Isolation of Novel Microbial 3\(\alpha\)-, 3\(\beta\)-, and 17\(\beta\)-Hydroxysteroid Dehydrogenases

PURIFICATION, CHARACTERIZATION, AND ANALYTICAL APPLICATIONS OF A 17\(\beta\)-HYDROXysteroid DEHYDROGENASE FROM AN ALCALIGENES sp.*

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By selecting for growth on testosterone or estradiol-17\(\beta\) as the only source of organic carbon, we have isolated a number of soil microorganisms which contain highly active and novel, inducible, NAD-linked 3\(\alpha\)-, 3\(\beta\)-, and 17\(\beta\)-hydroxysteroid dehydrogenases. Such enzymes are suitable for the microanalysis of steroids and of steroid-transforming enzymes, as well as for performing stereoselective oxidations and reductions of steroids. Of particular interest among these organisms is a soil species of Alcaligenes containing 17\(\beta\)-hydroxysteroid dehydrogenase, easily separable from 3\(\beta\)-hydroxysteroid dehydrogenase. Unlike any of the other isolated organisms, this Alcaligenes sp. contained no 3\(\alpha\)-hydroxysteroid dehydrogenase activity. A large-scale purification (763-fold) to homogeneity of the major induced 17\(\beta\)-hydroxysteroid dehydrogenase was achieved by ion-exchange, hydrophobic, and affinity chromatographies. The enzyme has high specific activity for the oxidation of testosterone (\(V_{\text{max}}\) = 303 \(\mu\text{mol/min/mg}\) of protein; \(K_m\) = 3.6 \(\mu\text{M}\)) and reacts almost equally well with estradiol-17\(\beta\) (\(V_{\text{max}}\) = 356 \(\mu\text{mol/min/mg}\); \(K_m\) = 6.4 \(\mu\text{M}\)). It consists of apparently identical subunits (\(M_z\) = 32,000) and exists in polymeric form under nondenaturing conditions (\(M_z\) = 68,000 by gel filtration and 86,000 by polyacrylamide gel electrophoresis). The isoelectric point is pH 5.1. The enzyme is almost completely specific for 17\(\beta\)-hydroxysteroids which may be \(\Delta^2\)-olefins or ring A phenols or have cis or trans A/B ring fusions. Substituents at other positions are tolerated, although the presence of a 16\(\alpha\)- or 16\(\beta\)-hydroxyl group blocks the oxidation of the 17\(\beta\)-hydroxy group. 3\(\beta\)-Hydroxysteroids (A/B ring fusion trans, but not cis, or \(\Delta^2\)-olefins) are very poor substrates. The application of this highly active, specific, and stable 17\(\beta\)-hydroxysteroid dehydrogenase to the microestimation of steroids by enzymatic cycling of nicotinamide nucleotides and for the stereospecific oxidation of steroids is demonstrated.

Although the enzymatic estimation of steroid hormones by means of nicotinamide nucleotide-dependent hydroxysteroid dehydrogenases was proposed many years ago (Talalay, 1969), the sensitivity of these methods remained inadequate for the analysis of tissue and plasma levels of steroids until the introduction of enzymatic cycling techniques (Harkonen et al., 1974; Nicolas et al., 1979; Payne et al., 1982). The scope of these methods is, however, limited by the availability of relatively few purified, specific, and catalytically highly active hydroxysteroid dehydrogenases. Microbial 3\(\alpha\)- and 3\(\beta\)-hydroxysteroid dehydrogenases are available for analytical purposes (Shikita and Talalay, 1979), but a major problem has been the lack of a suitable 17\(\beta\)-hydroxysteroid dehydrogenase for the measurement or stereospecific oxidoreductions of testosterone and related steroids. In an effort to extend the library of analytically useful hydroxysteroid dehydrogenases, we undertook a systematic search for such enzymes among soil microorganisms capable of growing on steroids as the sole source of carbon. Enzymes of high specific activity are required for analytical purposes for two reasons: (a) to permit the oxidations and reductions of substrates with low maximum velocities to go to completion; and (b) to minimize the quantities of enzymes added to the analytical systems, and thereby to reduce the blank values which arise from the amplification by cycling of contaminating nicotinamide nucleotides (Payne et al., 1982).

In this report, we demonstrate that many soil microorganisms capable of growing on steroids as their only source of carbon contain inducible hydroxysteroid dehydrogenases with a variety of specificities. We describe the purification to homogeneity and the characterization of the properties of a very active 17\(\beta\)-hydroxysteroid dehydrogenase from an Alcaligenes sp. The enzyme reacts with the 17\(\beta\)-hydroxyl groups of C\(\alpha\) and C\(\alpha\) steroids and shows comparable reaction velocities and low \(K_m\) values for testosterone and estradiol-17\(\beta\). The application of this enzyme to the sensitive measurement of steroids and to the stereospecific oxidation and reduction of these compounds is illustrated.

**EXPERIMENTAL PROCEDURES**

Enzyme Activities in a Variety of Soil Microorganisms—Our search for novel bacterial hydroxysteroid dehydrogenases, by

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Portions of this paper (including "Experimental Procedures" and Figs. 3–9) are presented in miniprint at the end of this paper. The abbreviations used are: CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; DTT, dithiothreitol; HSD, hydroxysteroid dehydrogenase, e.g. 17\(\beta\)-HSD, 17\(\beta\)-hydroxysteroid dehydrogenase; SC 1.1.1.92 and 1.1.1.63; HPLC, high pressure liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; S-NAD, thiaminotetraanalog of NAD; BSA, bovine

