istry 11, 4974.
Gold, P., and Freedman, S. O. (1965a), *J. Exp. Med. 121,* 439.
Gold, P., and Freedman, S. O. (1965b), *J. Exp. Med. 122,* 467.
Häkkinen, I. P. T. (1972), *Immunochemistry 9,* 1115.
Hakomori, S. (1964), *J. Biochem. 55,* 205.
Heath, E. C. (1971), *Annu. Rev. Biochem. 40,* 29.
Hughes, K. W., and Clamp, J. R. (1972), *Biochim. Biophys. Acta 264,* 418.
Krupey, J., Gold, P., and Freedman, S. O. (1968), *J. Exp. Med. 128,* 387.
Kupchik, H. Z., Zamcheck, N., and Saravis, C. A. (1973), *J. Nat. Cancer Inst. 51,* 1741.
Lee, Y. C., and Montgomery, R. (1961), *Arch. Biochem. Biophys. 95,* 263.
Liu, T.-Y., and Chang, Y. H. (1971), *J. Biol. Chem. 246,* 2842.
Mach, J.-P., and Pusztaszeri, G. (1972), *Immunochemistry 9,* 1031.
Reinhold, V. N. (1972), *Methods Enzymol. 25B,* 244.
Smith, F., and Unrau, A. M. (1959), *Chem. Ind. (London),* 881.
Spiro, R. G. (1960), *J. Biol. Chem. 235,* 2860.
Spiro, R. G. (1970), *Annu. Rev. Biochem. 39,* 599.
Stellner, K., Saito, H., and Hakomori, S. (1973), *Arch. Biochem. Biophys. 155,* 464.
Terry, W. D., Henkart, P. A., Coligan, J. E., and Todd, C. W. (1972), *J. Exp. Med. 136,* 200.
Terry, W. D., Henkart, P. A., Coligan, J. E., and Todd, C. W. (1974), *Transplantation Rev. 20,* 100.
Thomson, D. M. P., Krupey, J., Freedman, S. O., and Gold, P. (1969), *Proc. Nat. Acad. Sci. U.S. 64,* 161.
Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950), *Nature (London) 166,* 444.
Von Kleist, S., Chavanel, G., and Burtin, P. (1972), *Proc. Nat. Acad. Sci. U.S. 69,* 2492.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.

Partial Characterization of the Cytosol 3α-Hydroxysteroid: NAD(P)$^+$ Oxidoreductase of Rat Ventral Prostate†

Joel D. Taurog, Ronald J. Moore, and Jean D. Wilson*

ABSTRACT: The cytosol 3α-hydroxysteroid dehydrogenase of rat ventral prostate has been partially purified. The rates of both the oxidation and reduction by crude and partially purified enzymes have been measured with a variety of radioactive substrates, and the effects of several inhibitor steroids have been assessed. Four conclusions have been drawn from the study. First, no detectable 3β-androstanediol was formed from dihydrotestosterone, and the oxidation of 3β-androstanediol was undetectable. Second, the cytosol enzyme exhibits a distinct and unique substrate specificity in that steroids with keto or hydroxyl substitution on the 11th carbon of the steroid cannot serve as substrates or as inhibitors of the enzyme. Third, either 5α or 5β reduction of Δ^4,3-keto steroids must take place before the steroids can serve as substrates of the enzyme. Fourth, many Δ^4,3-keto steroids that cannot act as substrates for the enzyme inhibit the enzyme competitively and may well serve as physiological regulators of the reaction in the intact cell.

Enzymes that interconvert the 3-keto group of steroids containing saturated A,B rings with both 3α- and 3β-hydroxysteroids have been characterized in a variety of bacteria (Talalay and Marcus, 1954, 1956; Marcus and Talalay, 1956; Ringold *et al.,* 1967) and in several mammalian tissues (Tomkins, 1956; Koide, 1963, 1965a,b, 1969; Gustafsson *et al.,* 1968; Unhjem, 1970; Rommerts and van der Molen, 1971; Shimazaki *et al.,* 1972; Björkhem *et al.,* 1973; Martin and Nicholas, 1973; Levy *et al.,* 1974; Mowszowicz and Bardin, 1974). Either di- or triphosphopyridine nucleotides can serve as coenzymes, and in most instances the enzymes are located predominantly in the cytosol (soluble) fraction of the cell. In addition to their role in the metabolism of steroids the enzymes may participate in transhydrogenase reactions in some tissues (Hurlock and Talalay, 1958; Baron *et al.,* 1963; Koide *et al.,* 1962; Koide, 1964).

In androgen target tissues, such as the ventral prostate of the rat, the 3α-hydroxysteroid oxidoreductase[1] may play a

† From the Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas 75235. *Received September 30, 1974.* This work has been aided by Grant AM03892 from the National Institutes of Health.

[1] Trivial names used are: testosterone, 17β-hydroxy-4-androsten-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; androsterone, 3α-hydroxy-5α-androstan-17-one; 3α-androstanediol, 5α-androstane-3α,17β-diol; 3β-androstanediol, 5α-androstane-3β,17β-diol; androstenedione, 4-androstene-3,17-dione; androstanedione, 5α-androstane-3,17-dione; epitestosterone, 17α-hydroxy-4-androsten-3-one; progesterone, 4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; cortexolone, 17α,21-dihydroxy-4-pregnene-3,20-dione; 19-nortestosterone, 17β-hydroxyestren-3-one; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; cortisone, 17α,21-dihydroxy-4-pregnene-3,11,20-trione; methyldihydrotestosterone, 17α-methyl-17β-hydroxy-5α-androstan-3-one; allopregnanedione, 5α-pregnane-3,20-dione; 5α-dihydrodeoxycorticosterone, 21-hydroxy-5α-pregnane-3,20-dione; 5α-dihydrocortexolone, 17α,21-dihydroxy-5α-pregnane-3,20-dione; 5α-dihydrocorticosterone, 11β,21-dihydroxy-5α-pregnane-3,20-dione; 5α-dihydrocortisol, 11β,17α,21-trihydroxy-5α-pregnane-3,20-dione; 5α-dihydrocortisone, 17α,21-dihydroxy-5α-pregnane-3,11,20-trione; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; 5β-androstanedione, 5β-androstane-3,17-dione; epidihydrotestosterone, 17α-hydroxy-5α-androstan-3-one; 17-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; 17-hydroxyallopregnanedione, 17α-hydroxy-5α-pregnane-3,20-dione; 3α-hydroxysteroid oxidoreductase, 3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase (EC 1.1.1.50).

critical role in androgen action. On the one hand, dihydrotestosterone, which is probably the active mediator of the intracellular effects of testosterone, is a substrate for the enzyme, and the rate of 3-keto reduction may serve to regulate the steady-state concentration of the hormone within the cell. On the other, 3α-androstanediol, the major product of the 3-keto reduction of dihydrotestosterone, is a potent androgen (Moore and Wilson, 1973), and although it has been thought that in prostate it exerts its physiological effects after conversion to dihydrotestosterone (Bruchovsky, 1971), 3α-androstanediol itself may play a unique role in mediating certain androgenic actions (Springer and Eckstein, 1971; Lubicz-Nowrocki, 1973).

