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Enzymatic Transformation of Morphine by Hydroxysteroid Dehydrogenase from *Pseudomonas testosteroni*

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Enzyme preparations from *Pseudomonas testosteroni* containing α- and β-hydroxysteroid dehydrogenases catalyzed the oxidation of morphine and codeine by nicotinamide adenine dinucleotide. Morphine was converted in relatively low yield into 14-hydroxymorphinone probably via morphinone as an intermediate. Codeine was converted to codeinone and 14-hydroxycodeinone. Only the conversions at the 6-position were carried out by the hydroxysteroid dehydrogenase. Hydroxylation at the 14-position did occur spontaneously (or enzymatically with a contaminating enzyme) after oxidation at the 6-position.

We have isolated organisms that act on morphine. The organisms belong to the genera *Arthrobacter* (7) and *Pseudomonas*, and species identification shows them to be similar to the bacteria involved in steroid transformation. We therefore wondered whether the steroid-transforming organisms would also act on morphine and its derivatives, and we used for this purpose the hydroxysteroid dehydrogenase (1.1.1.50 and 1.1.1.51) preparations from *Pseudomonas testosteroni*. Detailed analyses and characterization of this enzyme are described in the literature (1, 3, 8, 10-15).

MATERIALS AND METHODS

Determination of enzyme activity. Steroid dehydrogenase activity was measured by the spectrophotometric assay of Marcus and Talalay (8, 13, 15), using a DB or a Gilford spectrophotometer (Gilford 410 digital absorbance, model 222A). The activity of the α- and β-hydroxysteroid dehydrogenases was obtained by using androsterone and testosterone, respectively, as substrates. Standard solutions containing 250 μg of steroid per ml dissolved in reagent-grade methanol were prepared. Alternatively, alkaloid drugs (morphine or codeine) at 100- to 250-μg/ml concentration were used as substrates. The reaction mixture consisted of 0.1 ml of a solution of nicotinamide adenine dinucleotide (NAD^+) (30 mM) and 0.01 to 0.1 ml of the appropiate enzyme dilution. The volume was made up to 3.0 ml by the addition of 20 mM sodium pyrophosphate buffer, pH 8.95. Unless otherwise indicated, the reaction was initiated by the addition of enzyme. Measurements of the optical density were made against a blank cell containing all components except the appropriate steroid or alkaloid. One unit of either α or β enzyme activity was defined as a change in optical density at 340 nm of 0.001 per min in a 1-cm cell at 24 C and pH 8.95. The initial reaction rates are given in the kinetic experiments. Data on the enzyme activity will be in terms of the β enzyme only (using testosterone as the reference substrate).

Alternatively, the action of hydroxysteroid dehydrogenase on alkaloid drugs was followed by studying the decrease of *N*-[*methyl*-^{14}C]morphine. A typical reaction mixture contained 1 mM NAD^+, 100 to 250 μg of the alkaloid substrate per ml, 0.6 to 250 U of enzyme per ml, 0.01 to 0.1 μCi of *N*-[*methyl*-^{14}C]morphine, and 20 mM pyrophosphate buffer, pH 8.95, to make a total volume of 3 ml. The mixture was placed in a 50-ml Erlenmeyer flask and allowed to shake at 200 rpm on a gyratory shaker, model G2 (New Brunswick Scientific Co., Inc., New Brunswick, N.J.), at room temperature. Unless otherwise indicated the reaction was initiated by the addition of the enzyme.

Separation and purification of the enzymes. The hydroxysteroid dehydrogenase enzyme of *P. testosteroni* is an intracellular enzyme. Consequently, the enzyme had to be released by sonication when the grade I enzyme (crude dried cells) was used. A preparation of the grade I enzyme suspended in pyrophosphate buffer (20 mM, pH 8.95) was sonicated for 3 min at approximately 80 W in a Sonifier cell disrupter, model W185D (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.). The beaker containing the enzyme solution was surrounded by ice at all times. The preparation was then centrifuged for 20 min at 15,000 rpm at 4 C in a Sorvall Superspeed RC2-B. The enzyme activity of the supernatant was determined.