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means of enrichment culture of soil microorganisms, yielded 27 pure cultures capable of growing on testosterone and/or estradiol-17β as the sole source of organic carbon. Sonically prepared extracts from 17 of these cultures (which had been induced by addition of progesterone (200 μg/ml) in late logarithmic phase of growth) were examined for NAD-linked hydroxysteroid dehydrogenase activity. Eight of the 12 microorganisms isolated by growth on media containing testosterone and/or estradiol-17β showed varying amounts of 17β-hydroxysteroid dehydrogenase activity (for testosterone and estradiol-17β), 3α-hydroxysteroid dehydrogenase activity (for androsterone), or 3β-hydroxysteroid dehydrogenase (for dehydroepiandrosterone) (Fig. 1). The remaining microorganisms did not contain hydroxysteroid dehydrogenase activities. Furthermore, no dehydrogenase activity was observed in any of the microorganisms with cortisol as a substrate or when NADP was used in place of NAD. All of these microorganisms, except M21, contained a 3α-hydroxysteroid dehydrogenase (which was invariably the most active enzyme). M2 was unusual in that it contained only a 3α-hydroxysteroid dehydrogenase. The findings with this somewhat random sampling of soil microorganisms which can grow on steroids confirm that microbial hydroxysteroid dehydrogenases are generally NAD-specific and suggest that soil is an abundant source of microorganisms that contain a wide variety of such enzymes. It seems likely that hydroxysteroid dehydrogenases with other steric and positional specificities could be found by selective enrichment culture of soil microorganisms in media containing the appropriate steroids as carbon sources.

This initial screening procedure (Fig. 1), however, does not distinguish the number of enzymes responsible for these activities. For example, Pseudomonas testosteroni contains several species of (3 and 17)β-hydroxysteroid dehydrogenase (Talalay, 1963) which have been purified to homogeneity (Schultz et al., 1977), as well as distinct 3β- and 17β-hydroxysteroid dehydrogenases which are, however, difficult to resolve (Benson et al., 1974; Schultz et al., 1977). The substrate specificities of hydroxysteroid dehydrogenases present in each of the isolated organisms were determined by submitting crude centrifuged cell extracts to electrophoresis in native gels and by staining for enzyme activity with testosterone, estradiol-17β, dehydroepiandrosterone, or androsterone as substrates. This procedure showed that most of the isolated organisms contained several hydroxysteroid dehydrogenases with differing mobilities and substrate specificities. Fig. 2 shows the gels stained for hydroxysteroid dehydrogenase activity obtained with extracts of organism M21 which is a new species of Alcaligenes that can grow on estradiol-17β or testosterone as the sole source of organic carbon. This organism was the most promising source of a highly active 17β-hydroxysteroid dehydrogenase. It contained no 3α-hydroxysteroid dehydrogenase activity (Fig. 1 and Fig. 2, lane A), and the two or more 3β-hydroxysteroid dehydrogenase isoenzymes (Fig. 2, lane D) are well separated by electrophoresis from three 17β-hydroxysteroid dehydrogenase isoenzymes (Fig. 2, lanes E and T). These 17β-hydroxysteroid dehydrogenase isoenzymes oxidize both testosterone and estradiol-17β and

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**A Novel 17β-Hydroxysteroid Dehydrogenase**

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**Fig. 1. Inducible hydroxysteroid dehydrogenase activities in sonic extracts of various microbial cultures.** NAD-linked enzyme activity with four different steroids (A, androsterone; T, testosterone; E, estradiol-17β; D, dehydroepiandrosterone) was assayed in supernatant fractions of sonically disrupted organisms which were grown, induced with progesterone, and assayed as described under “Experimental Procedures.” Typical specific activities are shown for P. testosteroni (P. TESTO.) (ATCC 11996; Talalay et al., 1952) and for the Teller-Bongiovanni (TB) variant (Teller and Bongiovanni, 1963; Shikita and Talalay, 1979), as well as for bacteria which were isolated by enrichment culture from soil (M1B–M21, see “Experimental Procedures”). M21 is the Alcaligenes sp. from which a 17β-hydroxysteroid dehydrogenase was purified as described in this paper.

Serum albumin. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-0829, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

1 Progesterone has been shown to be an effective and inexpensive inducer of Δ4-3-ketosteroid isomerase in P. testosteroni, and most steroid-metabolizing enzymes appear to be induced coordinately (Shikita and Talalay, 1968).

2 ACTIVITY (units/mg protein) 3

**Fig. 2. Polyacrylamide gel electrophoresis of hydroxysteroid dehydrogenase activities in sonic extracts from an Alcaligenes sp.** Crude sonicates of progesterone-induced Alcaligenes sp. cultures were prepared, subjected to electrophoresis on 12.5% native polyacrylamide gels, and stained for activity as described under “Experimental Procedures.” Each gel contained 175 μg of total protein and at least 0.038 unit of hydroxysteroid dehydrogenase activity. Activity stains contained androsterone (lane A), dehydroepiandrosterone (lane D), estradiol-17β (lane E), or testosterone (lane T) as substrates.
consist of a major component \((R_s = 0.22)\), which was purified as described below, and two minor components \(R_s = 0.65\) and 0.72).

**Purification of 17β-Hydroxysteroid Dehydrogenase from Alcaligenes sp.**—Alcaligenes sp. is an abundant source of several enzymes. The large scale (150 liter) culture yielded approximately 20,000 units of 17β-hydroxysteroid dehydrogenase (testosterone as substrate) and 10,600 units of 3β-hydroxysteroid dehydrogenase (dehydroepiandrosterone as substrate) in the crude sonic extract at the time of harvest (42 h of growth), representing an induction of enzyme activity of about 25-fold. It was later found that greater yield of enzyme could have been obtained by earlier harvesting of cells. Sonic extracts from Alcaligenes sp. also contained large quantities of Δ⁵-3-ketosteroid isomerase activity (500,000 units).

A summary of the purification of 17β-hydroxysteroid dehydrogenase is presented in Table I. From a 150-liter fermentation, yielding 987 g of cells, wet weight, a 763-fold purification provided 14.5 mg of purified 17β-hydroxysteroid dehydrogenase with a specific activity of 335 μmol/min/mg of protein. The yield of purified 17β-hydroxysteroid dehydrogenase was 25%.

