Dubin, D. T., and Brown, R. E. (1967), *Biochim. Biophys. Acta 145*, 538.
Eagle, H. (1959), *Science 130*, 432.
Galibert, F., Larsen, C. J., Lelong, J. C., and Boiron, M. (1965), *Nature 207*, 1039.
Knight, E., Jr. (1969), *Biochim. Biophys. Acta 182*, 562.
Knight, E., Jr., and Darnell, J. E. (1967), *J. Mol. Biol. 28*, 491.
Knight, E., Jr., and Sugiyama, T. (1969), *Proc. Natl. Acad. Sci. U. S.* (in press).
Loening, U. E. (1967), *Biochem. J. 102*, 251.
Nass, M. M. K., and Buck, C. A. (1969), *Proc. Natl. Acad. Sci. U. S. 62*, 506.
O'Brien, T. W., and Kalf, G. F. (1967), *J. Biol. Chem. 242*, 2180.
Penman, S., Scherrer, K., Becker, Y., and Darnell, J. E. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 654.
Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34*, 49.
Perry, R. P. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 2179.
Radloff, R., Bauer, W., and Vinograd, J. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1514.
Sabatini, D. D., Tashiro, Y., and Palade, G. E. (1966), *J. Mol. Biol. 19*, 503.
Scherrer, K., and Darnell, J. E. (1962), *Biochem. Biophys. Res. Commun. 9*, 451.
Smith, L. (1955), *Methods Biochem. Anal. 2*, 427.
South, D. J., and Mahler, H. R. (1968), *Nature 218*, 1226.
Vesco, C., and Penman, S. (1969), *Proc. Natl. Acad. Sci. U. S. 62*, 218.
Waring, M. J. (1966), *Biochim. Biophys. Acta 114*, 234.
Weinberg, R. A., Leoning, U., Willems, M., and Penman, S. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1088.
Weinberg, R. A., and Penman, S. (1968), *J. Mol. Biol. 38*, 289.
Zylber, E., Vesco, C., and Penman, S. (1969), *J. Mol. Biol. 44*, 195.

Purification and Substrate Specificities of Bacterial Hydroxysteroid Dehydrogenases*

Charles R. Roe† and Nathan O. Kaplan‡

ABSTRACT: Four highly specific hydroxysteroid dehydrogenases have been purified from bacteria. Substrate specificity studies reveal them to be (a) ring A/B *trans* 3β-hydroxysteroid dehydrogenase, (b) 3α-hydroxysteroid dehydrogenase unable to oxidize 3α-hydroxysteroids ring A/B *cis* with an 11-keto group, (c) ring A/B *trans* 3α-hydroxysteroid dehydrogenase, and (d) an axial 3-hydroxysteroid dehydrogenase.

The possible application of these enzymes for the quantitative determination of specific steroid groups is presented.

The highly specific DPN-dependent hydroxysteroid dehydrogenases would appear to serve as ideal analytical tools for selective microquantitation of steroid substrates in biologic specimens (Stempfel and Sidbury, 1964; Hurlock and Talalay, 1957; Carstensen, 1966). The wild-type *Pseudomonas testosteroni* (ATCC 11996) has been shown to contain two DPN-dependent hydroxysteroid dehydrogenases. β-Hydroxysteroid dehydrogenase specifically oxidizes hydroxyl groups oriented above the plane of the molecule, regardless of the carbon atom to which the hydroxyl is bound. The 3β-, 17β-, and 16β-hydroxysteroids are all oxidized to their corresponding ketosteroids (Talalay and Dobson, 1953). Steroids bearing hydroxyl groups on other carbons are not substrates. α Enzyme activity is represented by the DPN-dependent oxidiation of 3α-hydroxysteroids to corresponding ketosteroids. This specificity includes those steroids having rings A/B *trans* or *cis* in either the androstane or pregnane derivatives.

Purification and characterization of the β and α enzyme from wild-type *Ps. testosteroni* have been carried out (Delin *et al.*, 1964; Squire *et al.*, 1964; Boyer *et al.*, 1965).

Because the α and β enzymes of the wild-type *Ps. testosteroni* are so nonselective, a search for organisms with more specific hydroxysteroid dehydrogenases was attempted. Emphasis has been placed on selective specificity for 3α-hydroxysteroids. These compounds are of extreme importance in the differentiation of the known varieties of congenital adrenal hyperplasia.

Three organisms have been examined in the present work. The first organism, designated STDH-m, is a presumed mutant of *Ps. testosteroni*. It was originally obtained from a stock culture of wild-type *Ps. testosteroni*. An actual relationship between the wild type and the mutant has never been established. Teller and Bongiovanni (1963) reported this organism as having the capacity for oxidation of dehydroepiandrosterone, but not testosterone. It was suggested that

* Publication No. 680 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received May 22, 1969.* Aided by grants from the American Cancer Society and National Cancer Institute, National Institutes of Health.

† Present address: Department of Pediatrics, Duke University Medical Center, Durham, N. C. 27706.

‡ Present address: Department of Chemistry, University of California, San Diego, La Jolla, Calif. 92037.

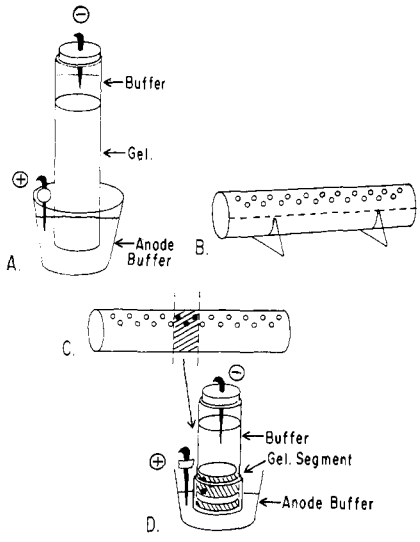

FIGURE 1: Schematic diagram indicating method of preparative polyacrylamide electrophoresis. (A) Preparative column, (B) apparatus for suctioning holes in surface of gel column, (C) dark stained holes represent site of enzyme, (D) elution apparatus into which the cut out disk is placed for electrophoretic elution of enzyme.

the "β enzyme" of this organism had lost ability to oxidize 17β-hydroxysteroids while retaining 3β activity. No purification or further characterization of the enzyme was carried out.

The second organism is strain 28 of *Ps. testosteroni* which was generously donated by Professor R. Y. Stainer of the University of California.

The third organism, which we have examined, M_3, is considered to be a partial revertant obtained from prolonged fermentation of the Teller–Bongiovanni organism STDH-m. It has numerous similarities to the group *Ps. testosteroni*, but is different from the wild type and Stanier strain 28.

The purification and substrate specificities of enzymes from each of the three organisms are the subjects of the present paper.

Methods

Experimental Procedures. GROWTH OF BACTERIA. The culture medium used was identical with that described by Marcus and Talalay (1956). The procedure was as follows: an overnight inoculum representing one-tenth of the final culture volume was transferred to the remaining medium. The culture was then grown from 4 to 6 hr at 30° with aeration at 1 l. of air/l. of medium per min. The steroid-inducing agent, testosterone (either sonicated or dissolved in a small volume of acetone), was added to a final concentration of 0.4 g/l. of medium. Growth was continued under the conditions stated above from 10 to 18 hr. The organisms were then harvested by the use of the Sharples centrifuge. In order to obtain large preparative cultures, the facilities of the Tufts New England Enzyme Center were used. We wish to thank Doctor Stanley Charm and his associates at the center for their cooperation in this endeavor.

