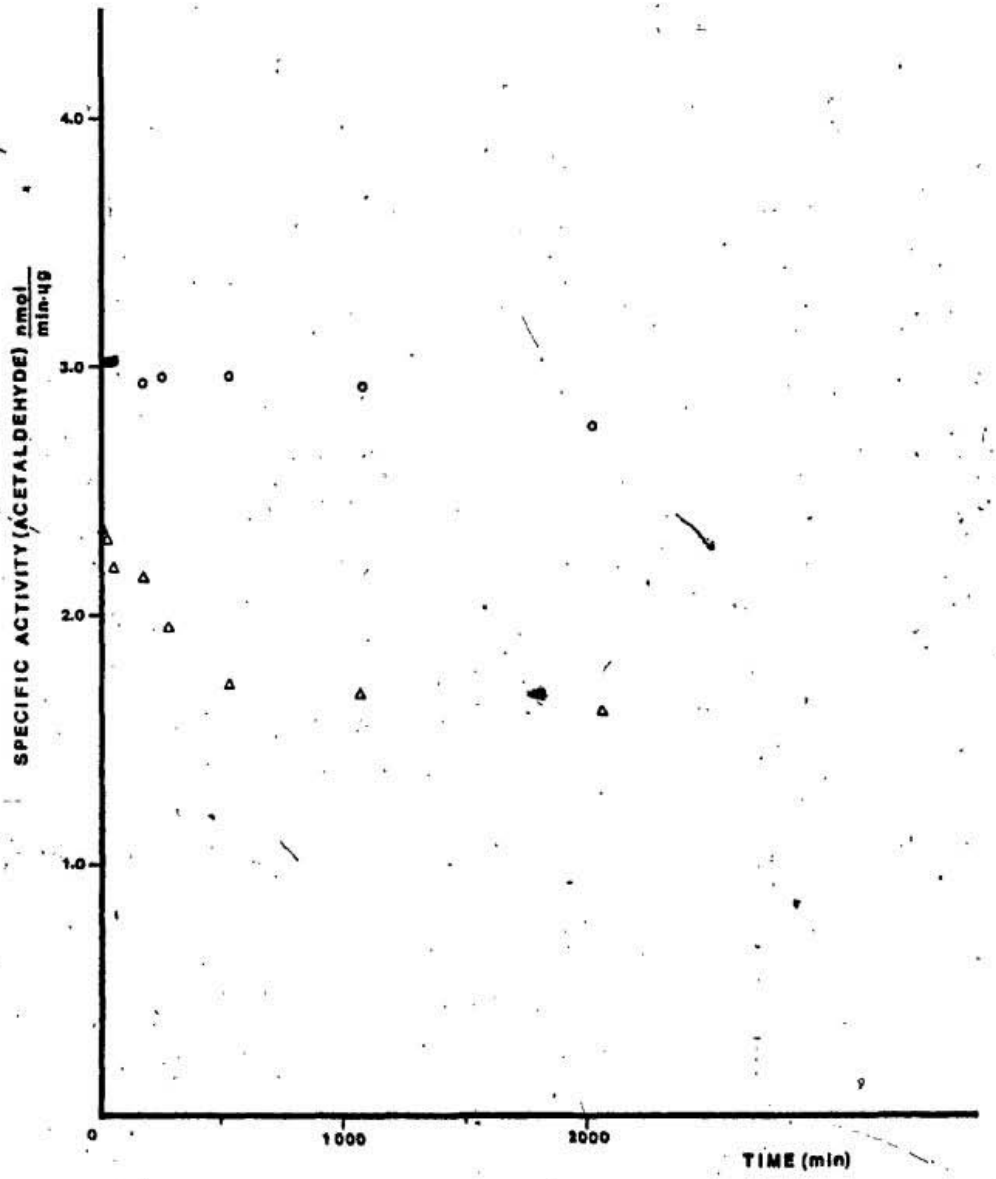

SPECIFIC ACTIVITY (ACETALDEHYDE) nmol/min-ug
4.0
3.0
2.0
1.0
0
1000
2000
TIME (min)

Fig. 24. Inactivation of ES Isozyme.

□ - 5β-DHT assay ("no steroid" control)

○ - 5β-DHT assay (experimental)

(Buffer, pyrophosphate, 0.05 M, pH = 9.0; Enzyme, 0.047 mM; NAD, 8.10 mM; Steroid, 0.261 mM; Incubation Temperature, 25.0°C)