The key steps in the purification of 17β-hydroxysteroid dehydrogenase were anion-exchange (Fig. 3) and affinity (Fig. 4) chromatography (see Miniprint). The initial DEAE-cellulose chromatography step (Fig. 3 and Table I, Step 4) generally gave a 6-10-fold purification with at least 90% recovery of activity. Activity with testosterone and estradiol-17β co-migrated in this and all other chromatographic steps, indicating the presence of a single 17β-hydroxysteroid dehydrogenase. 3β-Hydroxysteroid dehydrogenase activity was eluted immediately following the 17β-hydroxysteroid dehydrogenase (Fig. 3) but was not further purified. Preliminary studies indicated that the two minor 17β-hydroxysteroid dehydrogenase isoenzymes (see Fig. 2) were eluted much later in the gradient. These have not been studied further.

An additional DEAE-cellulose chromatography at a higher pH (Step 5) and hydrophobic chromatography on phenyl-Sepharose (Step 6) further purified the enzyme (Table I), but adsorption of 17β-hydroxysteroid dehydrogenase to Reactive Red 120-agarose proved the most effective step, providing an 8-fold purification over the preceding step with 98% recovery of activity (Fig. 4 and Table I, Step 7). This affinity adsorbent is quite specific since the enzyme activity is not adsorbed by other immobilized dyes, e.g. Blue-, Green-, and Orange-agarose from Amicon Corp. (Lexington, MA) or by the affinity adsorbent 5'-AMP-Sepharose 4B (Pharmacia PL Biochemicals, Piscataway, NJ). The enzyme which was eluted from the Reactive Red 120-agarose oxidized both testosterone and estradiol-17β and co-migrated with a distinct protein peak. Pool A (Fig. 4) was considered to be pure 17β-hydroxysteroid dehydrogenase. Pool B (Fig. 4) was rechromatographed on the same column, after which its specific activity was identical with that of Pool A, i.e. 325 units/mg of protein. This very high specific activity is typical of most other bacterial hydroxysteroid dehydrogenases which have been purified to homogeneity. For example, (3 and 17β)-hydroxysteroid dehydrogenase from P. testosteroni has approximately 200 units/mg (Schultz et al., 1977) and 3α-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase from P. testosteroni \(TB\) have 130 and 90 units/mg, respectively (Shikita and Talalay, 1979). In contrast, purified mammalian hydroxysteroid dehydrogenases such as placental 17β-hydroxysteroid dehydrogenase (Jarabak et al., 1962; Karavolas et al., 1970; Strickler and Tobias, 1980) and rat liver 3α-hydroxysteroid dehydrogenase (Penning et al., 1984) have specific activities of less than 10 units/mg of protein.

**Purity of 17β-Hydroxysteroid Dehydrogenase**—The purity of the final preparations of 17β-hydroxysteroid dehydrogenase was monitored by polyacrylamide electrophoresis on sodium dodecyl sulfate, native, and isoelectric focusing gels. Except for a very faint band \((M_1 \sim 45,000)\) which may represent aggregated enzyme, sodium dodecyl sulfate electrophoresis (Fig. 5A) of denatured 17β-hydroxysteroid dehydrogenase yielded a single band of protein \((M_1 = 32,000)\). Electrophoresis of native 17β-hydroxysteroid dehydrogenase at two different concentrations of acrylamide (12.5% acrylamide shown; Fig. 5B) revealed a single protein band associated with enzymatic activity. Isoelectric focusing (not shown) of pure 17β-hydroxysteroid dehydrogenase on polyacrylamide gels also yielded a single band of protein associated with enzymatic activity (pI 5.1). Since crude sonicates from Alcaligenes sp. contained significant quantities of 3β-hydroxysteroid dehydrogenase and Δ⁵-3-ketosteroid isomerase, we measured these enzymes (with dehydroepiandrosterone and 5-androsten-3,17-dione as substrates, respectively) in the final purified preparation of 17β-hydroxysteroid dehydrogenase and found each of these activities to represent only 0.01% of the 17β-hydroxysteroid dehydrogenase activity.

**Kinetics and Nicotinamide Nucleotide Specificity**—As shown in Table II, 17β-hydroxysteroid dehydrogenase from Alcaligenes sp. is an NAD-linked enzyme that promotes oxidoreductions of C₁₈ and C₁₉ steroids with \(K_m\) values in the micromolar range. The thionicotinamide analog of NAD (S-

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Supernatant fraction after sonic treatment</td>
<td>45,195</td>
<td>19,809</td>
<td>0.44</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Polymyxin F supernatant</td>
<td>13,047</td>
<td>17,560</td>
<td>1.3</td>
<td>89</td>
<td>3.1</td>
</tr>
<tr>
<td>3. Dialyzed ammonium sulfate precipitate (30-78%)</td>
<td>9,768</td>
<td>10,191</td>
<td>1.0</td>
<td>51</td>
<td>2.4</td>
</tr>
<tr>
<td>4. First DEAE-cellulose chromatography</td>
<td>1,620</td>
<td>11,579</td>
<td>7.1</td>
<td>59</td>
<td>16</td>
</tr>
<tr>
<td>5. Second DEAE-cellulose chromatography</td>
<td>588</td>
<td>9,858</td>
<td>16.8</td>
<td>50</td>
<td>38</td>
</tr>
<tr>
<td>6. Hydrophobic chromatography (phenyl-Sepharose)</td>
<td>217</td>
<td>7,990</td>
<td>36.8</td>
<td>40</td>
<td>8</td>
</tr>
<tr>
<td>7. Affinity chromatography (Red-agarose) total</td>
<td>33</td>
<td>7,895</td>
<td>239</td>
<td>40</td>
<td>544</td>
</tr>
<tr>
<td>Pool A</td>
<td>8.9</td>
<td>2,919</td>
<td>326</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool B (after chromatography)</td>
<td>9.4</td>
<td>3,062</td>
<td>328</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. Final (after concentration)</td>
<td>14.5</td>
<td>4,871</td>
<td>233*</td>
<td>25</td>
<td>763</td>
</tr>
</tbody>
</table>

* Mean value for pools A and B.
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Enzymatic activity was assayed as described under “Experimental Procedures.” Assays were performed at pH 9 with 5.7 ng of pure 17β-hydroxysteroid dehydrogenase except for the reduction of 4-androstene-3,17-dione (pH 7; 1.3 ng of 17β-hydroxysteroid dehydrogenase). Values for \( K_a \) and \( V_{max} \) were calculated from a double reciprocal plot of 7–11 assay points by use of a weighted linear regression analysis (Wilkinson, 1961).