EXTRACTION TECHNIQUE. The harvested wet cells were suspended in twice the volume of 0.05 M potassium phosphate buffer (pH 7.0) containing 10^{-3} M EDTA. The suspension was then processed into an acetone powder by pouring it in a thin stream, into 10–15 volumes of cold (−20°) acetone. After filtration, the material was resuspended in five volumes of cold butanol. Subsequent treatment included resuspension in acetone, and finally cold anhydrous ether, after which a fine talc-like powder was obtained.

This powder was routinely extracted with 5–10 volumes of 0.05 M potassium phosphate buffer (pH 7.0) with 20% glycerol and 10^{-3} M EDTA. This crude extract was then processed for further purification.

ENZYMATIC ASSAYS. Oxidation of Hydroxysteroids. All steroid substrates were obtained from the Mann Research Laboratories. The enzymatic assay was essentially as described by Talalay (Marcus and Talalay, 1956). The 3-ml system consists of 1.0 ml of 0.1 M sodium pyrophosphate buffer (pH 8.9), 0.1 ml of DPN (1.4×10^{-2} M), 0.1 ml of steroid in methanol (100 μg), 1.75 ml of distilled water, and 0.05 ml of enzyme extract. An enzyme unit equals the change in optical density at 340 mμ/min. The blank cuvet lacked only the steroid substrate for which methanol was substituted.

Reduction of Ketosteroids. A 3-ml system contains 1.0 ml of sodium phosphate buffer (pH 6.4), 0.1 ml of DPNH (1.4×10^{-2} M), 0.1 ml (100 μg) of steroid in methanol (5α-androstane-3,17-dione or 5β-androstane-3,17-dione), 1.75 ml of distilled water, and 0.05 ml of enzyme extract. The blank cuvet again contained no steroid substrate. The reaction was followed by quantitating DPNH oxidation at 340 mμ. Enzyme activity units are the same as those for oxidation of hydroxysteroids.

PROTEIN DETERMINATIONS. All protein determinations were carried out by the Lowry method (Lowry *et al.*, 1951) except in column eluates where optical density at 280 mμ was used.

THIN-LAYER CHROMATOGRAPHY. The Brinkmann–Desaga apparatus was used. Silica gel GF was applied to the 20 × 20 cm glass plates, activated at 105° for 30 min, and thereafter stored in a desiccator. This technique was employed in order to demonstrate the presence of the products of the reactions of various steroid dehydrogenases. Analytical grade chloroform and methanol were used as the solvent system in the ratio of 98:2, respectively.

Samples were frequently prepared from enzyme assays after the enzymatic reaction was completed. These reaction mixtures were extracted with 5 ml of chloroform, which was then removed, evaporated, and the sediment redissolved in 2 to 3 drops of methanol. This sample was then applied to the thin-layer plates and chromatographed with the appropriate standards.

ELECTROPHORETIC TECHNIQUES. Agar Gel. A system of 1% Ionagar–1.5% hydrolyzed starch in 0.02 M barbital buffer (pH 8.6) was used, on 8 × 10 cm glass plates. These plates had a capacity for five 1-cm wide slits for the samples. Electrophoresis was carried out for 40 to 60 min at 35 mA and 200 V.

After electrophoresis, each of the five lanes of agar was cut off and placed on standard microscope slides. These slides were then placed in a five-chambered plastic box, each chamber containing a different steroid substrate in the staining solution.

The staining solution used was composed of 70 ml of 0.1 M sodium phosphate (pH 8.0), 2.0 ml of DPN solution (1.4×10^{-2} M), 1 ml of phenazine methosulfate (5 mg/ml), and 4 ml of Nitro BT (10 mg/ml). This mixture was divided into

five portions in the staining tray, and specific steroid substrates were added to each compartment. After sufficient staining of the enzyme activity had occurred, the agar strips were removed and dried, making a permanent record on the glass slides by covering them with Whatman No. 1 filter paper impregnated with 5% glacial acetic acid.

Acrylamide Electrophoresis. The small disc electrophoretic method was essentially that described by Nerenberg (1966). In order to determine the size relationships between enzymes, a series of acrylamide concentrations were used to increase the sieving capacity of the gel.

The preparative acrylamide columns were also according to Nerenberg (Figure 1A–D). (The column gel volume was 400 ml of a 5 or 7% acrylamide gel mixture in a glass column measuring 5.1 × 36 cm.) Enzyme activities were located on the surface of the gel, by applying the same staining solution, as described above, to small holes suctioned out along the length of the column (B). The tetrazolium dye was precipitated wherever the specific substrate was oxidized (C). The zone of activity was cut out of the column, and this disk was then electrophoretically eluted. An apparatus designed by Nerenberg (D) allowed elution into a small buffer-containing well, the bottom of which was covered by a UM 3 ultra-membrane (Amicon Corp., Cambridge, Mass.). This allowed concentration of the enzyme in a relatively small volume and represented about a 40% recovery of the initial activity.

COLUMN CHROMATOGRAPHY. Ion-exchange chromatographic steps were carried out using DEAE-cellulose (Whatman DE-11) equilibrated in 0.05 M Tris (pH 8.6) with 20% glycerol and 10^{-3} M EDTA. Column size varied according to the amount of protein to be chromatographed. Care was taken not to exceed 10% of the theoretical capacity of the resin. Linear sodium chloride gradients were employed to elute the enzyme activity.

Sephadex G-100 was used essentially as described by Delin *et al.* (1964) except that the equilibrating buffer was 0.03 M potassium phosphate (pH 7.2) with 20% glycerol, and 10^{-3} M EDTA. The column volumes varied from 3 to 5 l., depending on the amount of protein used.

Results

Properties and Purification. STDH-m (PRESUMED MUTANT). The organism STDH-m was found to grow quite well in the same medium used by others for the wild-type *Ps. testosteroni*. The harvested organisms were converted into an acetone powder and extracted as described in Methods. The prime motive was to demonstrate the types of steroid dehydrogenases present in the crude extract. This was accomplished by preparing assays to illustrate the oxidation of an hydroxysteroid representative of the following classes: 17β-hydroxysteroid (testosterone), 3β-hydroxysteroid (epiandrosterone), ring A/B *trans* 3α-hydroxysteroid (androsterone), and ring A/B *cis* 3α-hydroxysteroid (etiocholanolone). Initial enzymatic assay confirmed the results of Teller and Bongiovanni (1963), that testosterone is not oxidized. Chloroform extracts of these reaction mixtures were obtained for examination by thin-layer chromatography. This further confirmed the oxidation of all the substrates to the corresponding ketosteroid except for testosterone as indicated in Figure 2. Steroid ring A dehydrogenase activities are readily demonstrable in

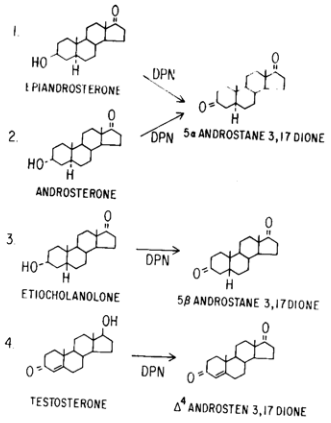

FIGURE 2: Hydroxysteroid dehydrogenase activities in STDH-m. Testosterone is not oxidized.

extracts of wild-type *Ps. testosteroni* (Levy and Talalay, 1959; Davidson and Talalay, 1966). These reactions are characterized by the formation of double bonds between carbons 1 and 2 or between carbons 4 and 5 in ring A of the steroid nucleus. These activities were absent in extracts of STDH-m cultures.

Electrophoretic study was carried out first using the agar gel technique described. The 3α- and 3β-hydroxysteroid dehydrogenase activities were each represented by only one band of activity, both of which were very negatively charged (Figure 3). Testosterone was not oxidized. Further characterization was carried out using numerous α- and β-hydroxysteroids. There were no bands of activity in the STDH-m extract other than those described for androsterone and dehydroepiandrosterone, in all of the reactive 3α- and 3β-hydroxysteroids used as substrates.