The 3-hydroxysteroid oxidoreductase of rat ventral prostate has been studied by Shimazaki and his colleagues (1972) who concluded that the rate of the reaction in rat prostate is not under endocrinological control by androgens and by Levy *et al.* who have described both 3α- and 3β-hydroxysteroid oxidoreductase activity in the tissue (1974). To gain further insight into the regulation of this reaction, the enzyme of rat ventral prostate has been partially purified, and the rates of both oxidation and reduction by crude cytosol and the partially purified enzyme have been measured with a variety of substrates and in the presence and absence of several inhibitors. The data indicate that this enzyme has several distinctive properties that make it a potentially important site for the regulation of androgen metabolism in the prostate.

Experimental Procedure

Preparation of Prostate Cytosol. Homogenization and preparation of prostate cytosol fractions were performed as described previously (Moore and Wilson, 1972). Mature male rats of the Sprague-Dawley strain weighing 350–450 g were killed by decapitation, and the ventral prostates were removed, dissected free of fat and connective tissue, and weighed; 25% w/v homogenates were prepared in 0.88 M sucrose–1.5 mM $CaCl_2$ using 30 strokes of a Dounce homogenizer fitted with a loose pestle (clearance 0.15 mm). The homogenates were filtered over eight layers of gauze, and the filtrates were rehomogenized by 20 strokes of a tight pestle (clearance 0.09 mm). Cytosol fractions were prepared by centrifugation of the filtered, rehomogenized preparations at 100,000*g*. The cytosol fractions containing approximately 15 mg of protein/ml were stored at −20°. Enzyme activity in this preparation was stable for at least 2 years. For these experiments, 1600 ml of prostate cytosol obtained from approximately 400 g wet weight of prostate was used.

Routine Enzyme Assay. 3α-Hydroxysteroid oxidoreductase was assayed by measuring the formation of [^{3}H]-3α-androstanediol from [1,2-^{3}H]dihydrotestosterone utilizing a thin-layer chromatographic separation. Enzyme assays contained, except where noted, 0.5 μM [1,2-^{3}H]dihydrotestosterone (4.3 Ci/mmol), 0.5 mM NADPH, 0.02 M Tris-Cl or 0.10 M potassium phosphate buffer (pH 7.2) as indicated, and 0.5 mg or less protein in a total volume of 0.5 ml. Incubations were carried out at 37° for 30 min, and the reaction was stopped by the addition of 5 volumes of chloroform–methanol (2:1). Aliquots of the organic phase containing approximately 3 × 10^5 dpm were evaporated to dryness, reconstituted in 20 μl of chloroform containing 10 μg each of authentic dihydrotestosterone and 3α-androstanediol, and applied to 1.5 cm wide lanes on precoated plastic sheets of silica gel (Sil G-Hy). Chromatography was performed using the solvent system chloroform–methanol (95:5) at 20°. The steroids were visualized by spraying with anisaldehyde reagent containing 100 ml of glacial acetic acid, 2 ml of H_2SO_4, and 1 ml of *p*-anisaldehyde and heating at 100° for 15 min. The zones corresponding to dihydrotestosterone and androstanediol were cut out with scissors, transferred to scintillation vials, and assayed for radioactivity following the addition of 10 ml of 0.4% 2,4-diphenyloxazole in toluene–methanol (10:1). As demonstrated in Figure 1, the remainder of the plate contained little radioactivity, and, therefore, only the dihydrotestosterone and androstanediol areas of the plate were counted for routine purposes. Enzyme units are expressed as picomoles of dihydrotestosterone converted to androstanediol per 30 min.

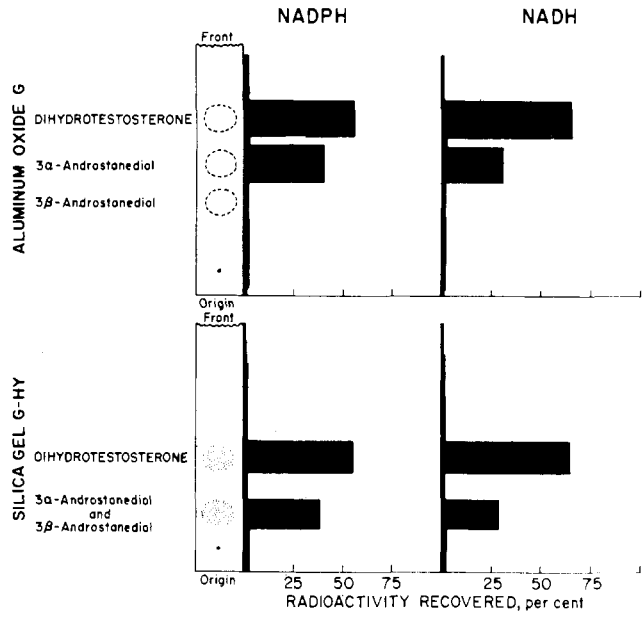

FIGURE 1: Thin-layer chromatography of 3α- and 3β-androstanediols on silica gel G-Hy and alumina G. Cytosol (0.9 mg of protein) was incubated with 0.5 μM [1,2-^{3}H]dihydrotestosterone (1 × 10^6 cpm), 0.5 mM NADH or 0.5 mM NADPH, and 0.1 M potassium phosphate (pH 6.6) in a total volume of 1 ml. After 30 min at 37°, the reaction was stopped by the addition of 5 ml of chloroform–methanol (2:1), and the steroids were extracted, mixed with carrier steroid, and spotted on thin-layer chromatography plates as described in the text. For the chromatography on aluminum oxide, each plate was developed two times at room temperature with benzene–ethanol (96:4), and the steroids were visualized under an ultraviolet lamp after spraying with 0.2% dichlorofluorescein. In the case of the silica gel plates, the developing solvent was chloroform–methanol (97.5:2.5), and after two ascents the steroids were visualized by spraying with anisaldehyde reagent. In both instances the entire lane from the origin to the solvent front was marked, and the plates were either cut or scraped into counting vials and assayed for radioactivity in a liquid scintillation counter.

Characterization of the Stereospecificity of the Enzyme Reaction. The products of the enzyme reaction were characterized by thin-layer chromatography on aluminum oxide, by recrystallization, and by digitonin precipitation.