To purify and separate the α- and β-hydroxysteroid dehydrogenases, the method described by Marcus and Talalay was followed (8): 25 mg of commercial hydroxysteroid dehydrogenase grade II (containing approximately 50% activity each of the α and β enzymes) was dissolved at 1-mg/ml concentration in phosphate buffer, 30 mM, pH 7.2, containing ethylenediaminetetraacetic acid (EDTA) (0.15%) and

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NAD^+ (0.1 mM), and ammonium sulfate was added stepwise. The success of the purification depends upon the careful elimination of heavy metals through the use of deionized distilled water, washing all glassware with 0.2% solutions of EDTA, and using purified ammonium sulfate recrystallized from a saturated 0.2% EDTA solution. The ammonium sulfate precipitates were resuspended in 5 ml of the same buffer and dialyzed overnight. The β enzyme is precipitated preferably between 35 and 40% ammonium sulfate saturation, and the α enzyme between 55 and 60%. The final preparation of α or β enzyme used had no more than 4 to 8% of contamination of the other enzyme. Protein was determined by the method of Warburg and Christian (16).

Enzymes and reagents. Commercially available grades I, II, and III of NAD^+-dependent hydroxysteroid dehydrogenase preparations from *P. testosteroni* were obtained from Sigma Chemical Co. (St. Louis, Mo.), as were U.S.P.-grade testosterone, androsterone, NAD^+, grade III, and NAD phosphate. Reduced NAD (NADH) was obtained from P-L Biochemicals, Inc. (Milwaukee, Wisc.), and flavin adenine dinucleotide, B grade, was obtained from Calbiochem (Los Angeles, Calif.). All the above-mentioned cofactors were prepared by using pyrophosphate buffer (NaH_2PO_4-$Na_4P_2O_7$), 20 mM, pH 8.95, unless otherwise indicated. Reduced glutathione was purchased from Nutritional Biochemical Corp. (Cleveland, Ohio). The source of the alkaloid drugs and *N*-[*methyl*-^{14}C]morphine has been already described (6, 7).

Determination of products. Extraction of the alkaloids and their determination by gas or thin-layer chromatography (TLC), or by scintillation counting of labeled drugs, has been described elsewhere (6, 7).

RESULTS

Action of hydroxysteroid dehydrogenase on morphine. When morphine was incubated in the presence of hydroxysteroid dehydrogenase grade II for 3 to 6 h in the conditions indicated above, a decrease in the amount of morphine occurred. When the extracts of the incubation mixture were chromatographed in thin layer, using the solvent system ethyl acetate-methanol-ammonium hydroxide (86:10:4), besides morphine (R_f = 0.22), two new substances with R_f = 0.63 (substance S_1) and 0.28 (substance S_2) appeared. When *N*-[*methyl*-^{14}C]morphine was added to the reaction mixture, all of the spots visualized in the I_2 vapor were radioactive. A substance running immediately behind the solvent front did not contain radioactivity and was found to be due to the enzyme preparation. The relative amount of the two products formed (substances S_1 and S_2) varied according to the grade of enzyme used and conditions of assay. Usually the amount of substance S_1 was between two and three times higher than the amount of substance S_2. Up to 20% of the morphine added was transformed in 4 h. Various additions to and changes in the reaction mixture were attempted in order to optimize the formation of transformation products. Neither NAD phosphate, NADH, nor flavin adenine dinucleotide could replace NAD^+ as the cofactor for the enzyme. The enzyme was very labile in the presence of divalent metal ions (Fe^{2+}, Cu^{2+}, or Zn^{2+}) at 1 mM concentration. As a result of this finding, EDTA was used for the remainder of the study as chelating agent. Talalay and Marcus (14) reported the necessity of free sulfhydryl groups for enzyme activity. However, the addition to the system of sulfhydryl reducing agents as cysteine or glutathione failed to enhance enzyme activity. Substituting tris(hydroxymethyl)aminomethane buffer for pyrophosphate buffer also inhibited the transformation of morphine.

Although determinations of nicotinamide-linked dehydrogenases are readily done by measuring the optical density at 340 nm, such spectrophotometric assays on extracts of crude cells of steroid dehydrogenase grade I showed very little activity. Kersters and DeLey (4) have pointed out that assays with NAD^+ as cofactor cannot be carried out with crude extracts due to the usual presence of high NADH oxidase activity. The grade I enzyme showed 4 to 10 times more NADH oxidase activity than did grade III. Grades II and III of the enzyme contained only small amounts of the NADH oxidase activity that did not interfere with the spectrophotometric assay and were used in the rest of the study.