The rather marked difference in charge between β and α enzymatic activities of STDH-m and *Ps. testosteroni* (ATCC No. 11996—Stanier strain No. 78) is indicated in Figure 4. The β activity is compared using dehydroepiandrosterone while androsterone (andro) is used for α activity detection. In both cases, the activities in the wild-type *Ps. testosteroni*

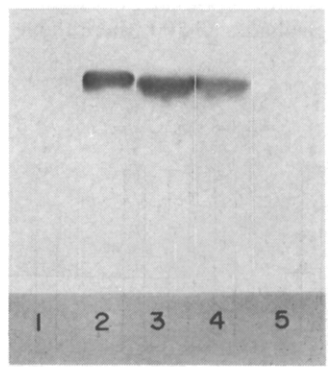

FIGURE 3: Agar gel electrophoresis of crude extract of STDH-m with specific straining as follows: (1) testosterone (17β), (2) dehydroepiandrosterone (Δ^5-3β), (3) androsterone (3α A/B *trans*), (4) etiocholanolone (3α A/B *cis*), (5) methanol solvent blank + DPN. The anode is at the top of all electrophoretograms.

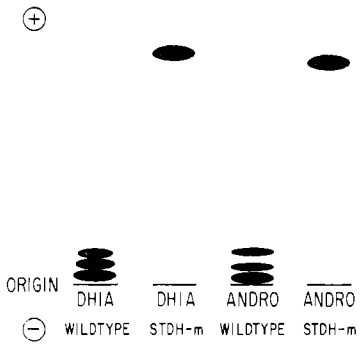

FIGURE 4: Agar gel comparison of extracts of the wild-type *Ps. testosteroni* (ATCC No. 11996) and STDH-m for α and β enzyme activity. The "mutant" activity zones have a far greater negative charge. 35 mA, 200 V × 60 ft.

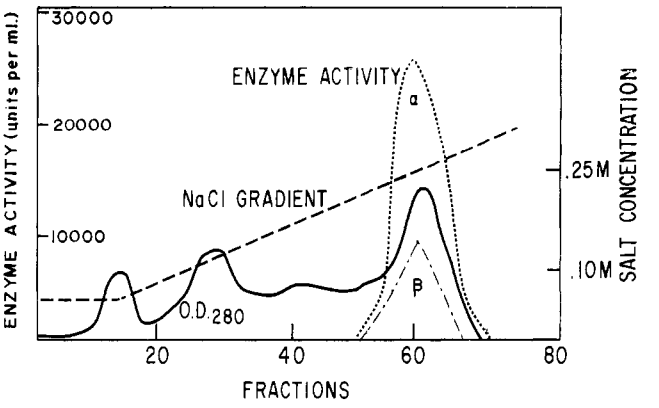

FIGURE 6: Elution pattern of STDH-m on DE-11 equilibrated with 0.05 M Tris (pH 8.6), 20% glycerol, and 10^{-3} M EDTA. Both α and β activity obtained in single peak.

are represented by multiple electrophoretic forms in contrast to those of STDH-m.

Since the two activities in STDH-m were so similar in charge, they were examined for size differences by using a series of acrylamide gels of different concentrations. Examination of Figure 5 demonstrates that in the 7% acrylamide gel, maximum sieving is achieved, thereby resulting in substantial separation of the α and β activities. (Chromatography on Sephadex G-100 confirms this electrophoretic finding.) Substitution of epiandrosterone and etiocholanolone for dehydroepiandrosterone and androsterone as β and α substrates, respectively, gave identical results.

From the preceding electrophoretic information, a purification procedure was devised, utilizing the similarity in charge and dissimilarity in size of these two enzyme activities. An acetone powder was extracted with 10:1 (volume–weight) of 0.05 M potassium phosphate buffer (pH 7.0) with 20% glycerol, and 10^{-3} M EDTA. After the total protein of the extract was measured, a neutral 4% solution of protamine sulfate, consisting of one-fifth the total protein weight as solid protamine sulfate, was added to the crude extract and stirred overnight. The copious precipitate of nucleic acids was removed, and ammonium sulfate fractionation was carried out. After dialysis, the 20–60% ammonium sulfate cut was subjected to ion-exchange chromatography (DE-11) as described (Figure 6). The two enzyme activities were eluted at approximately 0.25 M NaCl. This peak was concentrated and applied to Sephadex G-100 where the α and β activities were separated (Figure 7). It has consistently been found that the α activity exists in higher concentrations than the β enzyme in these preparations. In order to further purify the α and β enzyme activities, the concentrated peaks from Sephadex were individually applied to 7% preparative acrylamide columns. The enzymes isolated in this way, were found to be single protein bands by agar gel and acrylamide electrophoresis. The α peak, when analyzed by the Yphantis procedure (Yphantis, 1964), gave a molecular weight of 47,000, which is approximately the same as Squire *et al.* (1964) obtained for the wild-type α enzyme. Chromatography on Sephadex G-100 indicated elution volumes identical with those of the wild-type β and α enzyme corresponding to molecular weights of 100,000 and 47,000, respectively. The purification procedure is summarized in Table I.

STANIER STRAIN 28. In preliminary experiments, Stanier strain 28 of *P. testosteroni* was grown in 3-l. volumes, induced with testosterone, and harvested after 16–18-hr fermentation at room temperature. The average wet yield of 5–7 g/l. was adequate for the production of approximately 3 g of acetone powder. This was then extracted with 0.05 M potassium phosphate (pH 7.0), with 20% glycerol and 10^{-3} M EDTA. This crude extract could then be examined for hydroxysteroid dehydrogenase activity.

Assays were prepared to detect oxidation of 5α-androstan-

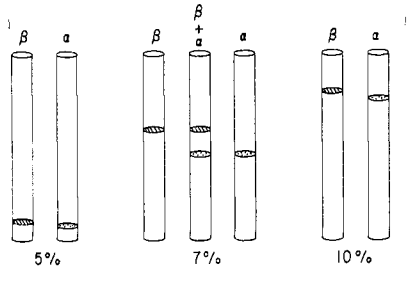

FIGURE 5: Acrylamide disc electrophoresis of crude extract of STDH-m applied to three different concentrations of acrylamide and run for the same time period. Dehydroepiandrosterone is the substrate for β activity and androsterone is used to detect α activity.

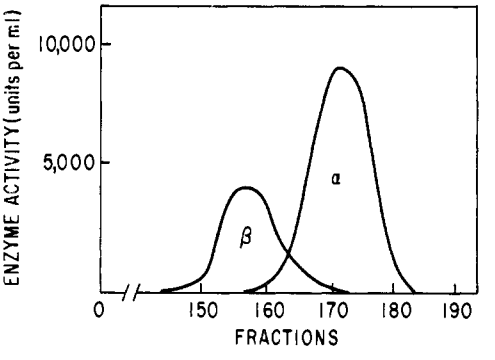

FIGURE 7: Elution pattern of STDH-m on Sephadex G-100 in 0.03 M potassium phosphate (pH 7.2), 20% glycerol, and 10^{-3} M EDTA demonstrating separation on the basis of size.

TABLE I: Purification Summary for STDH-m 3β- and 3α-Hydroxysteroid Dehydrogenases.