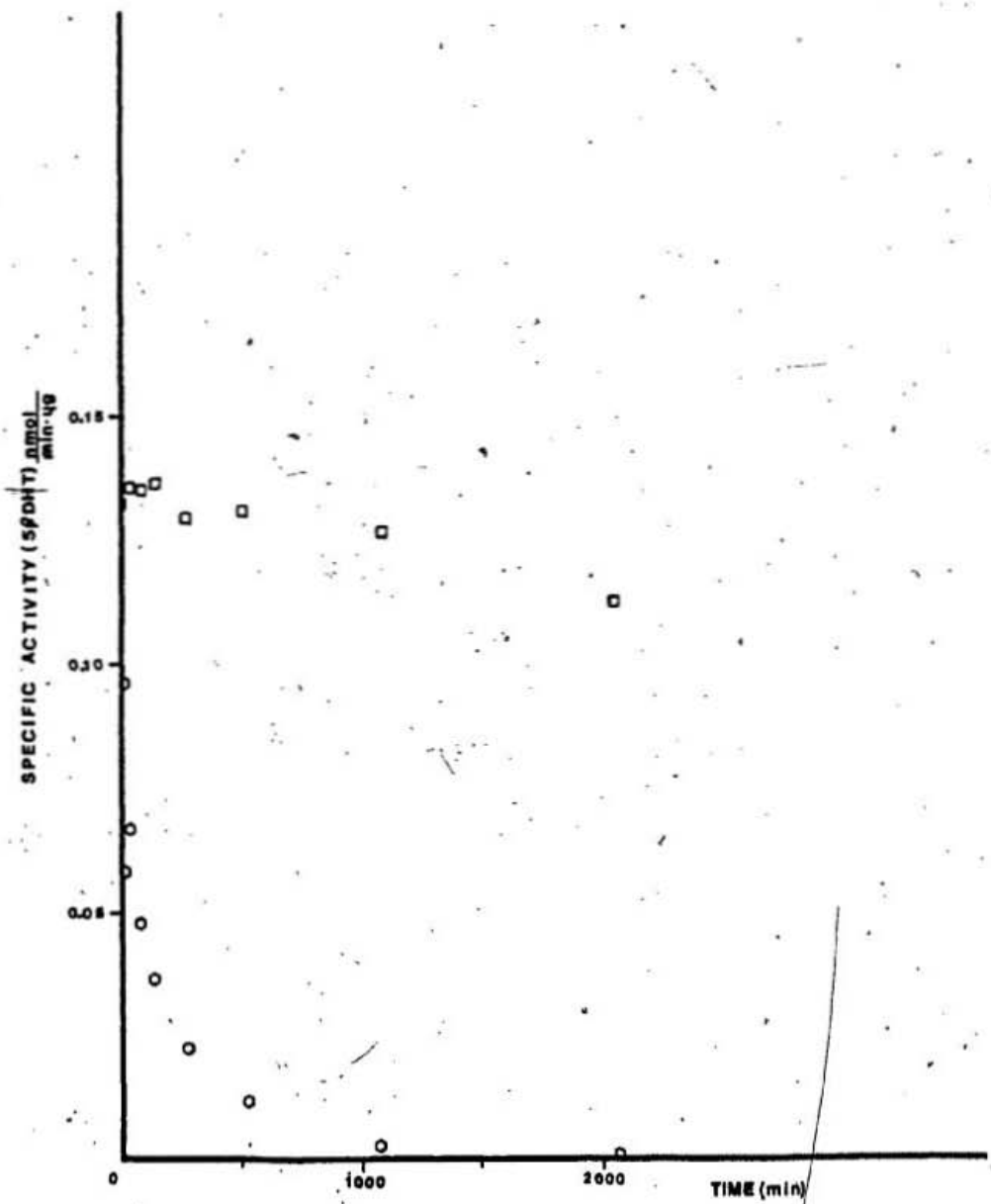
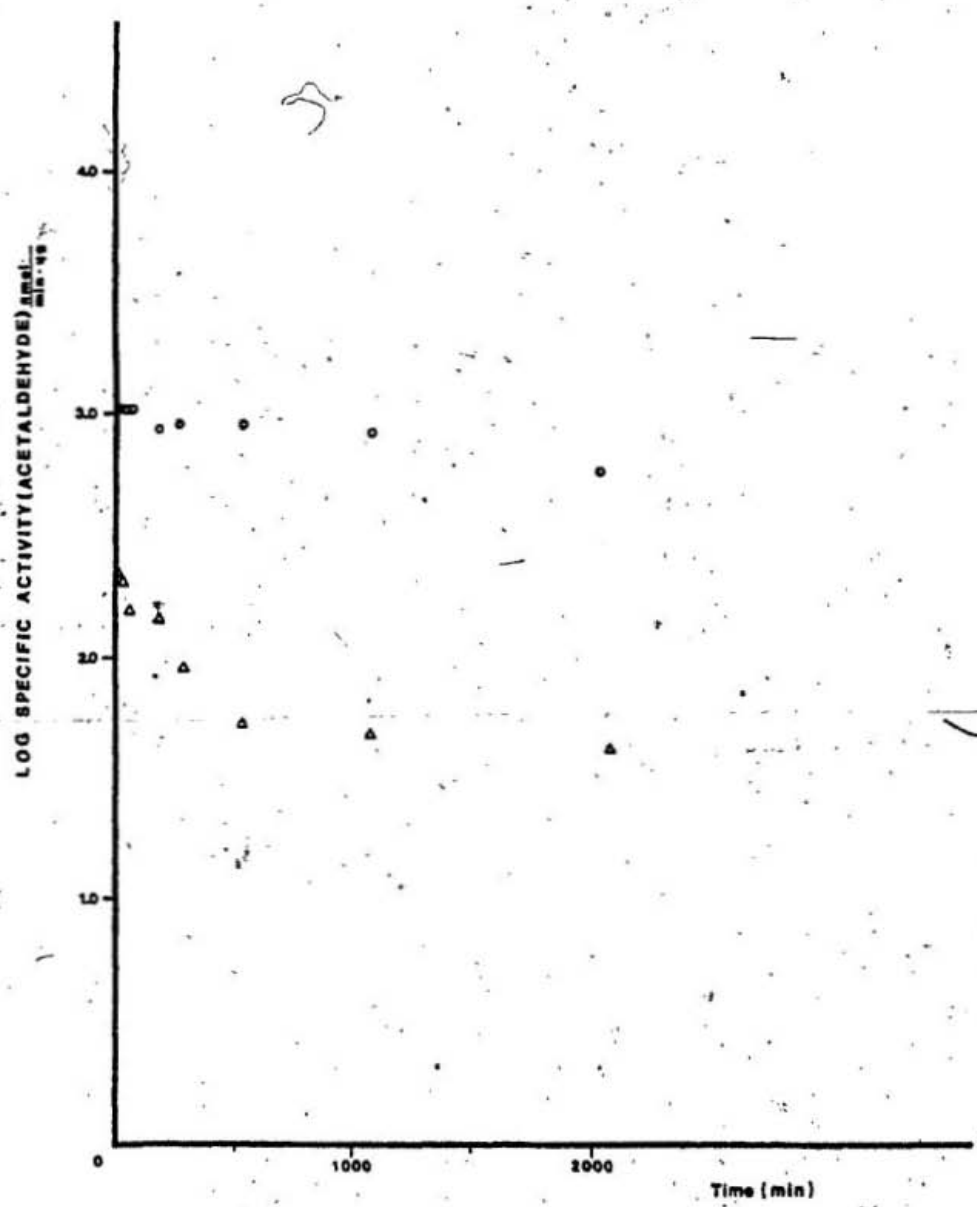