The influence of various parameters on the activity of hydroxysteroid dehydrogenase on morphine was investigated. Controls lacking the morphine substrate or lacking enzyme failed to show any reduction of the NAD^+. The effect of temperature on the hydroxysteroid dehydrogenase activity showed a plateau effect, with an optimal temperature range of between 27 and 40 C (Fig. 1A). The effect of increasing enzyme concentration with respect to activity showed a linear relationship, with a leveling off of activity at approximately 1,000 U/ml (Fig. 1B). The enzyme activity was markedly sensitive to changes in pH, as already pointed out for other reactions catalyzed by NAD^+-requiring enzymes (9). Increasing the pH of the solution favored higher transforming activity on morphine, with maximum activity at pH 9.0 (Fig. 1C).

After the addition of increasing amounts of NAD^+, one would expect to find a point where the enzyme activity levels off. Contrary to this assumption, experimental data showed an inhibitory effect at higher concentrations of

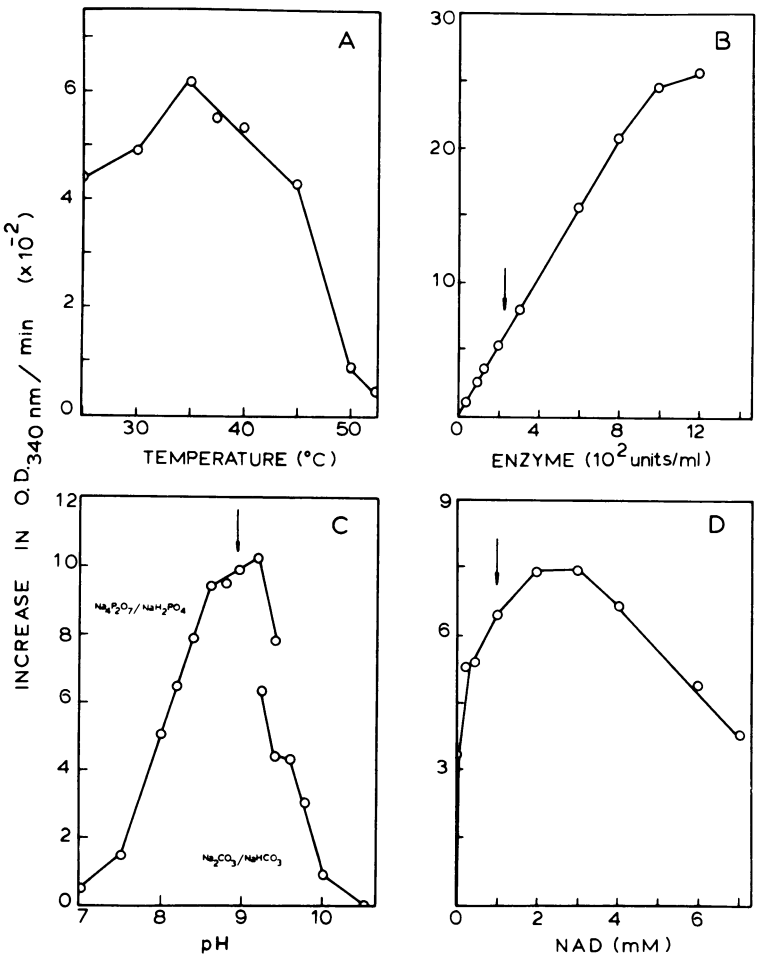

FIG. 1. *Effect of temperature (A), enzyme concentration (B), pH (C), and NAD⁺ concentration (D) on the hydroxysteroid dehydrogenase activity with respect to morphine. The velocity of the reaction was measured spectrophotometrically, using 80 μg of morphine per ml as substrate.*

NAD^+ (Fig. 1D). The grade III NAD^+ (obtained from Sigma Chemical Co.) contains a minor amount of an impurity that had been reported (2, 17) to be a competitive inhibitor in certain enzyme systems. The 1 mM concentration of NAD^+ used in these studies was well below the critical concentration of 4 mM required for the inhibitory effect. Based on the above findings, a typical reaction mixture consisted of 1 mM NAD^+, 250 U of enzyme per ml, 100 to 250 μg of morphine per ml, and pyrophosphate buffer, 20 mM, pH 8.95, up to 3 ml.

The Michaelis constants of morphine and codeine were calculated by using a Lineweaver-Burk plot (Fig. 2). The K_m of codeine was calculated as 1.92×10^{-3} M. The calculation of the K_m value for morphine was complicated by the low solubility of this drug (50 mg/ml in methanol), but an approximate value of 5.7×10^{-3} M was found by extrapolation using two different Lineweaver-Burk plots for two enzyme concentrations (Fig. 2). The K_m figures obtained can be compared with the K_m of β-hydroxysteroid dehydrogenase for testosterone, 5.5×10^{-5} M (15), and that of α-hydroxysteroid dehydrogenase for androsterone, 1.5×10^{-3} M (12). The enzyme had a higher K_m values for alkaloids than for steroids.