	Protein (mg)	Activity × 10^6 Units		Specific Activity (units/mg)	
		α	β	α	β
Crude extract	29,000.0	22.5	10.5	777	360
Protamine sulfate	12,500.0	22.5	9.0	1,800	720
Ammonium sulfate (20–60%)	2,120.0	14.1	9.0	6,564	4,236
DE-11 Column	238.0	8.85	1.8	37,260	7,578
Sephadex G-100 peak I	72.0	8.4	0	115,068	0
peak II	14.0	0	1.5	0	107,142
Preparative polyacrylamide[a]					
(7%) peak I	4.2	1.41	0	335,700	0
peak II	4.0	0	0.6	0	150,000

[a] To a 7% polyacrylamide preparative column was applied 50% of the α peak from the Sephadex column. Thus 4.5 × 10^6 units were applied and approximately 1.5 × 10^6 units were recovered (30%).

3α-ol-17-one (androsterone), 5β-androstan-3α-ol-17-one (etiocholanolone), Δ⁴-androsten-17β-ol-3-one (testosterone), and 5α-androstan-3β-ol-17-one (epiandrosterone). These compounds represent: A/B *trans* 3α-hydroxy-, A/B *cis* 3α-hydroxy-, 17β-, and A/B *trans* 3β-hydroxysteroid groups, respectively. The assays were carried out by monitoring enzymatic activity at 340 mμ: all four hydroxysteroids were oxidized. Confirmation of this oxidation was noted by thin-layer chromatography of the products of the reactions, thus demonstrating conversion of each of the substrates into the corresponding ketone.

Since both 3α-hydroxysteroids (A/B *trans* and *cis*) were oxidized, an examination of the extracts by agar gel electrophoresis was carried out. Figure 8 is an analysis staining specific lanes of the gel with different steroid substrates. The 17β- and 3β-hydroxysteroids (lanes 1 and 3) have identical patterns, and the blank lane (5) of methanol without steroid is characterized by one DPN-dependent band. In lane 2, there is a prominent activity zone close to the origin, representing an α-hydroxysteroid dehydrogenase. Another enzyme in lane 2, the most negatively charged, is specific for the ring A/B *trans* 3α-hydroxysteroid, androsterone. This enzyme is designated 28-α-*trans* because no reaction could be demonstrated with the ring A/B *cis* isomer, etiocholanolone. Another band of interest is represented by reaction with 5β-androstane-3,17-dione (lane 4). This is seen to be closer to the sample origin than the other bands of activity. This band is also seen as a late-staining component when etiocholanolone is used as substrate. The α enzyme oxidizes the etiocholanolone to 5β-androstane-3,17-dione which is in turn the substrate for the ring A Δ⁴-5β-steroid dehydrogenase. This enzyme has been demonstrated and isolated from the wild-type *Ps. testosteroni* (Davidson and Talalay, 1966). As expected, the steroid 5α-androstane-3,17-dione is not a substrate for this enzyme.

In order to confirm the role of the 28α-*trans* enzyme, a preparative acrylamide electrophoretic analysis was carried out. Approximately 100 mg of protein of a crude extract which had been dialyzed against the glycine-Tris buffer used in the technique was applied to the 5% gel column, and run for 5 hr at 40 mA and 200 V. The acrylamide gel was removed from the glass column and stained for activity as described previously. The zone of activity detected with androsterone and not etiocholanolone was cut out and eluted electrophoretically. Agar gel analysis of this fraction compared with the crude extract against androsterone and etiocholanolone indicated that 28α-*trans* is DPN dependent, and does not react with etiocholanolone. Routine assays and extracts of these reaction mixtures with the two 3α-hydroxysteroids, confirmed the reluctance of this enzyme to oxidize the A/B *cis* steroid, etiocholanolone.

Because there are other types of enzymes in the extracts of this organism which also oxidize androsterone, it was imperative that the purification procedure be followed at all steps by agar gel electrophoresis. The majority of the α activity was obviously (by agar gel inspection) due to the more nonspecific and less negatively charged α enzyme shown in Figure 8 (lane 2). This situation made purification indices impossible to follow on the basis of specific activity, until after the 28-*trans* was isolated from other α activities. Therefore, the only reliable index of purification is the deletion of extraneous protein noted in Table II.

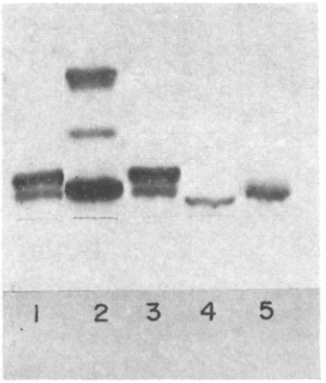

FIGURE 8: Agar gel electrophoresis of crude extract of strain No. 28 with specific staining: (1) testosterone (17β), (2) androsterone (3α A/B *trans*), (3) dehydroepiandrosterone (3β), (4) 5β-androstane-3,17-dione (etiocholanolone) (A/B *cis* diketone), (5) methanol solvent blank.

TABLE II: Purification Summary for 28α-*trans*-Hydroxysteroid Dehydrogenase.

	Total Protein (mg)	Total α Act.	Sp Act. α-*trans*	A_{280}/A_{260}	Purification (Protein Deletion)
Crude extract	26,600	45,600,000		0.59	
Protamine SO_4	15,840	48,000,000		0.75	1.6
$(NH_4)_2SO_4$ 20–60%	3,216	43,200,000		1.25	8
DEAE					
peak I	1,740	42,000,000		1.32	15
peak II	150	1,500,000	9,900	1.40	177
Sephadex G-100	37	366,000	9,900	1.33	719
De-11 column (α-*trans* only)	3.6	120,000	33,300	1.33	7,400

The New England Enzyme Center, once again, provided the means by which an 80-l. fermentation of this organism was carried out. Induction of the enzyme activity was accomplished using a final concentration of 0.3 g of testosterone/l. of medium. Somewhat less than 100 g of acetone powder was obtained from a culture of this size. For each gram of acetone powder, 10 ml of 0.05 M potassium phosphate buffer (pH 7.0) with 20% glycerol and 10^{-3} M EDTA was used for the initial extract. The crude extract contained approximately 45×10^6 units of α enzyme activity as measured with androsterone. To precipitate nucleic acid, a 4% solution of neutralized protamine sulfate was added as previously described. The copious precipitate was allowed to form and stir overnight. After centrifugation, the supernatant was fractionated with ammonium sulfate. The bulk of the activity remained in the 20–60% fraction.

Three grams of protein from this fraction were applied to a 2×65 cm column of DE-11 in 0.05 M Tris (pH 8.6) containing 20% glycerol and 10^{-3} M EDTA. Two major α activity peaks were obtained, as indicated in the diagram, Figure 9A. Agar gel electrophoresis of the elution pattern was necessary for the location of the 28α-*trans* and is indicated in the same figure (9B).

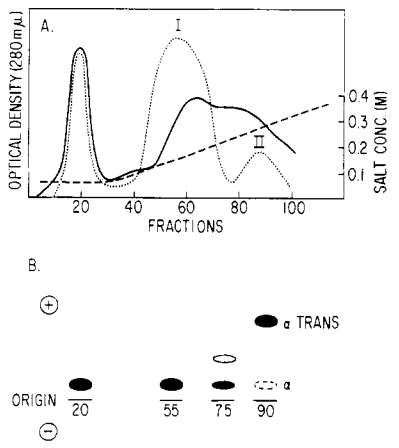

FIGURE 9: (A) Elution pattern of extract of Stanier No. 28 on DE-11 equilibrated with 0.05 M Tris (pH 8.6), 20% glycerol, and 10^{-3} M EDTA; (. . . .) α activity, (—) OD_{280}, (– –) NaCl gradients. (B) Agar gel electrophoretic analysis of fractions *vs.* androsterone is indicated at the bottom of the figure.