### Table II

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Co-substrate*</th>
<th>( K_a ) (µM)</th>
<th>( V_{max} ) (µmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>NAD</td>
<td>3.6 ± 0.5</td>
<td>135 ± 14</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>NAD</td>
<td>6.4 ± 1.1</td>
<td>65 ± 27</td>
</tr>
<tr>
<td>4-Androstene-3,17-dione</td>
<td>NADH</td>
<td>1.6 ± 0.3</td>
<td>17.6 ± 0.6</td>
</tr>
<tr>
<td>NAD</td>
<td>Testosterone</td>
<td>27 ± 3.5</td>
<td>333 ± 12.5</td>
</tr>
</tbody>
</table>

*Substrate concentrations were varied (1.6–30 µM for steroids; 10–830 µM for NAD).
*Co-substrate kept constant (330 µM for nicotinamide nucleotides; 34 µM for testosterone).
*Means ± S.E.

NAD is also reactive in catalyzing the oxidation of both testosterone and estradiol-17β. The 17β-hydroxysteroid dehydrogenase from Alcaligenes sp. has a \( K_a \) of 27 µM for NAD (in the oxidation of testosterone); NADP cannot serve as a coenzyme. In this respect, the 17β-hydroxysteroid dehydrogenase of Alcaligenes sp. resembles the hydroxysteroid dehydrogenases of \( P. testosteroni \) and other bacteria (Talalay and Williams-Ashman, 1960). In contrast, the 17β-hydroxysteroid dehydrogenase of human placenta (Jarabak et al., 1962; Karvovas et al., 1970) and other hydroxysteroid dehydrogenases from mammalian sources (Tomkins, 1956; Hurlock and Talalay, 1957; Langer and Engel, 1958; Liu and Kochakian, 1972; Michel et al., 1975) have dual nucleotide specificity.

The enzyme of Alcaligenes sp. also differs from other known 17β-hydroxysteroid dehydrogenases in that it catalyzes the oxidative deamination of both aromatic (e.g. estradiol-17β) and non-aromatic (e.g. testosterone) steroids at comparable rates, whereas the rate of oxidation of testosterone by the 3 and 17β-hydroxysteroid dehydrogenase of \( P. testosteroni \) is about 5–7 times that for estradiol-17β (Talalay and Dobson, 1963). Although it has been suggested that human placental 17β-hydroxysteroid dehydrogenase has an absolute requirement for an aromatic A ring (Karvovas and Engel, 1971), other evidence indicates that this enzyme does catalyze the very slow oxidation of nonphenolic 17β- and 20α-hydroxysteroids (Adams et al., 1962; Langer and Engel, 1958; Purdy et al., 1964; Strickler and Tobias, 1960; Mendoza-Hernández et al., 1984).

### Table III

Steroid substrate specificity of purified 17β-hydroxysteroid dehydrogenase of Alcaligenes sp.

The initial velocities of oxidation were measured in the standard enzyme assay system (0.33 mM NAD, 34 µM steroid, pH 9.0) with 5.7 ng of purified 17β-hydroxysteroid dehydrogenase. The velocities were normalized to a value of 100 for the oxidation of testosterone (specific activity; 236 units/mg of protein). The substrate concentrations were selected to be about 10 times the \( K_a \) for testosterone (3.6 µM). For substrates showing less than 2% of the oxidation velocity of testosterone, 50–150 times more enzyme was used. All values are the means of two to three determinations.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Substrate</th>
<th>Relative velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17β-Hydroxy-4-androsten-3-one (testosterone)</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>6β,17β-Dihydroxy-4-androsten-3-one</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>11α,17β-Dihydroxy-4-androsten-3-one</td>
<td>86</td>
</tr>
<tr>
<td>4</td>
<td>11β,17β-Dihydroxy-4-androsten-3-one</td>
<td>136</td>
</tr>
<tr>
<td>5</td>
<td>15α,17β-Dihydroxy-4-androsten-3-one</td>
<td>131</td>
</tr>
<tr>
<td>6</td>
<td>4-Androsten-3,17β-diol</td>
<td>75</td>
</tr>
<tr>
<td>7</td>
<td>5-Androsten-3,17β-diol</td>
<td>233</td>
</tr>
<tr>
<td>8</td>
<td>17β-Hydroxy-5α-androstan-3-one (di-hydrotestosterone)</td>
<td>133</td>
</tr>
<tr>
<td>9</td>
<td>17β-Hydroxy-5β-androstan-3-one</td>
<td>86</td>
</tr>
<tr>
<td>10</td>
<td>5α-Androstan-3β,17β-diol</td>
<td>72</td>
</tr>
<tr>
<td>11</td>
<td>5α-Androstan-3α,17β-diol</td>
<td>85</td>
</tr>
<tr>
<td>12</td>
<td>5β-Androstan-3α,17β-diol</td>
<td>57</td>
</tr>
<tr>
<td>13</td>
<td>1,3,5(10)-Estriatriene-3,17β-diol (estradiol-17β)</td>
<td>72</td>
</tr>
<tr>
<td>14</td>
<td>1,3,5(10)-Estriatriene-3,16α,17β-triol (estradiol)</td>
<td>72</td>
</tr>
<tr>
<td>15</td>
<td>1,3,5(10)-Estriatriene-3,16β,17β-triol</td>
<td>72</td>
</tr>
<tr>
<td>16</td>
<td>1,3,5(10)-Estriatriene-3,16α,17β-triol</td>
<td>72</td>
</tr>
<tr>
<td>17</td>
<td>17α-Hydroxy-4-androsten-3-one (epitestosterone)</td>
<td>72</td>
</tr>
<tr>
<td>18</td>
<td>1,3,5(10)-Estriatriene-3,17α-diol</td>
<td>72</td>
</tr>
<tr>
<td>19</td>
<td>11β,17α-21-Trihydroxy-4-pregnene-3,20-dione (cortisol)</td>
<td>72</td>
</tr>
<tr>
<td>20</td>
<td>3,17α-Dihydroxy-5-pregnene-20-one (17α-hydroxyprogrenolone)</td>
<td>72</td>
</tr>
<tr>
<td>21</td>
<td>3β-Hydroxy-5-androsten-17-one (dehydroepiandrosterone)</td>
<td>72</td>
</tr>
<tr>
<td>22</td>
<td>3β-Hydroxy-5-cholenoic acid</td>
<td>72</td>
</tr>
<tr>
<td>23</td>
<td>3β-Hydroxy-5β-androstan-3-one</td>
<td>72</td>
</tr>
<tr>
<td>24</td>
<td>3β-Hydroxy-5β-pregnen-20-one</td>
<td>72</td>
</tr>
<tr>
<td>25</td>
<td>3β-Hydroxy-5α-cholanoic acid</td>
<td>72</td>
</tr>
<tr>
<td>26</td>
<td>3β-Hydroxy-5α-androstan-3-one</td>
<td>72</td>
</tr>
<tr>
<td>27</td>
<td>5α-Androstan-3,17β-diol</td>
<td>0.8</td>
</tr>
<tr>
<td>28</td>
<td>5β-Androstan-3α,17β-diol</td>
<td>0.9</td>
</tr>
<tr>
<td>29</td>
<td>3α-Hydroxy-5α-androstan-17-one (androsterone)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Assay detection limit; equivalent to <0.3% of testosterone.