Reaction mixtures containing morphine and the hydroxysteroid dehydrogenase enzyme showed a measurable decrease in morphine within 4 h. There also occurred a simultaneous increase in the amount of transformation products detected, namely, substances S_1 and S_2. A comparison between the amount of morphine present and the amount of reduced NAD^+ over

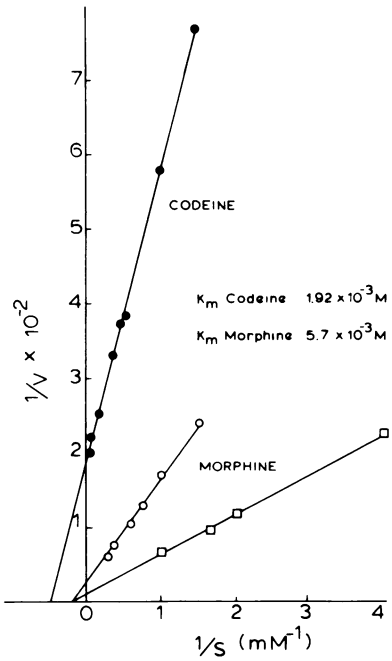

FIG. 2. *Lineweaver-Burk plot of the effect of substrate concentration on velocity of enzyme reaction. The velocity of the reaction was measured spectrophotometrically, using 225 (○, ●) or 80 U (□) of hydroxysteroid dehydrogenase grade II.*

a period of time showed the two substances to be inversely proportional to each other (Fig. 3). As the amount of morphine decreased with time, the absorption readings at 340 nm increased. No changes were noted in the control group lacking the enzyme. These data link the NAD^+-dependent hydroxysteroid dehydrogenase with the decrease in the amount of morphine and the production of transformation products.

Products formed by hydroxysteroid dehydrogenase acting on morphine and codeine. The action of hydroxysteroid dehydrogenase on morphine has been described as producing two major transformation products, one slow and one fast running. Similar results were obtained when codeine, the C-3 methyl ether of morphine, was used in place of morphine in the reaction mixture. Once again, two major spots were visualized in the I_2 vapor.

The identification of the morphine transformation products was attempted by comparing R_f and R_t values of the unknown products with known standards, using TLC and gas chromatography, respectively (6). Spot S_1 was identified as 14-hydroxymorphinone. It was postulated that spot S_2 could be either morphinone or 14-hydroxymorphine. Although morphinone was not available as a standard, the 14-hydroxymorphine standard failed to match with spot S_2 (in TLC) in the several solvent systems used, and in an experiment in which 14-hydroxymorphine was substituted for morphine in the reaction mixture, the enzyme was unable to act on this substrate and no transformation or degradation occurred. We therefore tentatively concluded that spot S_2 is morphinone.

Since the enzyme acts on codeine, it was hoped that the identification of the codeine transformation products might shed light on the reaction involving morphine. By again comparing R_f and R_t values of the unknown products with known standards, the fast-running spot was identified as 14-hydroxycodeinone and the slow-running spot as codeinone.

The fact that codeine was transformed to codeinone by the enzyme, together with the enzyme's inability to act on 14-hydroxymorphine, suggested that morphine, in being transformed to 14-hydroxymorphinone, was first changed to morphinone and that spot S_2 was indeed morphinone.

The transformation of morphine by the hydroxysteroid dehydrogenase enzyme into two products, one involving dehydrogenation and the other involving hydroxylation, seemed to contradict the notion of high specificity in the enzyme system. It appears that the hydroxysteroid dehydrogenase first acted upon the mor-

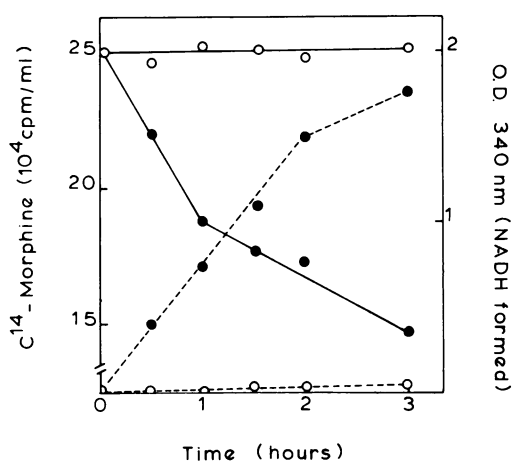

FIG. 3. *Comparation between the amount of morphine remaining and the amount of NAD^+ reduced over a period of time. Flasks contained in a 3-ml volume: 250 U of enzyme grade II; 250 μg (0.1 μCi) of morphine per ml; 1 mM NAD^+; and 20 mM pyrophosphate buffer, pH 8.95. [^{14}C]morphine, ——; NADH formed, – – –; control, ○; hydroxysteroid dehydrogenase, ●.*