For thin-layer chromatography, 6 g of aluminum oxide was mixed with water to make 13 ml of suspension, the mixture was applied to 20 × 20 cm glass plates at a thickness of approximately 0.3 mm, and the coated plates were air dried in an oven at 60°. Aliquots of the radioactive reaction products were added to a mixture of 10 μg each of authentic dihydrotestosterone, 3α-androstanediol, and 3β-androstanediol and spotted on the alumina coated plates.

The plates were developed twice in benzene–ethanol 96:4 (v/v), and air dried at room temperature. The dried plate

was sprayed with 0.2% dichlorofluorescein in ethanol, and the steroid areas were visualized under ultraviolet light and marked (Figure 1). The entire lane was divided into strips, scraped into counting vials, and assayed for ^3H.

For the recrystallization study, the chloroform–methanol extract was mixed with authentic 3α-androstanediol and spotted along the width of a silica G-Hy thin-layer plate. The silica plate was then developed at room temperature with one ascent of chloroform–methanol, 95:5. The edges and the middle 2 cm of the tlc plate were cut out, sprayed with anisaldehyde–H_2SO_4–glacial acetic acid, 1:2:100, and developed at 100° for 5 min. The horizontal zones corresponding to 3α-androstanediol on the unstained plate sections were scraped, placed in a centrifuge tube, and eluted with chloroform–methanol 2:1 (v/v). To this solution of presumed [^3H]-3α-androstanediol was added 185 mg of unlabeled authentic 3α-androstanediol that had been twice recrystallized from boiling methanol. The mixture was then dissolved in warm methanol and crystallized five times. The crystals and residues of the mother liquor from each crystallization were dried, weighed to 0.01 mg, dissolved in 10 ml of 0.4% 2,5-diphenyloxazole in toluene–methanol, 10:1 v/v, and assayed for radioactivity. The specific activity following the fifth crystallization (33,087 cpm/mg) was almost identical with that of the starting material (32,637 cpm/mg).

For the digitonin precipitation, chloroform–methanol extracts of the radioactive enzyme products were mixed with authentic 3β-androstanediol, and 3β-androstanediol digitonide was formed and washed by the method of Sperry and Webb (1950), dissolved in methanol, and assayed for radioactivity. In each of four experiments the 3β-androstanediol digitonide contained only 0.3% or less of the radioactivity.

Enzyme Purification Procedures. All operations were performed at 0–4°.

Ammonium Sulfate Fractionation. Cytosol preparations containing about 15 mg of protein/ml were allowed to thaw at 4°, and solid ammonium sulfate was slowly added with constant stirring to 50% saturation. After 1 hr the mixture was centrifuged at 20,000*g* for 30 min, and the supernatant was removed. To the supernatant solid ammonium sulfate was again slowly added with stirring to 80% saturation, and after 1 hr at 4° the mixture was centrifuged as before. The sediment (50–80% fraction) was dissolved in 0.02 M Tris (pH 7.20) at a concentration of 40–60 mg of protein/ml and stored at −20°. For 1500 ml of cytosol fractionated as above in two separate experiments, the recovery of protein and enzyme in the 50–80% ammonium sulfate fraction averaged 72 and 100%, respectively.

Gel Filtration Chromatography. Sephadex G-75 (40–120 μ particle size) was allowed to swell in water at room temperature, and fine particles were removed from the suspension by aspiration. A column with packed bed dimensions of 2.5 × 100 cm was equilibrated with 3 bed volumes of buffer. Samples of 50–80% ammonium sulfate sediment were dialyzed against the indicated buffer, and 10-ml aliquots were chromatographed at a constant pressure of 15 cm of H_2O and a flow rate of approximately 20 ml/hr; 5-ml fractions were collected and analyzed for protein and enzyme activity.

Fine particles were removed from Bio-Gel A 0.5m (100–200 mesh) as described above. A column with packed bed dimensions of 5 × 100 cm was equilibrated with 3 bed volumes of buffer. Samples (usually 50–80% ammonium sulfate precipitates) were dialyzed *vs.* the indicated buffer and chromatographed at a pressure of 80 cm of H_2O and a flow rate of approximately 120 ml/hr; 9–10-ml fractions were collected and analyzed for protein and enzyme activity.

Ion Exchange Chromatography. Microgranular DEAE-cellulose (Whatman DE 52) was slowly stirred at 20° into a volume of 0.2 M Tris (pH 7.2) containing 10 equiv of Tris/equiv of ion exchange resin. After 1 hr the settled resin was resuspended in 0.2 M Tris as above, and the fine particles were removed by aspiration. A column of resin having bed dimensions of 2.5 × 30 cm was prepared and equilibrated with 3 l. of 0.02 M Tris (pH 7.20) containing 0.5 mM dithiothreitol (T–DTT buffer). Equilibration was verified by measurements of conductivity and pH. Samples were applied and chromatographed at a flow rate of 60 ml/hr. Elution was carried out sequentially with T–DTT buffer (250 ml), a linear gradient of 0–0.25 M KCl in T–DTT buffer (800 ml), and 100 ml of 1.0 M KCl in T–DTT buffer. 5-ml fractions were collected and analyzed for protein, enzyme activity, and conductivity.

Kinetic Measurements. For the estimation of apparent K_m values for the various substrates, the standard assay mixtures contained, except where noted, 0.05 μM 1,2-^3H-labeled steroid substrate (35–50 Ci/mmol) and chromatographically pure nonradioactive substrate to provide the concentration range indicated, 0.5 mM NADPH or NADP, 0.2–0.4 mg of crude enzyme protein, 0.1 M potassium phosphate (pH 7.4), and 0.5 mM dithiothreitol in a final volume of 0.4 ml. To ensure steroid solubility, all assays contained 50 μg of Tween 40/μg of steroid. Initial velocities of the enzyme-catalyzed reactions were calculated from the percentage of 3-keto steroid reduced or formed as described above. Double reciprocal plots, analyzed by the method of least squares, were used to estimate the apparent K_m values.

For the measurement of apparent K_i values, the assay mixture contained, except where noted, 0.05–1 μM [1,2-^3H]dihydrotestosterone or 0.5–10 μM [1,2-^3H]-3α-androstanediol, 1 or 10 μM inhibitor steroid, 0.5 mM NADPH or NADP, 0.2 or 0.4 mg of crude enzyme protein, 0.1 M potassium phosphate (pH 7.4), and 0.5 mM dithiothreitol in a final volume of 0.4 ml. Initial velocities in the absence and presence of inhibitor were employed to estimate K_i values from double reciprocal plots. All assays contained 50 μg of Tween 40/μg of steroid.