Steroid Specificity—The substrate specificity of purified 17β-hydroxysteroid dehydrogenase from Alcaligenes sp. for 29 steroids is shown in Table III. As discussed above, both C<sub>19</sub> and C<sub>19</sub> steroids are excellent substrates for this enzyme. 17β-Hydroxysteroids (Table III, compounds 1–13) with unsaturated A or B rings, saturated rings (A/B cis or trans ring fusions), or a phenolic A ring are all good substrates. Compounds with a double bond at C-5 (compare compound 7 with 6) or which are A/B cis rather than trans (compare compound 9 with 8 and compound 12 with 10 and 11) appear to be somewhat less active. The presence of a 16α- or 16β-hydroxyl group blocks the oxidation of 17β-hydroxysteroids (compounds 14 and 15). However, two 16α-hydroxysteroids (compounds 15 and 16) are very poor substrates, indicating that the positional specificity of the enzyme is very high.

The stereospecificity of this enzyme is demonstrated by its inability to oxidize the 17α-hydroxyl groups of analogous compounds (compounds 17–19). Furthermore, 3α-, 6β-, 11α-, 11β-, or 15α-hydroxysteroids (compounds 2–5, 19, 20, and 29) are not substrates for this enzyme. When oxidations of compounds with more than one hydroxyl group (e.g. compounds 2–5) were allowed to go the completion (under conditions where complete oxidation was to be expected), the final absorbance of the assay solution indicated that only 1 mol of NADH was formed per mol of substrate added. This implies...
that only the 17β-hydroxyl group of each compound was oxidized.

Unlike the (3 and 17)β-hydroxysteroid dehydrogenase from P. testosteroni (Talalay and Marcus, 1956; Roe and Kaplan, 1969; Schultz, et al., 1977) for which the rates of oxidation of 3β-hydroxysteroids are as high as 75% of that of testosterone, the 17β-hydroxysteroid dehydrogenase from Alcaligenes sp. has negligible 3β-hydroxysteroid dehydrogenase activity. Most of the 3β-hydroxysteroids tested (compounds 20–25) were not substrates, although compounds 26–28 were oxidized very slowly. Examination of the reactivity of several 3β-hydroxysteroids suggests that Δ9-compounds (20–22) and A/B cis compounds (23–25) are not substrates, whereas A/B trans compounds (26–28) were oxidized very slowly. We studied the oxidation of epiandrosterone (3β-hydroxy-5α-androst-17-one) in detail. The activity could not be attributed to substrate impurity since the epiandrosterone was pure by thin-layer chromatography and since, under appropriate conditions, nearly stoichiometric quantities of NADH were produced when the oxidation of epiandrosterone was allowed to proceed to completion with large amounts of enzyme. Moreover, activity could not be attributed to contamination of our purified 17β-hydroxysteroid dehydrogenase preparation by the electrophoretically distinct, inducible 3β-hydroxysteroid dehydrogenase also present in Alcaligenes sp. since enzyme activity-stained bands (with either testosterone or epiandrosterone as substrate) co-migrated with the single Coomassie Blue-stained protein band during electrophoresis of purified 17β-hydroxysteroid dehydrogenase on native polyacrylamide gels. Furthermore, heat inactivation patterns for 17β-hydroxysteroid dehydrogenase with the use of either epiandrosterone (not shown) or testosterone (cf. Fig. 6A) were similar. We conclude that the pure 17β-hydroxysteroid dehydrogenase from Alcaligenes sp. has low reactivity with 3β-hydroxy-5α-steroids.

Isoelectric Point—17β-Hydroxysteroid dehydrogenase from Alcaligenes sp. is an acidic protein with an isoelectric point of 5.1 as determined by electrofocusing on polyacrylamide gels. The isoelectric point determined by chromatofocusing on ion exchange media (PBE 74, Pharmacia PL Biochemicals) with amphoteric buffers (Polybuffer 74, Pharmacia) was also 5.1.

Stability—17β-Hydroxysteroid dehydrogenase from Alcaligenes sp. is a remarkably stable enzyme. It is relatively insensitive to both heat and cold, and its stability is enhanced by glycerol. Dilute solutions (95 μg/ml) of enzyme are able to withstand temperatures of 50 °C for 1 h with only a 20% loss of activity if significant quantities of glycerol (52.5% by volume) are present (Fig. 6A). Under these conditions, the half-time for heat inactivation is 120 min. Activity is rapidly lost above 50 °C. Furthermore, this 17β-hydroxysteroid dehydrogenase is stable in the cold, retaining 80% of its activity when stored at pH 6.5–8.0 at 4°C for 1 week (Fig. 6B). This is in contrast to placental 17β-hydroxysteroid dehydrogenase which is cold-sensitive (Jarabak et al., 1966) and cannot be purified except in the presence of glycerol (Jarabak et al., 1962, 1966; Karavolas et al., 1970). Because glycerol also enhances the heat stability of 17β-hydroxysteroid dehydrogenase from Alcaligenes sp., we store our purified preparations at −20 °C in 50% glycerol and have observed no loss in activity for at least 1 year.