Peak II from the ion-exchange chromatography was concentrated and dialyzed against 0.05 M potassium phosphate (pH 7.2) with 20% glycerol and 10^{-3} M EDTA, and applied to a 5×140 cm column of Sephadex G-100. The single peak obtained from this column was found also to contain the nonspecific α enzyme (Figure 10). These impurities were then removed by a passage of this concentrated peak back over a small 50-ml column of DE-11. Following this, both agar gel and acrylamide disc electrophoresis revealed a single protein band, and a corresponding single enzyme activity band. Table II summarizes the purification procedure.

It is apparent from this that 28α-*trans* represents only about 3% of the total 3α-hydroxysteroid dehydrogenase activity of this organism.

M_3. The organism M_3 was obtained from a prolonged fermentation of the presumed mutant, STDH-m. The colonial characteristics of this organism are indistinguishable from either the wild type or Stanier strain No. 28, and is totally different from the golden colony of STDH-m. This organism was examined for steroid dehydrogenases in the same manner as the others. Small batch cultures (3 l.) were grown under the same conditions as the other organisms, and were processed to acetone powders. Preliminary assays revealed capacities for oxidation of both A/B *trans* and A/B *cis* 3β-, 17β-, and 3α-hydroxysteroids. Extracts in 0.05 M potassium phosphate (pH 7.0) with 20% glycerol were subjected to the agar gel electrophoretic screening technique (Figure 11).

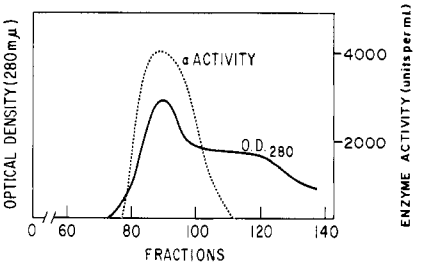

FIGURE 10: Sephadex G-100 elution pattern of peak II from ion-exchange-containing enzyme 28β-*trans*.

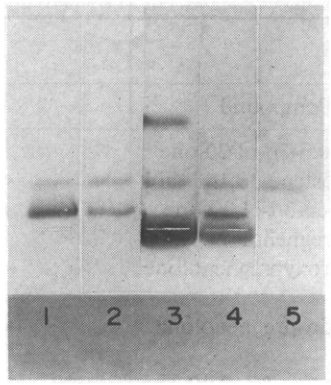

FIGURE 11: Agar gel electrophoresis of crude extract of M_3 with substrate staining: (1) testosterone, (2) dehydroepiandrosterone, (3) androsterone, (4) etiocholanolone, (5) solvent blank.

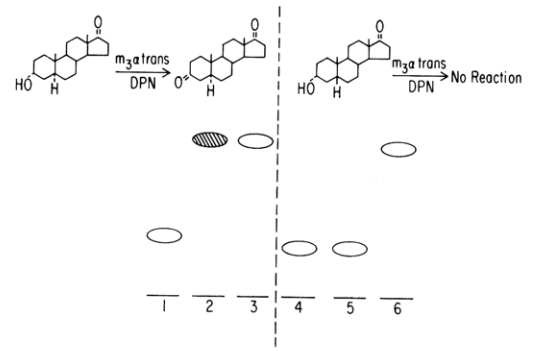

FIGURE 12: Thin-layer chromatogram demonstrating oxidation of androsterone by M_3 *α-trans* enzyme + DPN at pH 8.9. Cross-hatching indicates reaction product in all chromatograms; (1) androsterone, (2) androsterone and M_3 *α-trans* enzyme, (3) 5α-androstane-3,17-dione, (4) etiocholanolone, (5) etiocholanolone + M_3 *α-trans* enzyme, (6) 5β-androstane-3,17-dione.

Lanes stained with testosterone and dehydroepiandrosterone had a single major zone of enzyme activity, which was not associated with 3α-hydroxysteroids and is, in fact, a nonspecific β-hydroxysteroid dehydrogenase with an essentially identical substrate specificity to the wild-type β enzyme. The only other band present with the β-hydroxysteroids is seen in all lanes even in the absence of steroid substrate. In those lanes where androsterone and etiocholanolone are used as substrates, there is an α enzyme which remains at the origin. As in the case of Stanier strain No. 28 of *Ps. testosteroni*, there appears to be an enzyme specific for androsterone and not etiocholanolone. Similarly, it is very negatively charged and dependent upon the presence of DPN. Analysis with other 3α-hydroxysteroids indicated that the specificity was directed at the A/B *trans* ring junction and was therefore designated M_3 *α-trans*. Lane 4 reveals the ring A Δ^4-5β-steroid dehydrogenase in addition to the nonspecific α enzyme.

A concentrated extract of crude M_3 was put onto a preparative 5% acrylamide gel, and electrophoresed as described previously. Substrate tetrazolium staining located the area where the M_3 *α-trans* enzyme was located. This region was cut out for elution of the enzyme. For verification of its A/B *trans* specificity, reaction mixtures containing either 100 μg of androsterone or 100 μg of etiocholanolone were extracted following completion of the reactions. Figure 12, a thin-layer chromatogram, demonstrates that whereas androsterone (A/B *trans*) was oxidized by the M_3 *α-trans* enzyme, the A/B *cis* 3α-hydroxysteroid (etiocholanolone) was not. Table III summarizes the various electrophoretic mobilities of the enzymes studied.

Substrate Specificity Analyses. The steroid substrates were divided into three categories of increasing specificity. The first group were considered only according to α or β orientation of the reactive hydroxyl group. Within these a second subdivision was designated according to the junction of rings A and B. This results in the categories of 3α-hydroxysteroids ring A/B *trans* and A/B *cis* with analogous groups for the β-hydroxysteroids. The third classification is based on the influence of oxygenation at carbon 11 on the reactivity of the oxidizable hydroxyl group. Examples would be the 11-hydrogen parent compound androsterone, the 11-hydroxyl derivative, and the 11-keto derivative.

THE α AND β ENZYMES OF STDH-m. The 3β-hydroxysteroid dehydrogenase of STDH-m (M-3α) was checked by both assay and agar gel electrophoresis. The series of hydroxysteroids tested included representatives of the 3β- and 17β-hydroxysteroids classes all of which failed to react. Both the androstane and the pregnane derivatives of 3α-hydroxysteroids were oxidized regardless of ring A/B junction. A further subdivision was based on the degree of oxygenation at carbon 11. At concentrations where a reaction with a "parent" steroid (androsterone, etiocholanolone, or 5β-pregnane-3α,-17α,21-triol-20-one (THS)) was complete in 2 min, M-3α showed no reaction with a number of 3α-hydroxysteroids. These include 11-ketoetiocholanolone, 5β-pregnane-3α,17aα-21-triol-11,20-dione (THE), 11-ketopregnanetriol, and 5β-pregnan-3α-ol-11,20-dione. All of these compounds fall into the category of 3α-hydroxysteroids having rings A/B *cis* and a ketone function on carbon 11. The 11-hydroxylated derivatives of these compounds are oxidized at the three position. The oxidation of the 11-keto derivatives of these compounds can be detected only at extreme enzyme concentrations. Therefore the capacity for oxidation of this class

TABLE III: Agar Gel Electrophoretic Mobilities of Hydroxysteroid Dehydrogenases Relative to the Dye Bromophenol Blue.

	Stanier Strain 78	STDH-m	Stanier Strain 28	M_3
α-Hydroxysteroid dehydrogenase	0.08	0.77	0.21	0.06
β-Hydroxysteroid dehydrogenase	0.09	0.81	0.27	0.15
trans α-Hydroxysteroid dehydrogenase	*a*		0.73	0.56
Bromphenol blue	1.00	1.00	1.00	1.00

[a] An *α-trans* enzyme has been found in strain no. 78 with a mobility of 0.55. It is not included due to incomplete substrate specificity analysis.