SPECIFIC ACTIVITY (SPOHT) nmol/min·ug
0.15
0.10
0.05
0
1000
2000
TIME (min)

Fig. 25. Log. Plot: Inactivation of ES Isozyme.

o - Acetaldehyde assay ("no steroid" control)

Δ - Acetaldehyde assay (experimental)

(Buffer, pyrophosphate, 0.05 M, pH = 9.0; Enzyme, 0.047 mM; NAD, 8.10 mM; Steroid, 0.261 mM; Incubation Temperature, 25.0°C)


LOG SPECIFIC ACTIVITY (ACETALDEHYDE) nmol/min·mg
2.5
2.0
1.5
0
1000
2000
Time (min)

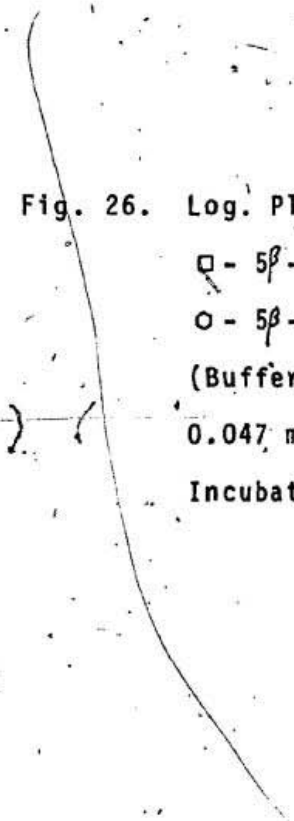


Fig. 26. Log. Plot: Inactivation of ES Isozyme.
□ - 5β-DHT assay ("no steroid" control)
○ - 5β-DHT assay (experimental)
(Buffer, pyrophosphate, 0.05 M, pH = 9.0; Enzyme, 0.047 mM; NAD, 8.10 mM; Steroid, 0.261 mM; Incubation Temperature, 25.0°C)

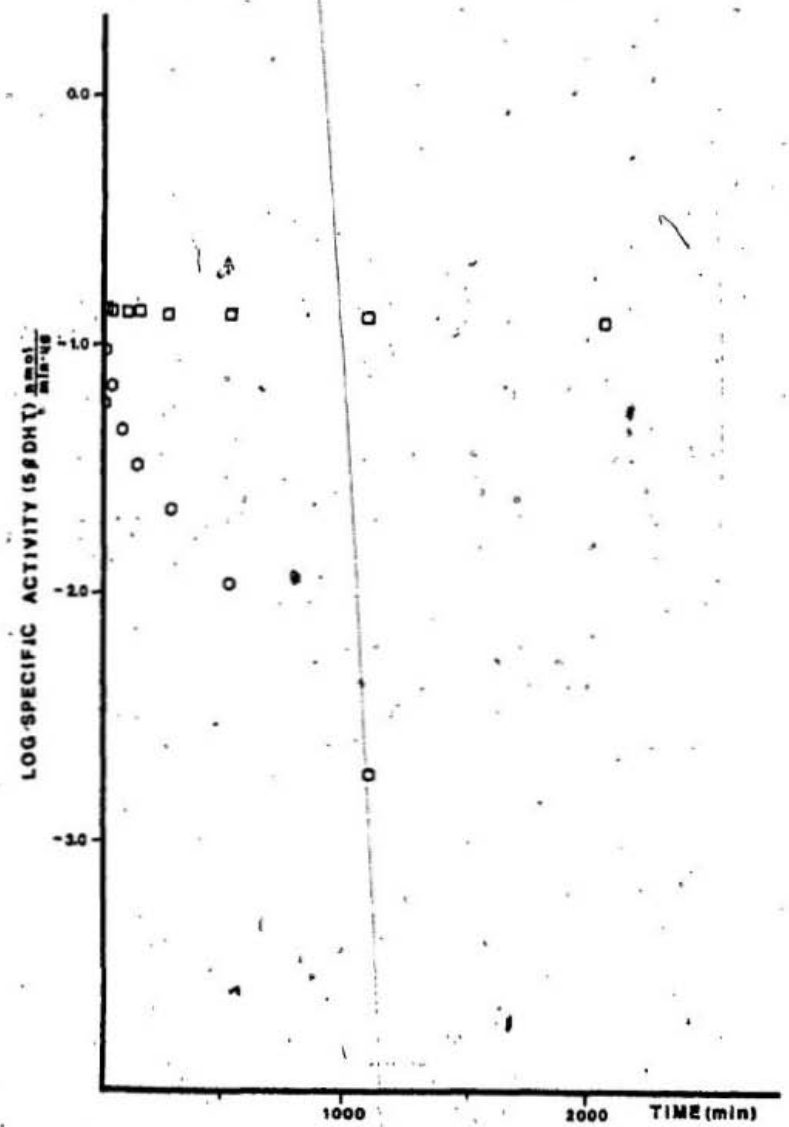

0.0
-1.0
-2.0
-3.0
LOG SPECIFIC ACTIVITY (5ΦDHT) amol/min-µg
1000
2000
TIME (min)

Fig. 27. Inactivation of SS Isozyme.

o - Acetaldehyde assay ("no steroid" control)

□ - 5β-DHT assay ("no steroid" control)

Δ - Acetaldehyde assay (experimental)

o - 5β-DHT assay (experimental)

(Buffer, pyrophosphate, 0.05 M, pH = 9.0; Enzyme, 0.028 mM; NAD, 6.95 mM; Steroid, 0.191 mM; Incubation Temperature, 25.0°C)