phine to form morphinone (Fig. 4). The subsequent transformation of the morphinone to 14-hydroxymorphinone could be due either to the action of a contaminating enzyme or to normal chemical oxidation of morphinone in the presence of air and high pH, since chemically the conjugated double-bond system found in morphinone made the C-14 position even more susceptible to attack.

Purification of the α and β forms of hydroxysteroid dehydrogenase and their action on morphine. The α- and β-hydroxysteroid dehydrogenases were separated and purified by ammonium sulfate fractionation according to the method described by Marcus and Talalay (8). Each collected fraction was then assayed for α and β activity using androsterone and testosterone, respectively, as substrates.

Reaction mixtures containing either the α- or β-hydroxysteroid dehydrogenase were assayed by using morphine as substrate in the spectrophotometer. Only the β enzyme produced increased optical absorbance values at 340 nm, indicating that the β and not the α enzyme was responsible for the morphine transformation (Fig. 5). Similar experiments showed decreases

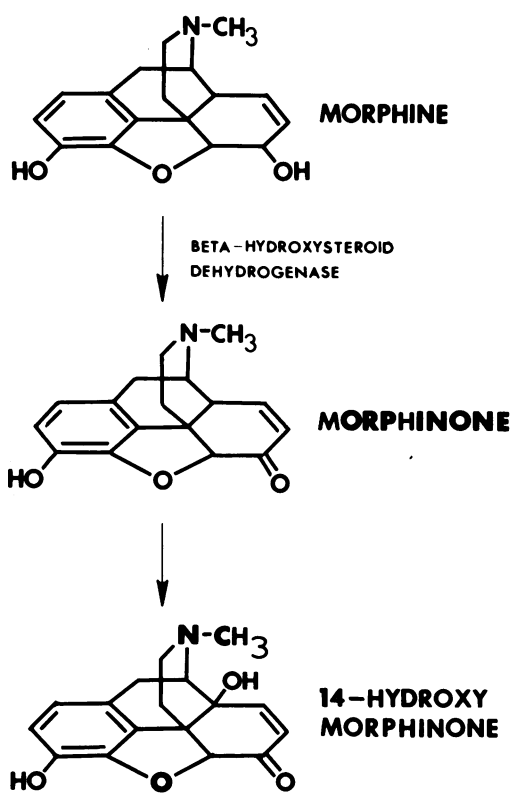

FIG. 4. *Hypothesized sequence of the transformation of morphine by hydroxysteroid dehydrogenase.*

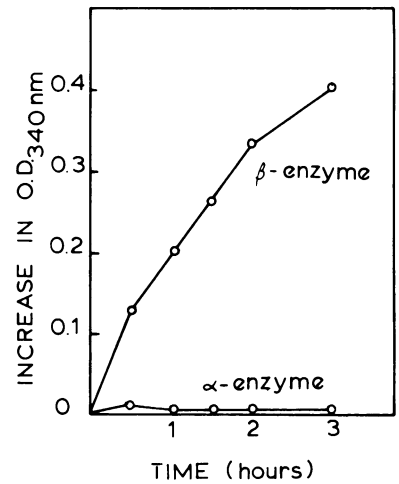

FIG. 5. *Reduction of NAD⁺ by purified α and β enzyme fractions of hydroxysteroid dehydrogenase, in the presence of morphine. Sixty units of α or β purified enzyme was used. Other conditions as in Fig. 3.*

in the amount of morphine and increases in the amount of transformation products when the β enzyme was incubated for 4 h with the drug, but no changes occurred in the morphine in incubations with the α enzyme.