TABLE IV: Substrate Specificity of the 3β Enzyme from STDH-m-Reacting Steroids.

Compound	Rate	Compound	Rate
Δ⁵-Androsten-3β-ol-17-one (DHIA)	1.00	5α-Pregnan-3β-ol-20-one	0.22
Δ⁵-Androstene-3β,17β-diol	0.79	Δ⁵-Androstene-3β,16β-diol	<0.2
Δ⁴-Androstene-3β,17β-diol[a]	0.61	Δ⁵-Pregnen-3β-ol-20-one (pregnenolone)	<0.2
5α-Androstan-3β-ol-17-one (epiandrosterone)	0.54	17α-Hydroxypregnenolone	<0.2
5α-Pregnane-3β,20β-diol	0.28	16-Dehydropregnenolone	<0.2
Nonreacting steroids			
Δ⁴-Androsten-17β-ol-3-one (testosterone)		5β-Androstan-3β-ol-17-one	
Δ¹-Testosterone		5β-Pregnan-3β-ol-20-one	
5β-Dihydrotestosterone		5β-Pregnane-3β,20β-diol	
5α-Androstane-3α,17β-diol		All 3α-hydroxysteroids	
17β-Estradiol			

[a] Thin-layer chromatography of reaction product reveals compound with the mobility of testosterone.

of steroids is greatly reduced in this enzyme, especially when compared with the α enzyme of the wild-type *Ps. testosteroni*.

The 3β-hydroxysteroid dehydrogenase (M-3β) was similarly examined. The original paper on this organism referred to only three hydroxysteroids: dehydroepiandrosterone, androsterone, and testosterone. In the present analysis, a series of 17β-hydroxysteroids were found not to be substrates. There was likewise no oxidation of the 3α-hydroxysteroids tested. The category of 3β-hydroxysteroids was then approached from the point of view of ring A/B junction. As many ring A/B *cis* 3β-hydroxysteroids as obtainable were tested, as well as their ring A/B *trans* counterparts. In both electrophoretic and routine assays procedures, none of the ring A/B *cis* 3β-hydroxysteroids were found to react while those having rings A/B *trans* were oxidized (Table IV).

To confirm that the 3β-hydroxysteroid was being converted into the corresponding ketosteroid, a series of assays were carried out and extracted after quantitative oxidation of the A/B *trans* compound had occurred. The diagram of the thin-layer chromatogram in Figure 13 demonstrates the remarkable substrate specificity of this enzyme. The A/B *trans* substrate 5α-androstan-3β-ol-17-one is readily converted into the corresponding ketone, 5α-androstane-3,17-dione. The analogous oxidation of the A/B *cis* substrate was not demonstrable.

THE 28α-*trans* ENZYME OF STANIER STRAIN NO. 28. A series of hydroxysteroids and ketosteroids were tested against 28α-*trans*, and reinforced the impression that the only 3α-hydroxysteroids which would react were those with ring A/B *trans*. It should also be noted that extremely weak reactions were detected with some β-hydroxysteroids. Although these reactions could not be detected by routine assay (ap-

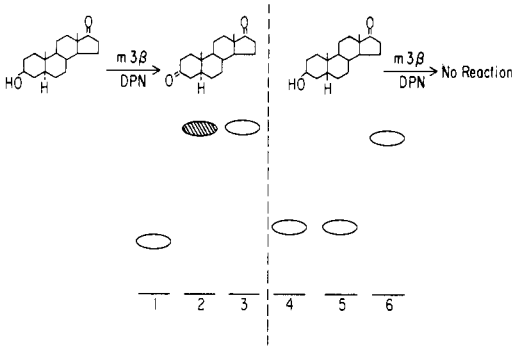

FIGURE 13: Thin-layer chromatogram demonstrating substrate specificity of purified STDH-m A/B *trans* 3β-OHSD. Lane 1 = epiandrosterone; 2, M 3β-OHSD + epiandrosterone + DPN; 3, 5α-androstane-3,17-dione; 4, 5β-androstan-3β-ol-17-one; 5, M 3β-OHSD + 5β-androstan-3β-ol-17-one + DPN; 6, 5β-androstane-3,-17-dione.

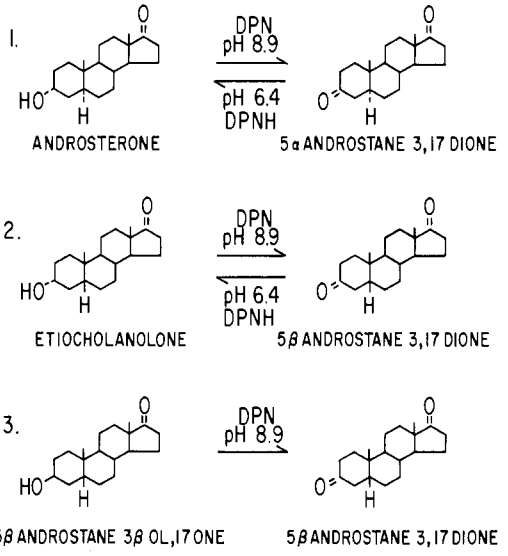

FIGURE 14: Three reactions of hydroxy- and ketosteroids with the 28α-*trans* enzyme evaluated by thin-layer chromatography.

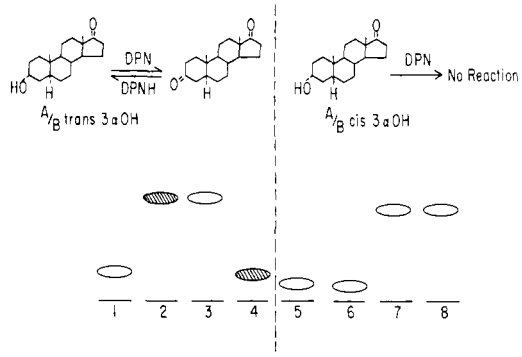

FIGURE 15: Thin-layer chromatogram of purified 28α-*trans* demonstrating substrate specificity: (1) androsterone; (2) 28α-*trans* + androsterone + DPN (pH 8.9); (3) 5α-androstane-3,17-dione; (4) 28α-*trans* + 5α-androstane-3,17-dione + DPNH (pH 6.4); (5) etiocholanolone; (6) 28α-*trans*- + etiocholanolone + DPN (pH 8.9); (7) 5β-androstane-3,17-dione; (8) 28α-*trans* + 5β-androstane-3,17-dione + DPNH pH 6.4. Cross-hatching represents products of reaction.

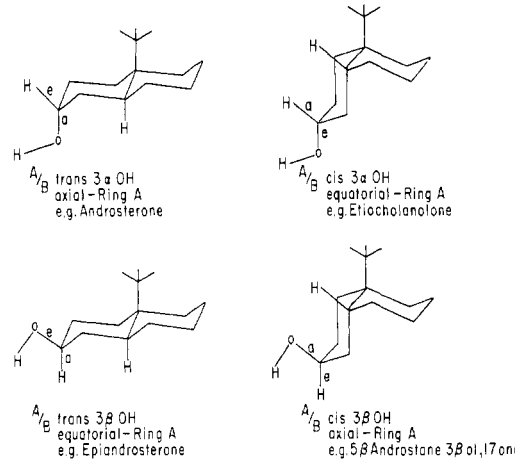

FIGURE 16: Steric configurations of rings A and B of the steroid nucleus in both *cis* and *trans* forms for 3α- and 3β-hydroxysteroids. Equatorial (e) and axial (a) designations refer to the band orientation relative to the plane of ring A.

parently because of dilution), overnight staining of agar gels did detect them.