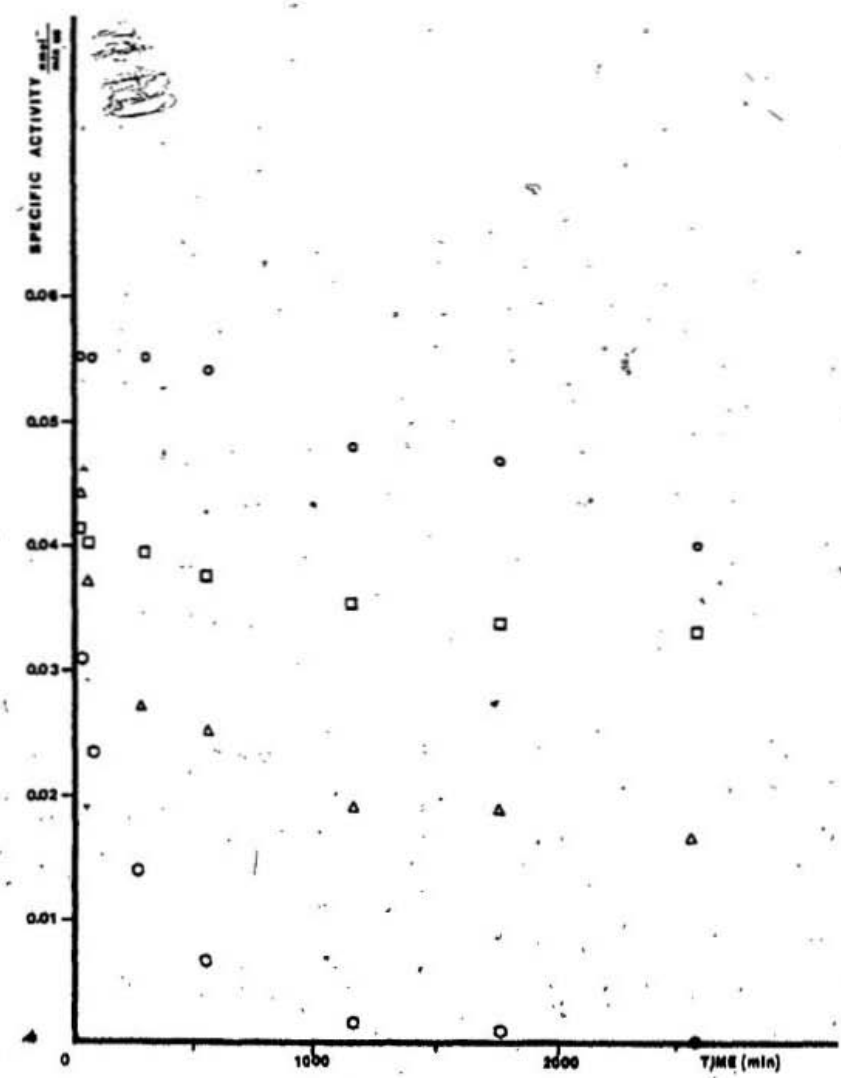

SPECIFIC ACTIVITY
0.06
0.05
0.04
0.03
0.02
0.01
0
1000
2000
TIME (min)

Fig. 28. Log. Plot: Inactivation of SS Isozyme.

○ - Acetaldehyde assay ("no steroid" control)

□ - 5β-DHT assay ("no steroid" control)

△ - Acetaldehyde assay (experimental)

○ - 5β-DHT assay (experimental)

(Buffer, pyrophosphate, 0.05 M, pH = 9.0; Enzyme, 0.028 mM; NAD, 6.95 mM; Steroid, 0.191 mM; Incubation Temperature, 25.0°C)