Chemical Analyses. Protein was measured by the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard. Conductivity was measured at 20° using a Radiometer conductivity meter, and KCl concentration was calculated from a standard curve of KCl in 0.02 M T–DTT buffer. Apparent molecular weights were determined by the elution patterns from Bio-Gel A 0.5m as described previously using bovine serum albumin and chymotrypsinogen as reference standards (Moore and Wilson, 1974).

Reagents. [1,2-^3H]Dihydrotestosterone (44 Ci/mmol), [1,2-^3H]-3α-androstanediol (44 Ci/mmol), [1,2-^3H]androsterone (40 Ci/mmol), [1,2-^3H]etiocholanolone (40 Ci/mmol), [1,2-^3H]testosterone (40 Ci/mmol), [1,2-^3H]androstenedione (46 Ci/mmol), [1,2-^3H]epitestosterone (49 Ci/mmol), [1,2-^3H]desoxycorticosterone (50 Ci/mmol), [1,2-^3H]cortexolone (35 Ci/mmol), [1,2-^3H]corticosterone (40 Ci/mmol), [1,2-^3H]cortisol (53 Ci/mmol), [1,2-^3H]cortisone (48 Ci/mmol), [1,2-^3H]-3β-androstanediol (44 Ci/mmol), [1,2-^3H]-17α-hydroxyprogesterone (45 Ci/nmol), and [1,2-^3H]progesterone (44 Ci/mmol) were obtained from New England Nuclear.

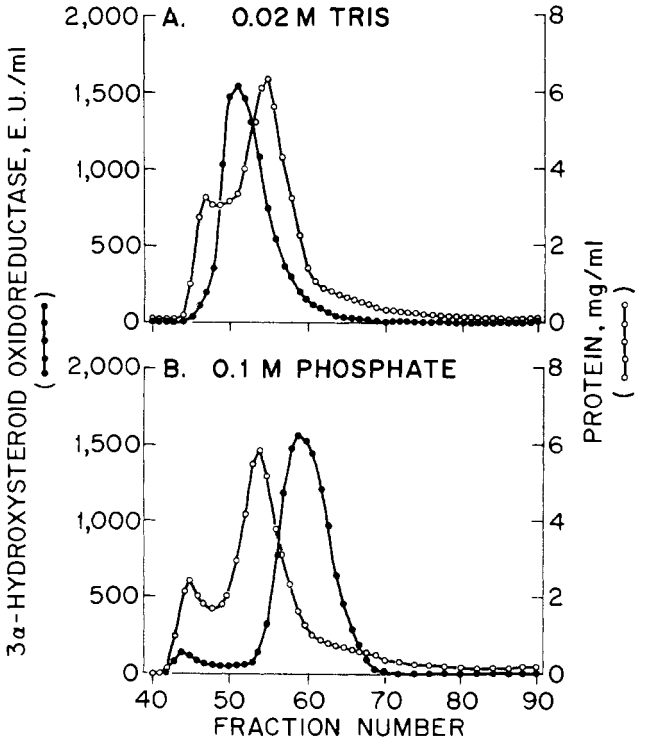

FIGURE 2: Gel filtration of 3α-hydroxysteroid oxidoreductase on Sephadex G-75: (A) 0.02 M Tris (pH 7.2); (B) 0.1 M potassium phosphate (pH 7.2); 50–80% ammonium sulfate precipitate of prostate cytosol (336 mg of protein in A and 270 mg of protein in B) was dialyzed in the cold overnight against the respective buffers indicated in the panels, each of which contained 0.5 mM dithiothreitol. The dialysate was applied to a Sephadex G-75 column (2.5 × 100 cm) that had been equilibrated in the same buffer. Flow rate was 21 ml/hr, and 78-drop fractions (5 ml) were collected and analyzed for enzyme and protein. Recovery of the enzyme was 77% in A and 82% in B.

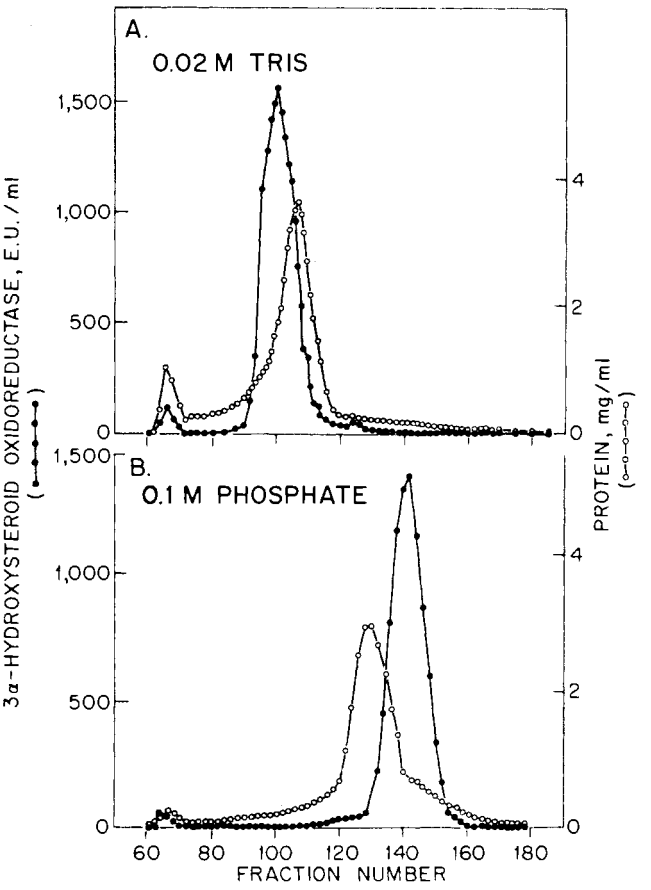

FIGURE 3: Gel filtration of 3α-hydroxysteroid oxidoreductase on agarose: (A) 0.02 M Tris (pH 7.2); (B) 0.1 M potassium phosphate (pH 7.2). Ammonium sulfate precipitate of prostate cytosol (50–80%) containing 767 mg of protein (A) or 630 mg of protein (B) was dialyzed in the cold overnight against the buffers shown in the respective panels, each of which was pH 7.2 and contained 0.5 mM dithiothreitol. The dialysate (18 ml) was applied to a Bio-Gel A 0.5m column (100–200 mesh, 5 × 100 cm) that had been equilibrated in the same buffer. Chromatography was at a pressure of 80 cm of water and a flow rate of approximately 120 ml/hr; 150-drop fractions (10 ml) were collected and analyzed for enzyme and protein. Recovery of enzyme was 86% in A and 99% in B.