Further evidence that the β enzyme was involved in morphine transformation was provided by studies of competition. The possible influence of androsterone or testosterone on the action of hydroxysteroid dehydrogenase on morphine was examined in an effort to evaluate whether the α and/or β enzymes were responsible for the transformation products of morphine. Since the C_6-hydroxyl group of morphine is in the α position, one might expect the androsterone to act as a competitive inhibitor with the morphine. The androsterone, despite its very much higher affinity for the α enzyme, had no inhibitory effect on the transformation of morphine by the crude enzyme (containing approximately 50% α and 50% β activity). Testosterone (which is the substrate for the β enzyme) was highly inhibitory to the morphine-transforming reaction (Fig. 6). The addition of as little as 0.25 μg of testosterone per ml in the presence of 250 μg of morphine per ml significantly inhibited the action of the enzyme on morphine. In the presence of 5 μg or more of testosterone per ml, no transformation products could be visualized in the I_2 vapor, and only an insignificant amount of radioactivity was detected in the appropriate regions of the TLC plate. At concentrations of less than 0.1 μg of testosterone per ml, morphine transformation was not inhibited. This inhibition of the mor-

phine dehydrogenation by testosterone could not be due to competition for NAD^+ of two different dehydrogenases in the preparation. In that case, androsterone would produce a similar inhibition. Thus it appears that the β enzyme was responsible for the transformation of morphine.

DISCUSSION

This study has demonstrated the ability of the hydroxysteroid dehydrogenase enzyme from *P. testosteroni* to transform morphine. Conditions favoring morphine transformation included a high pH of 8.95 and a temperature range of 27 to 40 C (Fig. 1). The K_m values of morphine and codeine were calculated to be 1.9 and 5.7×10^{-3} M, respectively, well above the K_m for androsterone or testosterone (12, 15).

Morphine is transformed by the enzyme to 14-hydroxymorphine, probably via a morphinone intermediate. The production of morphinone from morphine has been attributed to the hydroxysteroid dehydrogenase enzyme, whereas the subsequent transformation to 14-hydroxymorphinone is the result of either a contaminating enzyme present in the original hydroxysteroid dehydrogenase extract or the result of chemical transformation. The oxygen of the 14-hydroxyl group of the 14-hydroxymorphinone appears to originate from molecular oxygen, since in the absence of air this substance was not found (unpublished data).

The α-hydroxysteroid dehydrogenase was found incapable of reacting with the morphine, and activity was dependent upon the β enzyme (Fig. 5). β-Hydroxysteroid dehydrogenase oxidizes three β-hydroxysteroids of the C_{19} and C_{21} series, 17 β-hydroxysteroids of the C_{18}, C_{19}, and C_{21} series, and certain 16 β-hydroxysteroids (13, 15). Therefore, it is less specific in its action than the α enzyme, which is only able to oxidize the three α-hydroxysteroids of the C_{19}, C_{21}, and C_{24} series (15). However, since β-hydroxysteroid dehydrogenase has been found to act only on the β-hydroxyl groups, it appears that the β-hydroxysteroid dehydrogenase enzyme must attack the morphine molecule from the "back" side, so that the C_6 hydroxyl group is now in the β position. This would explain the lack of action of the α enzyme on 14-hydroxymorphine, in which the 14-hydroxyl group may not allow the binding of the enzyme. Addition of testosterone to the reaction mixture, in quantities as small as 1:1,000 (testosterone to morphine), was capable of inhibiting the transformation of morphine by the enzyme. On the other hand, the addition of androsterone to preparations containing both α- and β-hydroxysteroid dehydrogenases was unable to cause a similar inhibitory effect (Fig. 6).

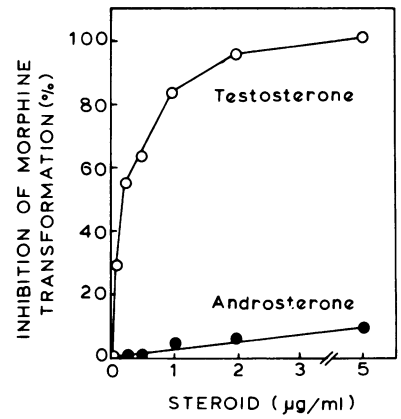

FIG. 6. *Inhibition of the transformation of morphine by hydroxysteroid dehydrogenase, by androsterone* (●) *and testosterone* (○). *Conditions as in Fig. 3.*

Thus, it appears that the highly specific steroid transforming enzyme β-hydroxysteroid dehydrogenase is able to act on an alkaloid. Still, the preparation of highly purified β-hydroxysteroid dehydrogenase is necessary to study its action on morphine. The transformation of the morphine alkaloids by the steroid enzyme system opens a new area in which to direct the search for new and better morphine-related compounds. The results of this study suggest that in addition to searching for new drug-transforming enzymes, one might also utilize previously isolated and documented enzymes. In the quest for a better analgesic, the use of established steroid enzymes seems to be a reasonable approach.

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