Effect of pH on Oxidoreduction Activity—Initial velocities for the forward and reverse reactions were studied as a function of pH in a range in which both enzyme and coenzyme are stable (Fig. 7). Activity rises with increasing pH reaching an apparent plateau between pH 9 and 10 in the direction of oxidation, whereas the reverse reaction velocity increases as the pH is reduced to about pH 6. The velocities for both reactions are equal at about pH 8. A similar inverse pH relationship between the forward and reverse reactions has been shown for (3 and 17)β-hydroxysteroid dehydrogenase from P. testosteroni (Talalay and Marcus, 1956). Since hydrogen ions participate in the reaction of NAD-linked enzymes, the pH profoundly affects equilibria as well as catalytic activity.

Molecular Weight Determination—The apparent molecular weight of native 17β-hydroxysteroid dehydrogenase (determined at neutral pH) was 68,000 ± 6,100 by gel filtration (Fig. 8) and 86,000 ± 9,500 by Ferguson analysis (Ferguson, 1964; Rodbard and Chrambach, 1970) on polyacrylamide gel electrophoresis (Fig. 9). Similar results (not shown) were obtained on gels at pH 8.5. Discrepancies between results from the two different sizing techniques have been previously reported (Carlson et al., 1977), and molecular weights are sometimes overestimated by methods which rely on the idealized molecular radius of the protein (cf. Payne et al., 1976). Since denatured 17β-hydroxysteroid dehydrogenase from Alcaligenes sp. yields one band of M, = 32,000 on sodium dodecyl sulfate gel electrophoresis (Fig. 5A), it seems likely that the native enzyme is dimeric with subunits of equal molecular weights.

Analytical Applications—With the availability of purified preparations of 17β-hydroxysteroid dehydrogenase from Alcaligenes sp., we are now able to measure by enzymatic assays the biologically important 17β-hydroxy- and 17-ketosteroids such as testosterone, dihydrotestosterone, and estradiol-17β. For example, minute quantities of testosterone (Fig. 10A) and
The selective oxidation of the 3α- and 17β-hydroxy groups of 5α-androstane-3α,17β-diol with 3α- and 17β-hydroxysteroid dehydrogenase. The selective oxidation of the 3α- and 17β-hydroxy groups of 34 μM 5α-androstane-3α,17β-diol by 0.019 unit of either 3α-hydroxysteroid dehydrogenase (HSD) (purified from P. testosteroni TB; Shikita and Talalay, 1979) or 17β-hydroxysteroid dehydrogenase (purified from Alcaligenes as described in text) was monitored in a 3-ml assay system as described under “Experimental Procedures.” The time of addition of the enzymes is indicated by the arrows. The horizontal dashed lines indicate the predicted fraction of NADH formed when one (bottom line) or two (top line) hydroxyl groups on the steroid have been completely oxidized. At each of the four plateaus shown, the steroid products were extracted from the assay mixture with ethyl acetate, subjected to normal-phase high pressure liquid chromatography, and detected by refractive index (R.I.) as described under “Experimental Procedures.” The actual chromatograms are shown in the insets. Elution times of appropriate standard steroids are indicated as follows: o, 5α-androstane-3,17-dione; O, androsterone; ©, dihydrotestosterone; @, 5α-androstane-3α,17β-diol.

17β-hydroxysteroids in urine (Fig. 10B) were assayed according to the scheme shown in Fig. 10 (top panel). The very small quantities of NAD(H) formed by the complete oxidation or reduction of the steroid are amplified at least 5000-fold by enzymatic cycling techniques coupled with fluorometric detection (Payne et al., 1982). This technique measures steroids with specific functional groups (e.g. 17β-hydroxysteroids). Thus, total 17β-hydroxysteroids were quantitated in fractions of a microliter of human urine (Fig. 10B). Individual steroids may then be assayed after separation by high pressure liquid chromatography (Payne et al., 1982). The specificity of the assay depends entirely on the substrate specificity of the hydroxysteroid dehydrogenase. In order to confirm the specificity of the measurement of 17β-hydroxysteroids by enzymatic cycling with 17β-hydroxysteroid dehydrogenase, we have assayed steroids in the presence of up to a 50-fold excess of other steroids that cannot serve as substrates. Androsterone and dehydroepiandrosterone which do not react with this enzyme (cf. Table III) gave no signal when assayed under these conditions. Episterone, which is a poor substrate for 17β-hydroxysteroid dehydrogenase (cf. Table III), gave only a 5% cross-reactivity in the assay.

Highly specific and active hydroxysteroid dehydrogenases are also useful reagents for performing stereoselective oxidoreductions of steroids (cf. Talalay and Levy, 1989). The rapid and complete oxidation of 5α-androstane-3α,17β-diol to three different products by the sequential use of 3α- and 17β-hydroxysteroid dehydrogenase is illustrated in Fig. 11. The first oxidation product is either dihydrotestosterone (17β-hydroxy-5α-androst-3-one) (Fig. 11A) or androsterone (Fig. 11B) depending on which of the two enzymes is used. The final product is 5α-androstane-3,17-dione (Fig. 11, A and B) when both enzymes are added. Under the specified assay conditions (pH 9.0; see “Experimental Procedures”), almost complete sequential oxidation of the two hydroxy groups of 5α-androstane-3α,17β-diol can be achieved. This example highlights the use of 17β-hydroxysteroid dehydrogenase as a potentially useful reagent in the stereoselective microsynthesis of steroids as for instance in the preparation of radioactive compounds.