[^{3}H]Androstanedione, [^{3}H]epidihydrotestosterone, [^{3}H]-17α-hydroxyallopregnanedione, [^{3}H]allopregnanedione, [^{3}H]-5α-dihydrodeoxycorticosterone, and [^{3}H]-5α-dihydrocortexolone were all synthesized by incubating the corresponding [1,2-^{3}H]-Δ^4,3-keto analogs (1 μM) with 0.5 mM NADPH and rat prostate microsomes (11 mg of protein) in a total volume of 2.5 ml for 1 hr at 25° (Moore and Wilson, 1973). The reaction mixture was extracted with chloroform–methanol 2:1 v/v, taken to dryness, and dissolved in chloroform, and the 5α-reduced derivatives were purified by preparative thin-layer chromatography as described by Frederiksen and Wilson (1971). $NADP^+$, NADPH, NAD^+, and NADH were purchased from P-L Biochemicals. Nonradioactive steroids were obtained from Steraloids, Inc., Pawling, N.Y. Digitonin was purchased from Nutritional Biochemicals. Precoated plastic plates of silica gel without gypsum (Polygram Sil G-Hy) were obtained from the Macherey-Nagel Co. (Düren). Bovine serum albumin was purchased from Armour, and chymotrypsinogen was obtained from Schwarz/Mann. Aluminum oxide GF-254, Type E, was obtained from Merck (Darmstadt).

Results

To determine whether detectable amounts of 3β-androstanediol were formed by the cytosol of rat ventral prostate under the conditions employed in these studies, three types of experiments were performed. First, following incubation of cytosol with [1,2-^{3}H]dihydrotestosterone in the presence either of NADPH or NADH, no significant radioactivity was recovered in the 3β-androstanediol area after thin-layer chromatography on aluminum oxide (Figure 1). Under the same circumstances, significant amounts of 3α-androstanediol were formed. Second, when the total [^{3}H]androstanediols recovered following such incubation were mixed with 3α-androstanediol and recrystallized repeatedly, there was no significant decrease in the specific activity of the crystals. Third, when 3β-androstanediol was isolated from such incubation mixtures as the digitonide, only 0.1–0.3% of the radioactivity of the mixture was recovered in the steroid digitonide under circumstances in which as much as 40% of the radioactivity was in 3α-androstanediol. Therefore, for all routine assays the radioactivity recovered in the combined 3α- and 3β-androstanediol area following chromatographic separation on silica gel G-Hy plates was assumed to represent 3α-androstanediol formation only (Figure 1).

When [1,2-^{3}H]dihydrotestosterone was incubated with prostate cytosol under the assay conditions employed in these studies, the formation of 3α-androstanediol was proportional to the protein added from 0.1 to 2 mg and was linear with time for as long as 2 hr. The rate of formation of androstanediol reached saturation at approximately 5 μM dihydrotestosterone and 0.2 mM NADPH. On the basis of

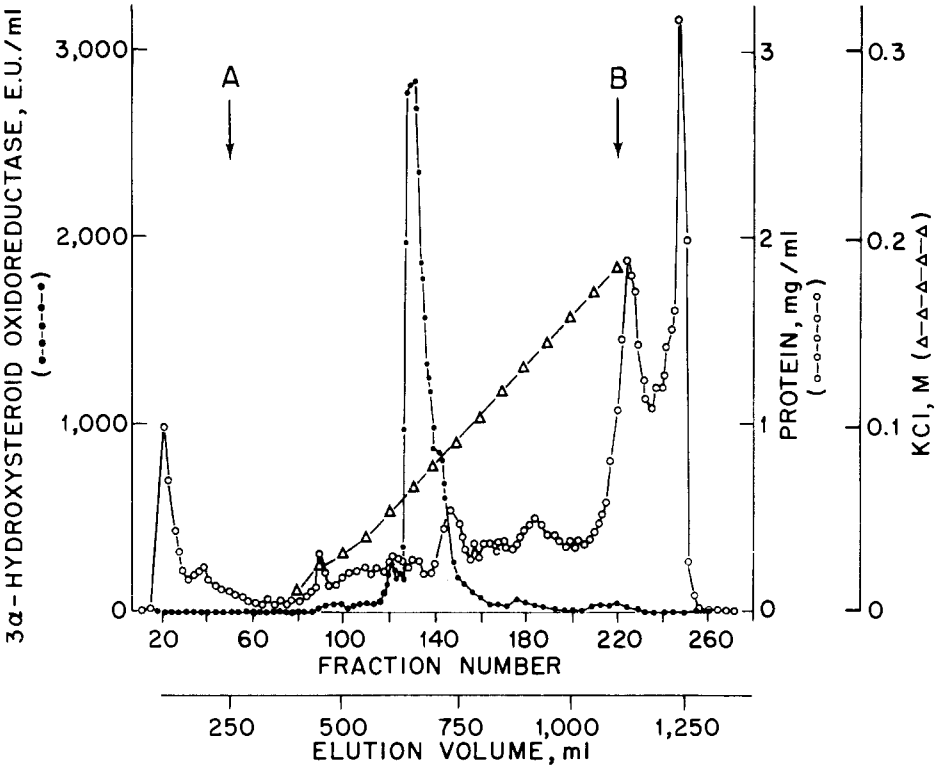

FIGURE 4: DEAE-cellulose chromatography of 3α-hydroxysteroid oxidoreductase. A 50–80% ammonium sulfate fraction of prostate cytosol containing 660 mg of protein was dialyzed overnight against 0.02 M Tris–0.5 mM dithiothreitol (pH 7.20) (T-DTT buffer). The dialysate was applied to a 2.5 × 50 cm column of microgranular DEAE-cellulose that had previously been equilibrated in the same buffer, and the column was eluted in 5-ml fractions at 60 ml/hr with 250 ml of T-DTT buffer (fractions 1–50) followed by an 800-ml linear KCl gradient (0–0.25 M) in T-DTT buffer (A) and then with 100 ml of 1 M KCl in T-DTT buffer (B). The fractions were analyzed for protein, enzyme, and KCl. Recovery of protein was 80%, and recovery of enzyme was 94%.

these studies, an assay for the purification procedure was designed to contain 0.5 μM [1,2-^{3}H]dihydrotestosterone, 0.5 mM NADPH, 0.02 M Tris or 0.1 M potassium phosphate (pH 7.2), and 0.5 mg or less protein in a total volume of 0.5 ml, and incubation was for 30 min at 37°. The concentration of dihydrotestosterone utilized was chosen arbitrarily because it allowed complete solubilization of the substrate in assay mixtures that contained little protein.