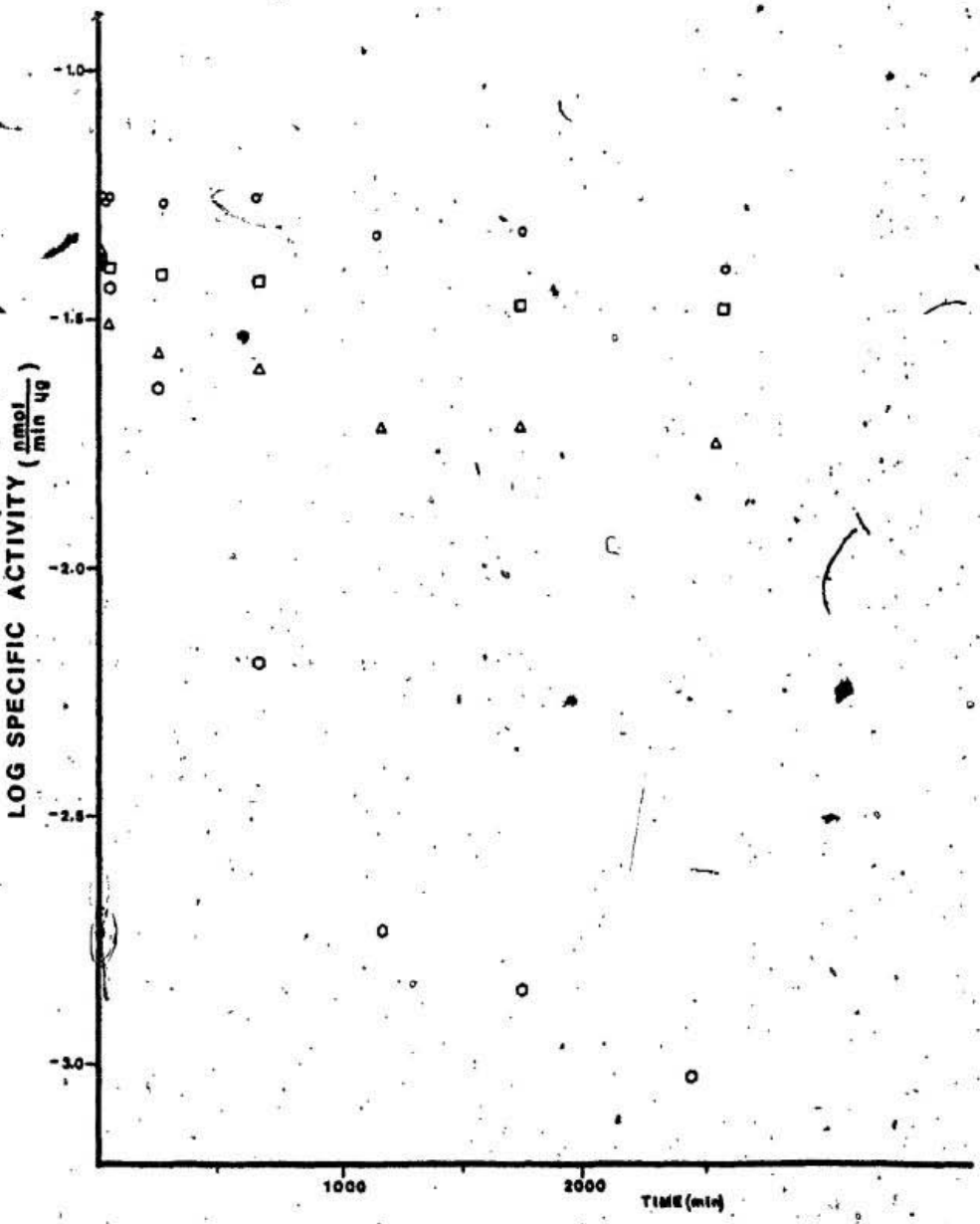

LOG SPECIFIC ACTIVITY (nmol / min ug)
-1.0
-1.5
-2.0
-2.5
-3.0
1000
2000
TIME (min)

Fig. 29. Inactivation of SS Isozyme.

□ - 5β-DHT assay ("no steroid" control)

△ - 5β-DHT assay ([steroid] / [active site] <1)

○ - 5β-DHT assay ([steroid] / [active site] =1)

(Buffer, pyrophosphate, 0.05 M, pH = 9.0; Enzyme, 0.028 mM; NAD, 6.95 mM; Steroid, △ = 0.041 mM, ○ = 0.058 mM; Incubation Temperature, 25.0°C)

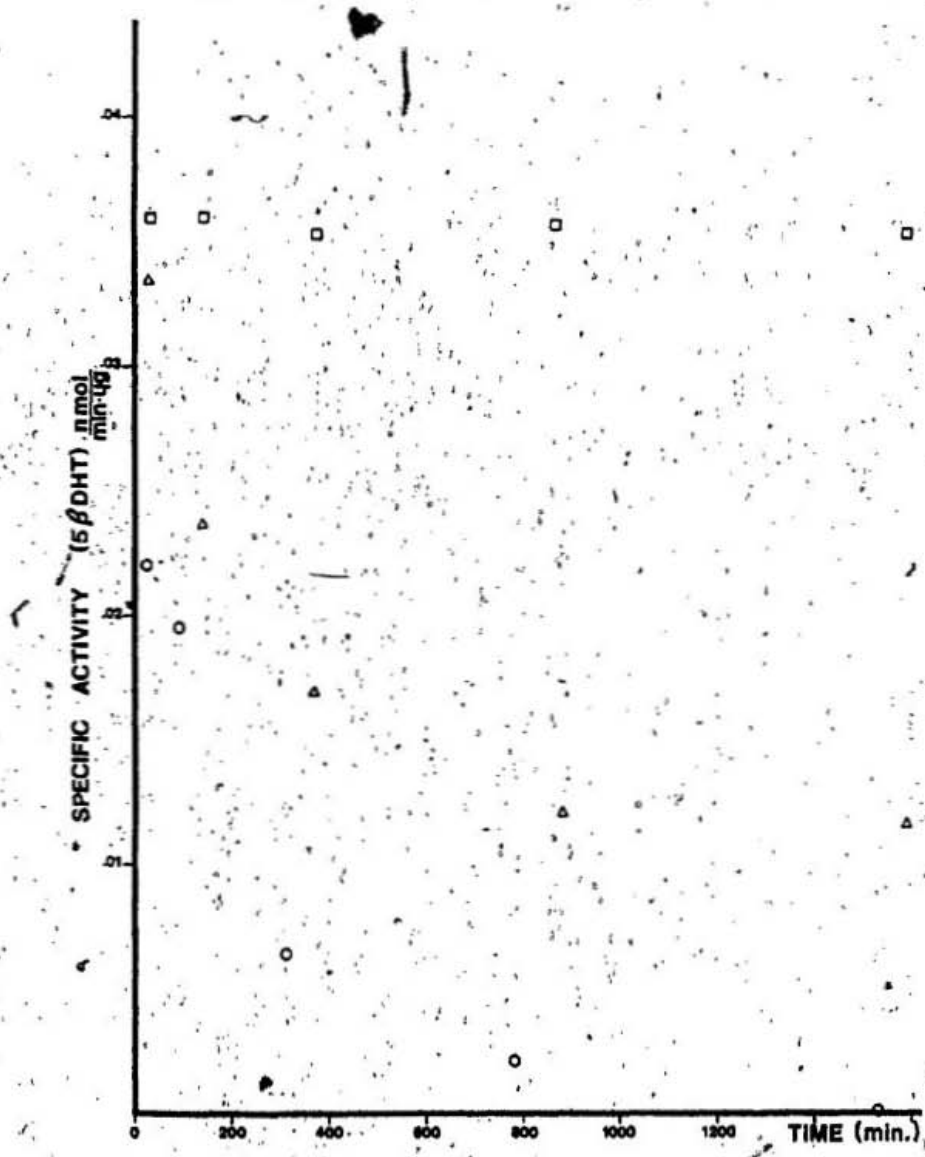

SPECIFIC ACTIVITY (5βDHT) nmol/min-ug
0
200
400
600
800
1000
1200
TIME (min.)

Fig. 30. Acetaldehyde Assay of ES Isozyme.

□ - "no steroid" control, + NAD.