In the initial substrate specificity analysis of the more crude 28α-*trans* it was noted that certain 3β-hydroxysteroids were also substrates although extremely weak ones. Having obtained a purified preparation of this enzyme, the substrate specificities were repeated. These results did not differ from the original findings. The extremely weak reactions with the 3β-hydroxysteroids were again demonstrated. Curiously, epiandrosterone (5α-androstan-3β-ol-17-one) and dehydroepiandrosterone (Δ^5-androsten-3β-ol-17-one) were not substrates. Electrophoretic attempts to separate the α from the β activities in the purified enzyme by varying concentrations of acrylamide were unsuccessful. Because of the extreme weakness of the β activity in 28α-*trans* (undetectable by routine assay) it was not considered to significantly interfere with the quantitative oxidation of 3α-hydroxysteroids of the enzyme.

Thin-layer chromatography of the chloroform extracts of the reactions occurring after 1-hr incubation with several substrates was performed. Three reactions as shown in Figure 14 were examined with the 28α-*trans* enzyme.

The assays were prepared as previously described. Chloroform extracts of these assays were evaporated and applied to silica gel GF plates, flanked by the appropriate standards, in order to determine the specificity of the 28α-*trans* enzyme. Figure 15 demonstrates the specificity for the oxidation of the A/B *trans* 3α-hydroxysteroid as well as the reduction of the corresponding diketone. The corresponding reactions with the A/B *cis* compounds, etiocholanolone and 5β-androstane-3,17-dione, could not be demonstrated (reaction 2, Figure 14).

A similar analysis was carried out with 3β-hydroxysteroids and the purified 28α-*trans* enzyme. Oxidation of certain A/B *cis* 3β-hydroxysteroids had been noted in earlier experiments. Thin-layer chromatography of extracted assays and appropriate standards, demonstrated a rather marked specificity for these compounds. Prolonged incubations (hours) were required to obtain enough product for detection by this method. Three reactions were examined: (1) oxidation of 5β-androstan-3β-ol-17-one, (2) oxidation of 5α-androstan-3β-ol-17-one (epiandrosterone), and (3) a control of oxidation of androsterone (5α-androstan-3α-ol-17-one). The A/B *cis* 3β-hydroxysteroid was oxidized to the corresponding diketone, whereas no oxidation could be demonstrated with the A/B *trans* compound, epiandrosterone.

The fact that 28α-*trans* was shown to react weakly with A/B *cis* 3β-hydroxysteroids was initially disconcerting. Further testing revealed oxidation of 5β-pregnane-3β,30β-diol instead of the 5α isomer. The same result occurred using 5β-pregnan-3α-ol-20-one. The two dihydrotestosterones, namely 5α and 5β, were unreactive. Therefore, it became clear that the specificity was related to the 3β-hydroxyl group of the A/B *cis* ring system. Figure 16 depicts Dreiding models of A/B *trans* and *cis* 3α- and 3β-hydroxysteroids. The axial (a) and equatorial (e) designations in the figure conform to bond orientation relative to ring A. If androsterone is compared with etiocholanolone, it is readily apparent that the hydroxyl group of the former is axial while in the latter it is equatorial to the plane of ring A. The corresponding 3β-hydroxysteroids, for example, epiandrosterone (A/B *trans*) and 5β-androstan-3β-ol-17-one, have a slightly different orientation. The hydroxyl group of the former is equatorial (e), while the latter is axial (a) to the plane of ring A. All of the reactive steroids have in common an hydroxyl group oriented axial to ring A on carbon 3. For a reaction to occur rapidly, the ring A/B junction must be *trans*. Therefore, it would seem that the enzyme is functionally an *trans* α-hydroxysteroid dehydrogenase, but its main specificity is directed by the axial orientation to ring A of the steroid rather than to the whole molecular plane.

SUBSTRATE SPECIFICITY ANALYSIS OF THE α-*trans* ENZYME FROM M_3. Specificities determined by routine assay at 340 mμ, agar gel electrophoresis, and thin-layer chromatography all confirmed the specificity for A/B *trans* 3α-hydroxysteroids. All A/B *cis* 3α-hydroxy compounds were not attacked by this enzyme. These compounds include etiocholanolone, 11-hydroxyetiocholanolone, 11-*O*-etiocholanolone, 5β-pregnane-3α,17α,21-triol-20-one (THS), 5β-pregnane-3α,11β,17α,21-tetrol-20-one (THF), and 5β-pregnane-3α,17α,21-triol-11,20-

TABLE V: Comparative Substrate Specificities of Different Steroid Dehydrogenases.

Compound	Wild-Type β	SDTH-m β	Wild-Type α	SDTH m α	28-α_{trans}	M_3 α_{trans}
Testosterone	+	0	0	0	0	0
5α-Dihydrotestosterone	+	0	0	0	0	0
5β-Dihydrotestosterone	+	0	0	0	0	0
5α-Androstan-3α-ol-17β-ol	+	0	+	+	+	+
5α-Androstan-3β-ol-17-one	+	+	0	0	0	0
5β-Androstan-3β-ol-17-one	+	0	0	0	±	0
5α-Pregnane-3β,-20β-diol	+	+	0	0	0	0
5β-Pregnane-3β,-20β-diol	+	0	0	0	±	0
5α-Pregnan-3β-ol-20-one	+	+	0	0	0	0
5β-Pregnan-3β-ol-20-one	+	0	0	0	±	0
DHIA[a]	+	+	0	0	0	0
Δ⁵-Pregnen-3β-ol-20-one	+	±	0	0	0	0
Androsterone	0	0	+	+	+	+
11-Hydroxyandrosterone	0	0	+	+	+	0
11-Ketoandrosterone	0	0	+	+	+	+
Allo-THS[a]	0	0	+	+	+	+
Etiocholanolone	0	0	+	+	0	0
11-Hydroxyetiocholanolone	0	0	+	+	0	0
11-Ketoetiocholanolone	0	0	+	±	0	0
THS[a]	0	0	+	+	0	0
THF[a]	0	0	+	+	0	0
THE[a]	0	0	+	±	0	0
5α-Androstane-3,17-dione	0	0	0	0	0	0
5β-Androstane-3,17-dione	0	0	0	0	0	0
Δ⁴-Androsten-3,17-dione	0	0	0	0	0	0

[a] DHIA = Δ⁵-androsten-3β-ol-17-one; Allo-THS = 5α-pregnane-3α,17α,21-triol-20-one; THS = 5β-pregnane-3α,17α,21-triol-20-one; THF = 5β-pregnane-3α,11β,17α,21-tetrol-20-one; THE = 5β-pregnane-3α,17α,21-triol-11,20-dione.

dione (THE). However, 5α-pregnane-3α,17α,21-triol-20-one (allo-THS) was oxidized to the three ketosteroids as would be expected since rings A/B are *trans* to each other. The most surprising aspects of specificity for 3α-hydroxysteroids was that whereas the 11-keto derivative of androsterone was readily oxidized, there was little, if any, capacity to oxidize the 11-hydroxy derivative. This represents greater specificity than the 28α-*trans* enzyme.

In examining the specificity of 28α-*trans*, it was noted that the specificity was directed at the axial orientation of the hydroxyl group to the plane of ring A. This phenomenon was examined with M_3 α-*trans* and found to be a feature of this enzyme. No reaction could be demonstrated with any 3β- or 17β-hydroxysteroid regardless of its ring A/B junction.

Table V summarizes the steroid group specificities of these enzymes.

Discussion

The methods used in this study permit a direct and rapid approach to enzymes which ordinarily would go undetected. The ease with which they can be isolated makes their use in quantitative techniques more than a theoretical possibility.