In attempting to purify the enzyme it was noted that when the ammonium sulfate preparation was passed through a gel filtration column using either Sephadex G-75 (Figure 2) or Bio-Gel A 0.5m (Figure 3), the enzyme exhibited different elution characteristics depending on the buffer utilized. In 0.02 M Tris (pH 7.2) enzyme activity eluted before the major protein peak of the cytosol and had an apparent molecular weight of 55,000–60,000, whereas in 0.1 M potassium phosphate (pH 7.2) the enzyme activity was recovered after the elution of the major protein peak corresponding to proteins with molecular weights of 25,000–30,000. The nature of this apparent size change has not been elucidated. On DEAE-cellulose chromatography the enzyme was eluted with 0.06–0.08 M KCl (Figure 4). When gel filtration and DEAE chromatography were performed sequentially, an enzyme purification of approximately 100-fold was achieved with a recovery of about 40% of the starting activity (Table I). The partially purified enzyme in contrast to the crude cytosol was unstable under every condition tested. Furthermore, when the partially purified enzyme was subjected to disc gel electrophoresis (results not shown), the enzyme activity in the gel did not correspond to any of the stainable protein bands. Since 100-fold purification did not result in the isolation of a homogeneous enzyme protein, it was concluded that complete purification of the enzyme from this tissue might well require an order of magnitude more tissue than was obtained from the 80 animals used in the experiment of Table I. Since this was impractical, no further attempts were made to purify the enzyme, and attention was directed toward the characterization of the reaction utilizing the partially purified enzyme.

Table I: Partial Purification of 3α-Hydroxysteroid Oxidoreductase of Rat Ventral Prostate.

Step	Volume (ml)	Protein (mg)	Enzyme		
			EU × 10^{-5}	EU/mg of Protein	Purification
Cytosol	148	2116	3.15	149	1.0
Ammonium sulfate	50	1395	3.07	220	1.5
Agarose (Tris)	133	628	2.18	348	2.3
DEAE-cellulose	123	24.7	1.58	6,310	42
Agarose (KP_i)	285	8.0	1.31	16,400	110

The pH curve for 3-keto steroid reduction was relatively flat with little difference observed between pH 6.2 and 7.8. In the case of the oxidation reaction, however, the pH curve was steeper, and the apparent optimum rate of the reaction took place at a pH of approximately 8.8. These apparent optima were similar in the crude cytosol and in the partially

Table II: Kinetic Properties of Crude and Partially Purified 3α-Hydroxysteroid Oxidoreductase in Relation to Steroid and Coenzyme Substrates.

Source of Enzyme	Fixed Substrate	Variable Substrate	Apparent K_m for Variable Substrate (μM)
Purified	Dihydrotestosterone, 0.5 μM	NADH	660
	3α-Androstanediol, 0.5 μM	NADPH	2.2
		NAD^+	1000
		$NADP^+$	5.0
Crude	NADH, 10 mM	Dihydrotestosterone	1.4
	NADPH, 0.5 mM		0.6
Purified	NADH, 10 mM		2.0
	NADPH, 0.5 mM		0.55
Crude	NAD^+, 10 mM	3α-Androstanediol	5.2
	$NADP^+$, 0.5 mM		5.2
Purified	NAD^+, 10 mM		2.5
	$NADP^+$, 0.5 mM		3.2

[a] The assays were performed using 0.4 mg of crude enzyme protein or 0.02 mg of purified enzyme protein in a total volume of 0.4 ml incubated 30 min at 37°. Substrates were varied as follows: NADH, 10 μM to 10 mM; NADPH, 1 μM to 1 mM; NAD^+, 10 μM to 10 mM; $NADP^+$, 1 μM to 1 mM; [1,2-^{3}H]dihydrotestosterone, 0.05–10 μM; [1,2-^{3}H]-3α-androstanediol, 0.05–10 μM. At the end of the incubation, the steroids were extracted and analyzed as described in the text. The K_m values were determined from double reciprocal plots.

Table III: Comparison of Various 3α- and 3β-Hydroxysteroids as Substrates and Competitive Inhibitors for 3α-Hydroxysteroid Oxidation.[a]

Expt	Substrate or Inhibitor Steroids	Apparent K_m (μM)	Apparent K_i (μM)
1	Androsterone	4.84	4.46
	3α-Androstanediol	5.17	
	Etiocholanolone	13.2	17.7
2	3α-Androstanediol	5.6	
	3β-Androstanediol	*b*	*c*

[a] The rate of enzyme catalyzed 3-keto oxidation of various radioactive steroids was measured in a total volume of 0.4 ml using crude enzyme (0.2 mg of protein), 0.5 mM NADP, and substrate steroids over a range of 0.5–10 μM, and K_m values were determined from double reciprocal plots. To test the ability of steroids to inhibit the conversion of androstanediol to dihydrotestosterone crude enzyme (0.2 mg of protein) was incubated in a total volume of 0.4 ml with 0.5 mM NADP, 10 μM inhibitor steroid, and radioactive androstanediol that varied from 0.5 to 10 μM, and competitive inhibition constants were calculated from double reciprocal plots. [b] Reaction velocity too low to yield meaningful K_m values. [c] Inhibition too low to yield meaningful K_i values.

purified enzyme. For comparative purposes in subsequent studies, both reactions were measured at a pH that approximated a physiological range (0.1 M potassium phosphate (pH 7.4)).

At pH 7.4, the apparent K_m values for substrates and cofactors for both the oxidation and reduction reactions are summarized in Table II. For both reactions triphosphopyridine nucleotide is the preferred cofactor (apparent K_m's of 2.2 and 5 μM for NADPH and $NADP^+$ as compared to 0.66 and 1 mM for NADH and NAD^+). The apparent K_m's for dihydrotestosterone (0.6 to 2 μM) and 3α-androstanediol (2.5–5.2 μM) did not vary significantly when triphosphopyridine nucleotide was replaced with diphosphopyridine nucleotide at saturating concentration. Since the ratio of NADPH to NADP is high in most tissues, it is likely that under physiological conditions the formation of 3α-androstanediol is favored.

The substrate specificity of the enzyme was investigated in two ways. First the effects of 17 oxidation, 3α and 3β reduction, and 5β reduction on the oxidative reaction were characterized by measuring the conversion of androsterone, 3α-androstanediol, and etiocholanolone to androstanedione, dihydrotestosterone, and 5β-dihydrotestosterone (Table III). Androsterone and 3α-androstanediol had similar apparent K_m's (4.8 and 5.2 μM), and when androsterone was added as inhibitor to reaction mixtures containing radioactive 3α-androstanediol, the apparent K_i was also similar (4.5 μM). Thus, 17 oxidation has no apparent effect on the oxidation of the 3α-hydroxyl. In contrast, when androsterone and etiocholanolone were compared both the apparent K_m and K_i values for etiocholanolone were about three times that of androsterone, indicating that the 5α-reduced steroid was a more effective substrate than the 5β analog. Furthermore, when 3α- and 3β-androstanediol were compared it is clear that the 3β-androstanediol is weakly oxidized if at all by the enzyme. The latter finding is in keeping with the observation that 3β-androstanediol could not be detected following incubation with dihydrotestosterone.