○ - steroid, + NAD

○ - "no steroid", no NAD

△ - steroid, no NAD

(Buffer, sodium phosphate, 0.1 M, pH = 7.0; NAD, 0.18 mM; Acetaldehyde, 0.12 mM; Steroid, 0.11 mM; 340 nm)

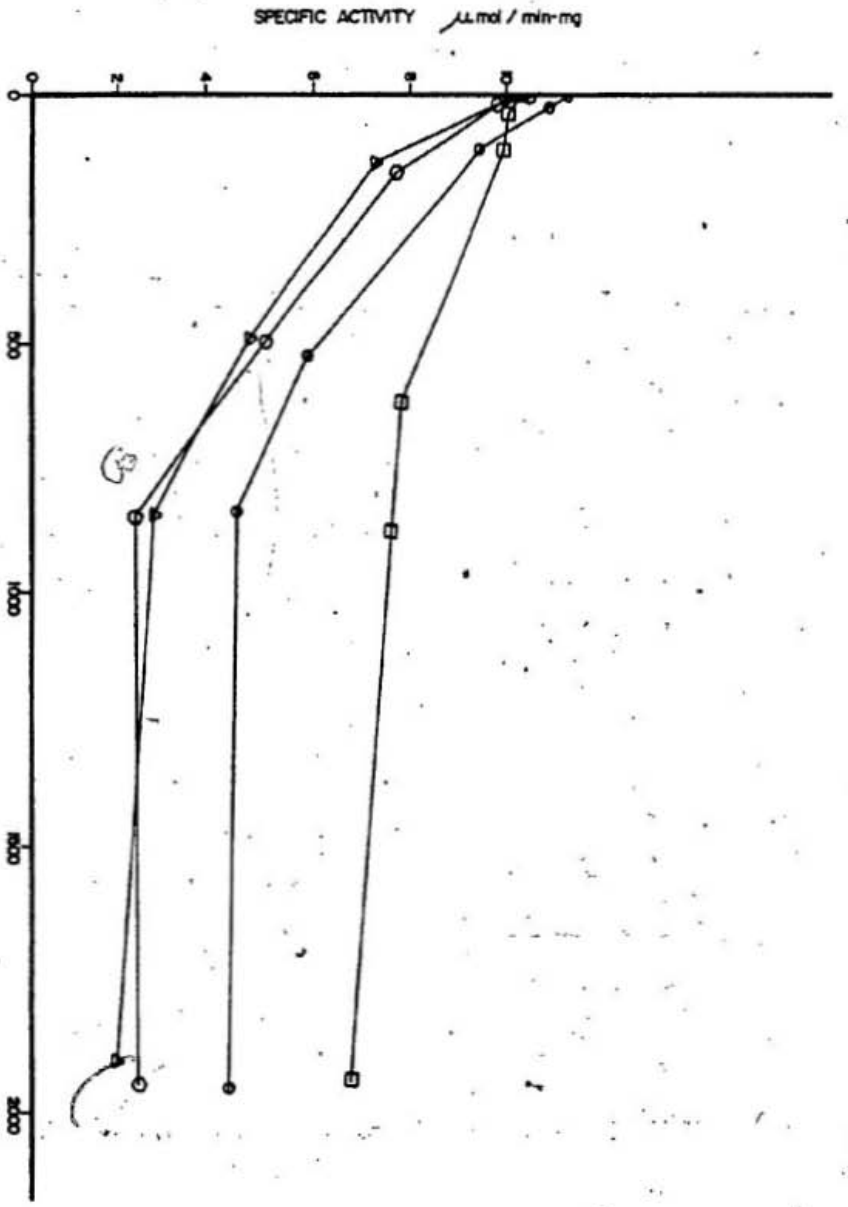

SPECIFIC ACTIVITY μ mol / min-mg
0
2
4
6
8
10
1000
1500
2000
TIME (min.)

Fig. 31. 5β-DHT Assay of ES Isozyme.

□ - "no steroid" control, + NAD

○ - steroid, + NAD

ȯ - "no steroid" , no NAD

△ - steroid, no NAD

(Buffer, sodium phosphate, 0.1 M, pH = 7.0; NAD, 0.18 mM; Acetaldehyde, 0.12 mM; Steroid, 0.11 mM; 340 nm)