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REFERENCES

The following solutions, all of which contained 1 mM DTT and 1 mM EDTA, were used:

**Solution A:** 30 mM potassium phosphate and 10% (v/v) glycerol, pH 7.0.

**Solution B:** 5 mM potassium phosphate, pH 7.0.

**Solution C:** 10 mM Tris-HCl, pH 8.0 (at 4°C).

**Solution D:** 1 M Tris-HCl, pH 8.0, 5 M urea (at 4°C).

**Solution E:** 30 mM potassium phosphate, pH 7.5.

**Step 1:** The washed cells were thawed, resuspended in 1.3 liters of Solution A and 0.5 ml of dithiothreitol (DTT) was added to the suspension buffer. The resuspended, washed cells were sonicated at 4°C for 3 minutes with 8-s bursts to achieve 95% saturation and the suspension was stirred at 4°C for 1 hour. The solution was then centrifuged at 4°C at 10,000 g for 30 minutes. The supernatant was carefully collected and stored at 4°C for further purification.

**Step 2:** The pooled fractions, containing a high activity of enzyme, were dialyzed against 0.1 M ammonium acetate in the cold and subjected to an anion exchange chromatography on a column of Reactive Red 120-210 potassium phosphate. After elution, the active fractions were collected and lyophilized.

**Step 3:** The lyophilized enzyme was redissolved in 0.1 M sodium phosphate buffer, pH 7.0, and applied to a Reactive Red 120-210 column of ammonium acetate. The enzyme was eluted using a linear gradient of potassium phosphate. Total protein was monitored at 280 nm.

**Step 4:** The dialysed enzyme was applied to a Reactive Red 120-210 column equilibrated with Solution E at a rate of 5.6 ml/min. Fractions containing 95% of the 17β-hydroxysteroid activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution E.

**Step 5:** The pooled fractions were applied to a Reactive Red 120-210 column equilibrated with Solution F. The column was then washed with Solution G at a rate of 5 ml/min. Fractions containing 95% of the enzyme activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.

**Step 6:** The pooled fractions were applied to a Reactive Red 120-210 column equilibrated with Solution H. The column was then washed with Solution I at a rate of 5 ml/min. Fractions containing 95% of the 17β-hydroxysteroid activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.

**Step 7:** The dialysed enzyme was subjected to an affinity chromatography on a Reactive Red 120-210 column. The leading half of the eluate was pooled (Fraction I) and concentrated (Fraction II). It contained 98% of the purified enzyme in a volume of 0.25 ml. The eluate was concentrated and stored at -20°C. The final 17β-hydroxysteroid activity was 90% of the original.

**Step 8:** The enzyme was then applied to a Reactive Red 120-210 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of potassium phosphate. The fractions containing 95% of the enzyme activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.

**Step 9:** The enzyme was then applied to an affinity chromatography on a Reactive Red 120-210 column. The leading half of the eluate was pooled (Fraction I) and concentrated (Fraction II). The enzyme was then applied to a Reactive Red 120-210 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of potassium phosphate. The fractions containing 95% of the enzyme activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.

**Step 10:** The enzyme was then applied to an affinity chromatography on a Reactive Red 120-210 column. The leading half of the eluate was pooled (Fraction I) and concentrated (Fraction II). The enzyme was then applied to a Reactive Red 120-210 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of potassium phosphate. The fractions containing 95% of the enzyme activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.

**Step 11:** The enzyme was then applied to an affinity chromatography on a Reactive Red 120-210 column. The leading half of the eluate was pooled (Fraction I) and concentrated (Fraction II). The enzyme was then applied to a Reactive Red 120-210 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of potassium phosphate. The fractions containing 95% of the enzyme activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.

**Step 12:** The enzyme was then applied to an affinity chromatography on a Reactive Red 120-210 column. The leading half of the eluate was pooled (Fraction I) and concentrated (Fraction II). The enzyme was then applied to a Reactive Red 120-210 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of potassium phosphate. The fractions containing 95% of the enzyme activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.

**Step 13:** The enzyme was then applied to an affinity chromatography on a Reactive Red 120-210 column. The leading half of the eluate was pooled (Fraction I) and concentrated (Fraction II). The enzyme was then applied to a Reactive Red 120-210 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of potassium phosphate. The fractions containing 95% of the enzyme activity were pooled, precipitated with ammonium sulfate (0-60% saturation), and dialyzed against Solution F.
A Novel 17β-Hydroxysteroid Dehydrogenase

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**Fig. 1:** Affinity chromatography of 17β-hydroxysteroid dehydrogenase (Fig 2). A dialyzed ammonium sulfate precipitate (217 mg of protein) was chromatographed on a Bio-Rad-120-agarose column and was eluted with a linear gradient of increasing salt and glycerol. Total protein was monitored at 280 nm. The fractions (5.5 ml) were assayed for enzymatic activity by measuring the oxidation of testosterone at pH 7.5, 52.5% of protein per ml in buffers containing ammonium phosphate, pH 7.5, 52.5% MES and 50°C. Initial activity (100%) for activity with testosterone as substrate. Each point represents the mean ± S.D. of 3-4 assays. The fractions were combined into pools A and B as shown. Pool A contains pure 17β-HSD; Pool B was subsequently rechromatographed on the same column.

**Fig. 2:** Effect of pH on stability of purified 17β-HSD (a) on the velocity of testosterone and estradiol-3-17β-diol by purified 17β-HSD. Affinity chromatography of 17β-hydroxysteroid dehydrogenase by gel filtration. Gel permeation HPLC of 17β-HSD (5-7 µg) was subjected to electrophoresis in native gels of 12% total acrylamide as described under "Experimental Procedures." Gels were stained for either 17β-HSD activity with testosterone (A) or for protein with Coomassie Blue B250.