The effects of Δ^4,3-keto steroids, 5β reduction, and various substitutions on the steroid molecule on the 3-keto reduction were then investigated by comparing the 3α reduction of various radioactive steroids and the ability of nonradioactive steroids to inhibit the conversion of radioactive dihydrotestosterone to 3α-androstanediol (Table IV). No Δ^4,3-keto steroid was converted to detectable amounts of the corresponding Δ^4,3α-hydroxy analog under the conditions of this assay. However, several Δ^4,3-keto steroids were potent competitive inhibitors of 3α-androstanediol formation, including C-19 steroids (testosterone, androstenedione, and epitestosterone), C-21 steroids (progesterone, deoxycorticosterone, and cortexolone), and one C-18 steroid (19-nortestosterone). Three C-21 steroids with substitutions in the 11 carbon were neither substrates nor inhibitors. In the 5α-reduced series, C-19 (androstanedione and epidihydrotestosterone) and C-21 steroids (allopregnanedione, 5α-dihydrodeoxycorticosterone, 5α-dihydrocortexolone, and 17-hydroxyallopregnanedione) were both effective substrates and potent inhibitors of the reaction. Again, 5α-reduced C-21 steroids with substitutions on the 11th carbon did not inhibit the conversion of dihydrotestosterone to 3α-androstanediol, implying that substitutions in this position block the capacity of steroids to serve as substrate or competitive inhibitors of the enzyme. The two 5β-reduced steroids examined, 5β-dihydrotestosterone and 5β-androstanedione, had apparent K_i's of 1.52 and 1.33 μM, values 2–3 times greater than those of the corresponding 5α analogs, indicating as in the case of the oxidation reactions that

Table IV: Comparison of Various 3-Keto Steroids as Substrates and Competitive Inhibitors for 3-Keto Steroid Reduction. [d]

Substrate or Inhibitor Steroids	K_m (μM)	K_i (μM)
A. Δ^4,3-Keto steroids		
Testosterone	*a*	0.93
Androstenedione	*a*	1.34
Epitestosterone	*a*	1.22
Progesterone	*a*	0.23
Deoxycorticosterone	*a*	0.22
Cortexolone	*a*	0.16
19-Nortestosterone	*a*	2.27
Corticosterone	*a*	*b*
Cortisol	*a*	*b*
Cortisone	*a*	*b*
B. 5α-Reduced steroids		
Dihydrotestosterone	0.60, 0.82	
Androstanedione	0.93	0.82
5α-Dihydrotestosterone hemisuccinate	*c*	0.44
Methyldihydrotestosterone	*c*	0.47
Epidihydrotestosterone	0.48	*c*
17-Hydroxyallopregnanedione	0.16	*c*
Allopregnanedione	0.27	0.33
5α-Dihydrodeoxycorticosterone	0.22	0.18
5α-Dihydrocortexolone	0.38	0.18
5α-Dihydrocorticosterone	*c*	*b*
5α-Dihydrocortisol	*c*	*b*
5α-Dihydrocortisone	*c*	*b*
C. 5β-Reduced steroids		
5β-Dihydrotestosterone	*c*	1.52
5β-Androstanedione	*c*	1.33

[a] Reaction velocities too low to yield meaningful K_m values. [b] Inhibition too low to yield meaningful K_i values. [c] Constants not measured. [d] The rate of enzyme-catalyzed 3-keto reduction of various radioactive steroids was measured in a total volume of 0.4 ml using crude enzyme (0.4 mg of protein), 0.5 mM NADPH, and substrate steroids over a range of 0.05–1.0 μM, and apparent K_m values were determined from double reciprocal plots. To test for competitive inhibition the rate of conversion of dihydrotestosterone to androstanediol was measured in a total volume of 0.4 ml using crude enzyme (0.4 mg of protein), 0.5 mM NADPH, 1 μM inhibitor steroid, and radioactive dihydrotestosterone that varied from 0.05 to 1.0 μM, and apparent competitive inhibition constants were calculated from double reciprocal plots.

5α-reduced steroids are better substrates than the 5β analogs.

Discussion

Attempts to obtain the 3α-hydroxysteroid oxidoreductase of rat ventral prostate in the completely pure state were unsuccessful, primarily because means have not been found to stabilize the enzyme during purification. Indeed, the 100-fold purification achieved here was no greater than has previously been reported for the enzyme of rat liver (Koide, 1963, 1965a).

The 3α-hydroxysteroid oxidoreductase of rat prostate cytosol shares many characteristics with the corresponding enzymes of liver and kidney (Tomkins, 1956; Koide, 1969; Mowszowicz and Bardin, 1974). All of these enzymes utilize NADPH and NADP preferentially as coenzymes, and they cannot utilize steroids that are not saturated in the A, B ring as substrates. In the present study no attempt was made to determine whether, as in liver, α,β-unsaturated steroids with electronegative substituents could serve as substrates for the formation of Δ^4-3-ol steroids (Ringold *et al.*, 1964). However, as in liver, Δ^4-3-keto steroids were shown to be potent competitive inhibitors of the enzyme (Koide, 1969).

The prostate enzyme does have distinctive characteristics, however. First, the enzyme of prostate cytosol appears to be independent of endocrinological control (Shimazaki *et al.*, 1973). Second, 5β-reduced steroids are less effective substrates than the corresponding 5α-reduced analogs, whereas the opposite is true in liver (Tomkins, 1956; Koide, 1969). Third, there is a unique substrate specificity for the enzyme in that steroids with ketone or hydroxyl groups on the 11th carbon can neither serve as substrates nor competitive inhibitors of the enzyme. It is interesting that another enzyme of sex hormone metabolism in this tissue, the 5α-reductase, exhibits almost identical substrate specificity (Frederiksen and Wilson, 1971). As a result of this substrate specificity, androgen metabolism in the prostate is independent of competitive inhibition by glucocorticoids, which are present in approximately 40-fold the concentration of circulating testosterone in the adult male.

The preferential reduction of 5α-reduced steroids by the enzyme suggests that in prostate 5α reduction must precede 3-keto reduction. However, in homogenates the maximal rate of the latter reaction is about ten times as rapid as the former, suggesting that any dihydrotestosterone formed would be rapidly converted to 3α-hydroxy compounds. Since dihydrotestosterone accumulation in the gland does take place *in vivo*, it is likely that the rate of 3α reduction in the cell cannot proceed at its maximal rate. Several possible control mechanisms deserve consideration. First, the reaction or its equilibrium in the steady state may be regulated by the relative amounts of pyridine nucleotides and by their redox state. Second, the fact that the enzyme is low or absent in the nucleus, the major site for the 5α-reductase of the tissues, may allow for compartmental accumulation of dihydrotestosterone (Wilson and Gloyna, 1970). Third, it is conceivable that in the cell, dihydrotestosterone-binding proteins protect the molecule from 3-keto reduction. Fourth, other 3-keto steroid oxidoreductase enzymes such as the enzyme in microsomal membranes may have kinetic characteristics that differ from those of the enzyme in cytosol (Levy *et al.*, 1974). Finally, the enzyme may be under regulation by some effector molecule. Possible candidates for such a role are Δ^4,3-keto steroids which might actually serve to regulate the rate of 3-keto reduction in the intact cell.