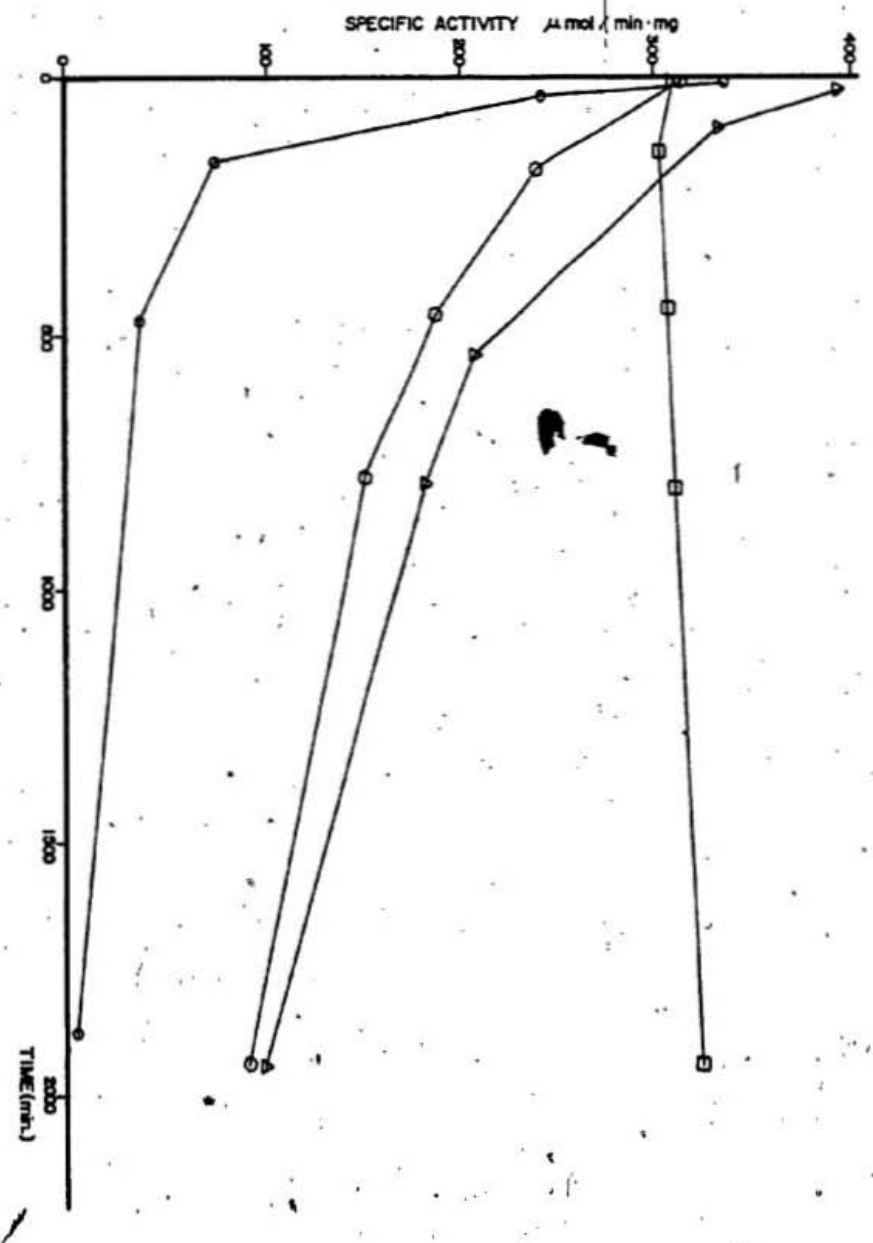

SPECIFIC ACTIVITY μmol / min·mg
100
200
300
400
500
1000
1500
2000
TIME(min.)

Fig. 32. Acetaldehyde Assay of SS Isozyme.

□ - "no steroid" control, + NAD

○ - steroid, + NAD

○ - "no steroid", no NAD

△ - steroid, no NAD

(Buffer, sodium phosphate, 0.1 M, pH = 7.0; NAD, 0.18 mM; Acetaldehyde, 0.12 mM; Steroid, 0.11 mM; 340 nm)

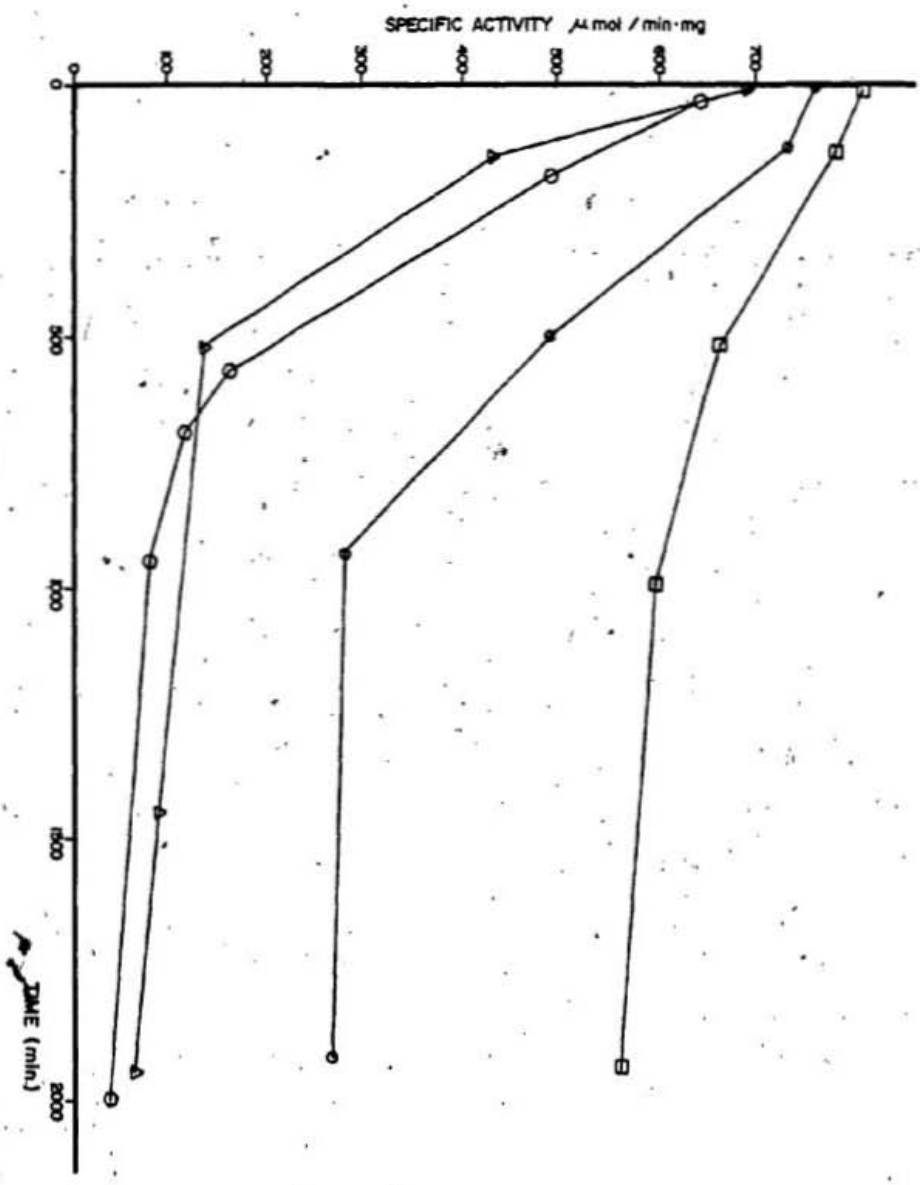

SPECIFIC ACTIVITY μmol / min·mg
0
100
200
300
400
500
600
700
500
1000
1500
2000
TIME (min.)