**Fig. 3:** Evidence for the purity of 17β-hydroxysteroid dehydrogenases

A. SDS-polyacrylamide gel electrophoresis. Standard proteins and purified 17β-HSD were subjected to electrophoresis in gels containing sodium dodecyl sulfate as described under "Experimental Procedures." Each lane contained 20 µg of protein. Standard proteins (molecular weight × 10^3) from top to bottom are: phosphorylase B, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, β-lactoglobulin. B. Pool A from a Sephadex G-100-equilibrated gel-filtration column (Fig 1A, Sepha 75). Pool B following repeat hydroxysteroid dehydrogenase affinity chromatography (Table 1, Step 7). Mean molecular weight of pure 17β-HSD (Giese et al, 1988) calculated from two such experiments is 11,000.

**Fig. 4:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by gel filtration. Gel permeation HPLC of 17β-HSD (5-7 µg) was subjected to electrophoresis in native gels of 12% total acrylamide as described under "Experimental Procedures." Gels were stained for either 17β-HSD activity with testosterone (A) or for protein with Coomassie Blue B250.

**Fig. 5:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by Ferguson analysis. Polyanalumine gel electrophoresis of purified 17β-HSD (250 observations shown) and of standard proteins (5 observations each, inset shown) were performed on gels of varying concentration as described in "Experimental Procedures." Ferguson plots (Ferguson, 1949) were constructed by a weighted linear regression of log R vs log weight (correlation coefficient, r = 0.96-0.99). As described earlier (Ferguson, 1949; Benoist & Chrambach, 1970), there is a linear relationship between Rg and the molecular radius (R) of protein. The inset shows such a plot for standard proteins. The line was established by unweighted linear regression (correlation coefficient, r = 0.99-1.00). The calculated molecular weight for 17β-HSD (X) from this regression is 66,000 ± 1,000 (S.E.).

**Fig. 6:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by Ferguson analysis. Polyanalumine gel electrophoresis of purified 17β-HSD (250 observations shown) and of standard proteins (5 observations each, inset shown) were performed on gels of varying concentration as described in "Experimental Procedures." Ferguson plots (Ferguson, 1949) were constructed by a weighted linear regression of log R vs log weight (correlation coefficient, r = 0.96-0.99). As described earlier (Ferguson, 1949; Benoist & Chrambach, 1970), there is a linear relationship between Rg and the molecular radius (R) of protein. The inset shows such a plot for standard proteins. The line was established by unweighted linear regression (correlation coefficient, r = 0.99-1.00). The calculated molecular weight for 17β-HSD (X) from this regression is 66,000 ± 1,000 (S.E.).

**Fig. 7:** Effect of pH on the velocity of interconversion of testosterone and estradiol-3-17β-diol by purified 17β-HSD. Determinations were made as described in "Experimental Procedures." Each point represents the mean ± S.D. of 3-4 assays. The fractions were combined into pools A and B as shown. Pool A contains pure 17β-HSD; Pool B was subsequently rechromatographed on the same column.

**Fig. 8:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by gel filtration. Gel permeation HPLC of 17β-HSD (5-7 µg) was subjected to electrophoresis in native gels of 12% total acrylamide as described under "Experimental Procedures." Gels were stained for either 17β-HSD activity with testosterone (A) or for protein with Coomassie Blue B250.

**Fig. 9:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by Ferguson analysis. Polyanalumine gel electrophoresis of purified 17β-HSD (250 observations shown) and of standard proteins (5 observations each, inset shown) were performed on gels of varying concentration as described in "Experimental Procedures." Ferguson plots (Ferguson, 1949) were constructed by a weighted linear regression of log R vs log weight (correlation coefficient, r = 0.96-0.99). As described earlier (Ferguson, 1949; Benoist & Chrambach, 1970), there is a linear relationship between Rg and the molecular radius (R) of protein. The inset shows such a plot for standard proteins. The line was established by unweighted linear regression (correlation coefficient, r = 0.99-1.00). The calculated molecular weight for 17β-HSD (X) from this regression is 66,000 ± 1,000 (S.E.).

**Fig. 10:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by Ferguson analysis. Polyanalumine gel electrophoresis of purified 17β-HSD (250 observations shown) and of standard proteins (5 observations each, inset shown) were performed on gels of varying concentration as described in "Experimental Procedures." Ferguson plots (Ferguson, 1949) were constructed by a weighted linear regression of log R vs log weight (correlation coefficient, r = 0.96-0.99). As described earlier (Ferguson, 1949; Benoist & Chrambach, 1970), there is a linear relationship between Rg and the molecular radius (R) of protein. The inset shows such a plot for standard proteins. The line was established by unweighted linear regression (correlation coefficient, r = 0.99-1.00). The calculated molecular weight for 17β-HSD (X) from this regression is 66,000 ± 1,000 (S.E.).

**Fig. 11:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by Ferguson analysis. Polyanalumine gel electrophoresis of purified 17β-HSD (250 observations shown) and of standard proteins (5 observations each, inset shown) were performed on gels of varying concentration as described in "Experimental Procedures." Ferguson plots (Ferguson, 1949) were constructed by a weighted linear regression of log R vs log weight (correlation coefficient, r = 0.96-0.99). As described earlier (Ferguson, 1949; Benoist & Chrambach, 1970), there is a linear relationship between Rg and the molecular radius (R) of protein. The inset shows such a plot for standard proteins. The line was established by unweighted linear regression (correlation coefficient, r = 0.99-1.00). The calculated molecular weight for 17β-HSD (X) from this regression is 66,000 ± 1,000 (S.E.).

**Fig. 12:** Estimation of the apparent molecular weight of purified 17β-hydroxysteroid dehydrogenase by Ferguson analysis. Polyanalumine gel electrophoresis of purified 17β-HSD (250 observations shown) and of standard proteins (5 observations each, inset shown) were performed on gels of varying concentration as described in "Experimental Procedures." Ferguson plots (Ferguson, 1949) were constructed by a weighted linear regression of log R vs log weight (correlation coefficient, r = 0.96-0.99). As described earlier (Ferguson, 1949; Benoist & Chrambach, 1970), there is a linear relationship between Rg and the molecular radius (R) of protein. The inset shows such a plot for standard proteins. The line was established by unweighted linear regression (correlation coefficient, r = 0.99-1.00). The calculated molecular weight for 17β-HSD (X) from this regression is 66,000 ± 1,000 (S.E.).