Acknowledgments

We are indebted to Dr. Michael R. Waterman who performed the disc gel electrophoresis. Mary Beth Neal provided able technical assistance.

References

Baron, D. N., Gore, M. B. R., Pietruszko, R., and Williams, D. C. (1963), *Biochem J. 88,* 19.

Björkhem, I., Danielsson, H., and Wikvall, K. (1973), *Eur. J. Biochem. 36,* 8.

Bruchovsky, N. (1971), *Endocrinology 89,* 1212.

Doman, E., and Koide, S. S. (1966), *Biochim. Biophys. Acta 128,* 209.
Frederiksen, D. W., and Wilson, J. D. (1971), *J. Biol. Chem. 246,* 2584.
Glock, G. E., and McLean, P. (1955), *Biochem. J. 61,* 388.
Gustafsson, J.-Å., Lisboa, B. P., and Sjövall, J. (1968), *Eur. J. Biochem. 6,* 317.
Hurlock, B., and Talalay, P. (1958), *J. Biol. Chem. 233,* 886.
Koide, S. S. (1963), *Arch. Biochem. Biophys. 101,* 278.
Koide, S. S. (1964), *Steroids 3,* 85.
Koide, S. S. (1965a), *Biochim. Biophys. Acta 110,* 189.
Koide, S. S. (1965b), *Steroids 6,* 123.
Koide, S. S. (1969), *Methods Enzymol. 15,* 651.
Koide, S., Chen, C., and Freeman, S. (1962), *Biochim. Biophys. Acta 63,* 186.
Levy, C., Marchut, M., Baulieu, E.-E., and Robel, P. (1974), *Steroids 23,* 291.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Lubicz-Nowrocki, C. M. (1973), *J. Endocrinol. 58,* 193.
Marcus, P. I., and Talalay, P. (1956), *J. Biol. Chem. 218,* 661.
Martin, C. W., and Nicholas, H. J. (1973), *Steroids 21,* 633.
Moore, R. J., and Wilson, J. D. (1972), *J. Biol. Chem. 247,* 958.
Moore, R. J., and Wilson, J. D. (1973), *Endocrinology 93,* 581.
Moore, R. J., and Wilson, J. D. (1974), *Biochemistry 13,* 450.
Mowszowicz, I., and Bardin, C. W. (1974), *Steroids 23,* 793.
Ringold, H. J., Graves, J. M. H., Clark, A., and Bellas, T. (1967), *Rec. Progr. Hormone Res. 23,* 349.
Ringold, H. J., Ramachandran, S., and Forchielli, E. (1964), *Biochim. Biophys. Acta 82,* 143.
Rommerts, F. F. G., and van der Molen, H. J. (1971), *Biochim. Biophys. Acta 248,* 489.
Shimazaki, J., Kato, N., Nagai, H., Yamanaka, H., and Shida, K. (1972), *Endocrinol. Jpn. 19,* 97.
Sperry, W. M., and Webb, M. (1950), *J. Biol. Chem. 187,* 97.
Springer, C., and Eckstein, B. (1971), *J. Endocrinol. 50,* 431.
Talalay, P., and Marcus, P. I. (1954), *Nature (London) 173,* 1189.
Talalay, P., and Marcus, P. I. (1956), *J. Biol. Chem. 218,* 675.
Tomkins, G. M. (1956), *J. Biol. Chem. 218,* 437.
Unhjem, O. (1970), *Acta Endocrinol. (Copenhagen) 65,* 525.
Wilson, J. D., and Gloyna, R. E. (1970), *Rec. Progr. Hormone Res. 26,* 309.

Stepwise Enzymatic Oligoribonucleotide Synthesis Including Modified Nucleotides†

Graham C. Walker‡ and Olke C. Uhlenbeck*,§

ABSTRACT: A method has been developed for the routine synthesis of 2′(3′)-*O*-monoacyl ribonucleoside 5′-diphosphates for stepwise synthesis of oligoribonucleotides with *Escherichia coli* polynucleotide phosphorylase. The use of triethyl orthoisovalerate allows the facile preparation of 2′(3′)-*O*-isovaleryl-UDP, -CDP, -ADP, -GDP, -IDP, -ϵ-ADP, -ϵ-CDP, and N^6-isopentenyl-ADP. The synthesis of N^6-isopentenyl-ADP from ADP by N^1-alkylation and the Dimroth rearrangement to N^6 is reported. The effects of several factors including the nature of the divalent cation, pH, salt concentration, and time on the efficiency of the polynucleotide phosphorylase catalyzed single additions of the 2′(3′)-*O*-isovaleryl ribonucleoside 5′-diphosphates to an oligoribonucleotide primer are reported. The syntheses of many tetranucleoside triphosphates and two pentanucleoside tetraphosphates in yields of 20–75% are reported. The 2′(3′)-*O*-isovaleryl derivatives of IDP, ϵ-ADP, ϵ-CDP, and N^6-isopentenyl-ADP were all accepted by polynucleotide phosphorylase as substrates for the monoaddition reaction. The extension of the method to include the syntheses of oligoribonucleotides containing modified nucleosides offers a means of studying the roles of these modifications by the use of relatively simple model compounds.

Oligoribonucleotides of defined sequence are useful model compounds for the study of the structure and function of RNA. Chemical synthesis of ribo oligomers has not developed as rapidly as their deoxyribo counterparts primarily due to the difficulty in ensuring the correct 3′ → 5′ internucleotide linkage. Although sequences as long as nine residues have been achieved chemically (Ohtsuka *et al.*, 1973), the syntheses are involved and time consuming. An alternate approach to oligoribonucleotide synthesis employs primer dependent polynucleotide phosphorylase from *Micrococcus luteus* (Thach and Doty, 1965). Although the enzymatic techniques are rapid and assure the correct inter-

† From the Departments of Chemistry and Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received April 15, 1974.* This work formed part of the Ph.D. Thesis of G.C.W., University of Illinois, 1974. This work was supported by grants from the National Institutes of Health, GM-05829 (to N. J. Leonard) and GM 19059 (to O.C.U.).

‡ Recipient of a University of Illinois Fellowship, 1972–1973.
§ Recipient of a Career Development Award (GM 70,498) from the National Institutes of Health.