Fig. 33. 5β-DHT Assay of SS Isozyme

□ - "no steroid" control, + NAD

○ - steroid, + NAD

○ - "no steroid", no NAD

△ - steroid, no NAD

(Buffer, sodium phosphate, 0.1 M, pH = 7.0; NAD 0.18 mM; Acetaldehyde, 0.12 mM; Steroid, 0.11 mM; 340 nm)

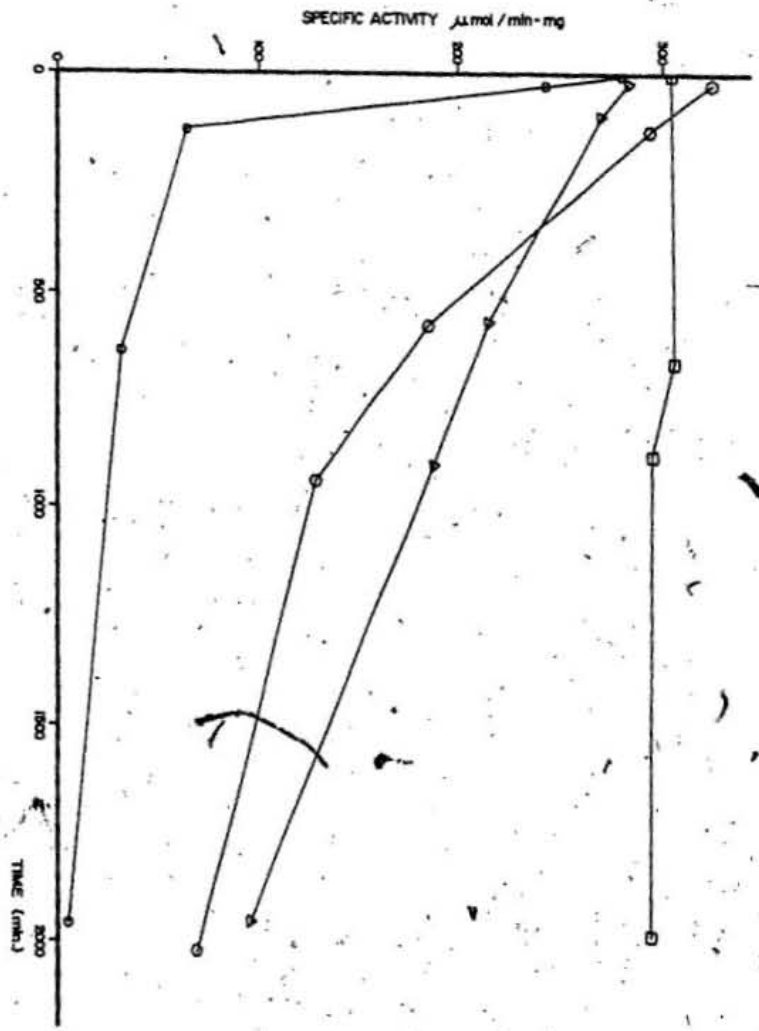
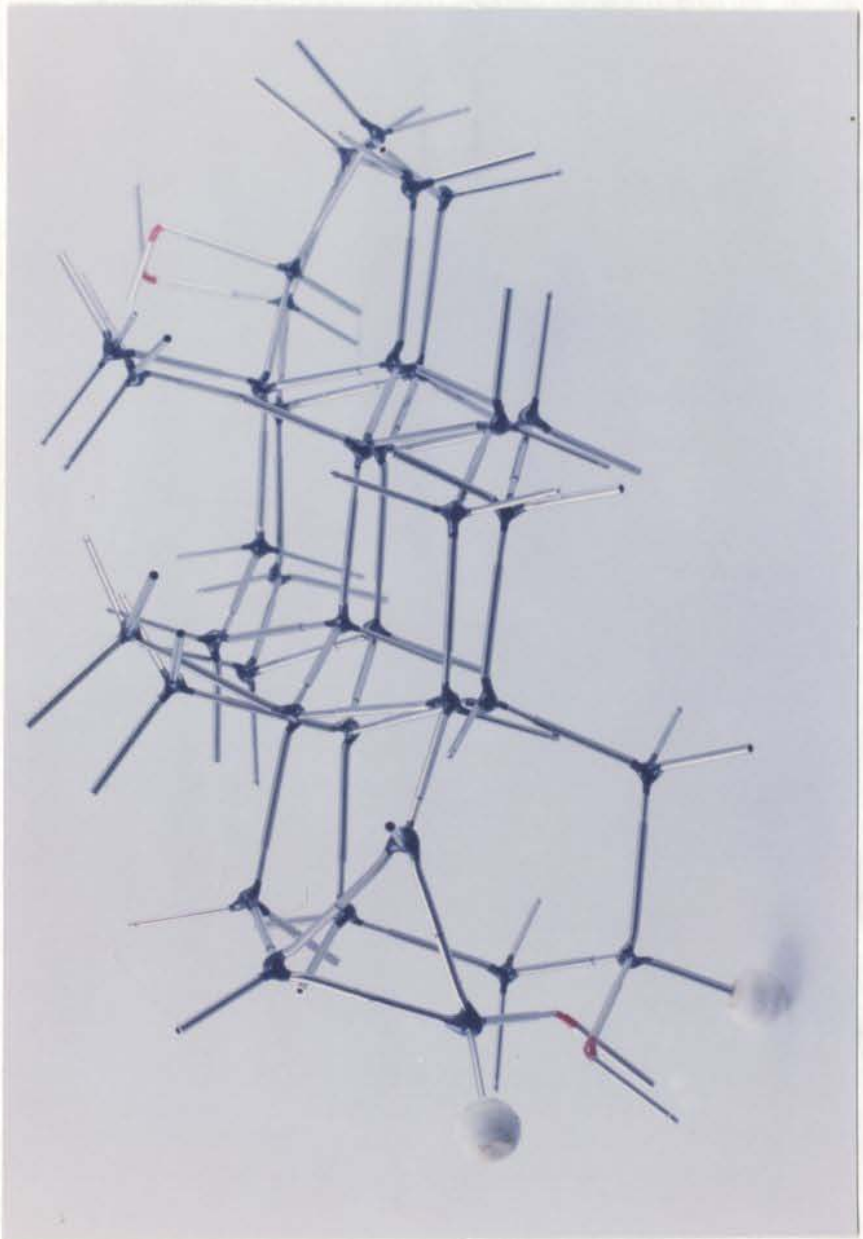

SPECIFIC ACTIVITY μmol / min-mg
0
500
1000
1500
2000
TIME (min.)

Fig. 34. Dreiding Models of 5β-androstane-3β,17β-diol and 2R,4S-2,4-cyclo-5α-androstane-3α,17β-diol.



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