The degree of specificity of these enzymes is, at present, greater than any other bacterial hydroxysteroid dehydrogenases (Capek *et al.*, 1966). A curious phenomenon thus far is that the more negatively charged the enzymes are at pH 8.6, the greater is their stereospecificity. Both strain 28 and M_3 have nonspecific α- and β-hydroxysteroid dehydrogenases as in the wild type. In all three of these organisms the nonspecific enzymes have low charges at pH 8.6. The more specific α-*trans* enzymes are always more negative in character. Specific studies are underway to establish the nature of this difference. Studies are also being attempted to analyze the extreme stereospecificity of $28\alpha_{trans}$ (axial hydroxysteroid dehydrogenase) and M_3 α_{trans}. The elegant method of Ringold *et al.* (1967) should be most revealing in such a study. The most recent and extensive review of the metabolic defects resulting in congenital adrenal hyperplasia is found in Stanbury *et al.* (1966). The result of a 3β-hydroxysteroid dehydrogenase defect is the accumulation of Δ⁵-3β-hydroxysteroids. These steroids are substrates specifically of the mutant 3β enzyme.

For specific evaluation of the 11β-hydroxylase deficiency, an absence of 11-hydroxylated 3α-hydroxysteroids would have to be demonstrated. The α-*trans* enzyme from M_3 would first measure all 3α-hydroxysteroids except 11-hydroxylated derivatives. If a complete 11-hydroxylase deficiency is present, then a second reaction with the α-*trans* enzyme from Stanier strain No. 28 would detect complete absence of 11-hydroxylated 3α-hydroxysteroids which are normally present. Techniques using these enzymes are being developed for fluorometric quantitation of steroids in the range of 10^{-9} to 10^{-10} mole. This type of sensitivity would allow quantitation even in small samples of blood plasma. The stereospecificity so far obtained could probably be extended further by use of different strains and mutants of *Ps. testosteroni*.

References

Boyer, J., Baron, D. N., and Talalay, P. (1965), *Biochemistry 4*, 1825.

Capek, A., Hanc, O., and Tadra, M. (1966), *in* Microbial Transformation of Steroids, Junk, Dr. W., Ed., The Hague, The Netherlands.

Carstensen, H. (1966), *Nature 212*, 1604.

Davidson, S. J., and Talalay, P. (1966), *J. Biol. Chem. 241*, 906.

Delin, S., Squire, P. G., and Porath, J. (1964), *Biochim. Biophys. Acta 89*, 398.
Hurlock, B. and Talalay, P. (1957), *J. Biol. Chem. 227*, 37.
Levy, H. R., and Talaley, P. (1959), *J. Biol. Chem. 234*, 2014.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marcus, P. I., and Talalay, P. (1956), *J. Biol. Chem. 218*, 661.
Nerenberg, S. T. (1966), Electrophoresis, Philadelphia, Pa., Davis.
Ringold, H. J., Graves, J. M. H., Clark, A., and Bellas, T. (1967), *Recent Progr. Hormone Res. 23*, 349.
Squire, P. G., Delin, S., and Porath, J. (1964), *Biochim. Biophys. Acta 89*, 409.
Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S. (1966), The Metabolic Basis of Inherited Disease, New York, N. Y.,McGraw-Hill, p 635.
Stempfel, R. S., and Sidbury, J. B. (1964), *J. Clin. Endocrinol. Metab. 24*, 367.
Talalay, P., and Dobson, M. M. (1953), *J. Biol. Chem. 205*, 823.
Teller, J. D., and Bongiovanni, A. M. (1963), *Nature 197*, 1112.
Yphantis, D. (1964), *Biochemistry 3*, 297.

Adenosine Triphosphate Requirement of Nitrogenase from *Azotobacter vinelandii**

K. Lamont Hadfield and William A. Bulen†

ABSTRACT: Knowledge of the amount of adenosine triphosphate hydrolyzed during the transfer of reducing electrons in the reactions catalyzed by nitrogenase (the adenosine triphosphate:$2e^-$ ratio) is requisite to understanding the energetics of the reactions and perhaps also the function(s) of adenosine triphosphate. Most reported adenosine triphosphate:$2e^-$ ratios range between 2 and 5, but both higher and lower values have been indicated. An extensive examination of adenosine triphosphate hydrolysis in the H_2 evolution and N_2 or C_2H_2 reductions catalyzed by the nitrogenase complex from *Azotobacter vinelandii* gave ratios approaching 5 at 30°. This ratio was independent of substrate, substrate concentration, H_2 inhibition, and pH but was temperature dependent.

Adenosine triphosphate:$2e^-$ values increased with temperature from 4.3 at 20° to 5.8 at 40°. The discussion includes an attempt to explain variations in the values observed with the clostridial and azotobacter enzymes. It is suggested that adenosine triphosphate is hydrolyzed in two ways, only one of which leads to electron transfer and that the concept of single-reaction stoichiometry is not applicable to this system.

A requirement for ATP in the reactions catalyzed by nitrogenase, the N_2-reducing enzyme complex, was suggested by arsenate and glucose inhibition experiments (McNary and Burris, 1962) and was subsequently established with *Clostridium pasteurianum* (Mortenson, 1964; Hardy and D'Eustachio, 1964) and *Azotobacter vinelandii* preparations (Bulen *et al.*, 1964). In addition to ATP, a source of electrons, such as dithionite (Bulen *et al.*, 1965) or enzyme systems providing reduced ferredoxin (Mortenson, 1964), is required for the reduction of N_2 or alternate substrates (N_3^-, N_2O, C_2H_2, CN^-, CH_3NC, etc.). As with other ATP-requiring enzymes, a divalent metal, preferably Mg^{2+}, is required (Burns and Bulen, 1965). The utilization of ATP is not restricted to N_2 (or alternate substrate) reduction since, in the absence of these, the enzyme from either *A. vinelandii* or *C. pasteurianum* catalyzes an ATP-dependent H_2 evolution (Bulen *et al.*, 1965; Burns and Bulen, 1965; Burns, 1965). Both the Fe–Mo–protein (I) and the Fe–protein (II) components of the nitrogenase complex from *A. vinelandii* are required for ATP hydrolysis and associated reactions (Bulen and LeComte, 1966). These proteins appear similar in terms of their requirements and general properties to those obtained from *C. pasteurianum* (molybdoferredoxin and azoferredoxin) (Mortenson *et al.*, 1967; Kennedy *et al.*, 1968) and from *A. chroococcum* (Kelly, 1969).

A lack of agreement exists about the quantity of ATP required in the nitrogenase-catalyzed reactions. Because of the nature of the electron distribution between H_2 evolution and substrate reduction, ATP utilization is conveniently referred to as the ATP:$2e^-$ ratio. With *A. vinelandii* preparations of either the nitrogenase complex or recombined Fe–Mo–protein and Fe–protein components, ATP:$2e^-$ ratios of *ca.* 5 were observed for both ATP-dependent H_2 evolution and N_2 reduction (Bulen and LeComte, 1966). With dialyzed, heated extracts or a resuspended pellet preparation obtained by prolonged high-speed centrifugation, ATP:$2e^-$ ratios of 2 were reported for H_2 evolution (Hardy and Knight, 1966).

* Contribution No. 360 from the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received June 12, 1969.* Preliminary reports of this research were presented at the Symposium on Control of Plant Growth at the 155th National Meeting of the American Chemical Society, San Francisco, Calif., April 1968, and at the Federation of American Societies for Experimental Biology Meetings, Atlantic City, N. J., April 1968 (*Federation Proc. 27*, 593).

† To whom to address reprint request.