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Catalysis of the Isomerization of Δ^5-3-Ketosteroids to Δ^4-3-Ketosteroids by Primary Amines: Evidence for an Imine Intermediate[1]

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The isomerization of 5-androstene-3,17-dione and 17β-hydroxy-5-androstene-3-one to 4-androstene-3,17-dione and 17β-hydroxy-4-androstene-3-one, respectively, is catalyzed by primary amines. In the case of the isomerization catalyzed by glycylglycine the reaction proceeds through an intermediate which absorbs maximally at 275 nm. Based on spectral similarities to appropriate model compounds and structural analysis of the intermediate after its reduction by sodium borohydride, the intermediate has been tentatively identified as the Δ^4-3-imine.

Isomerases which catalyze the conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids are found in several mammalian steroidogenic tissues (*1*) and in extracts of the bacterium *Pseudomonas testosteroni*. The former have been difficult to study at the molecular level since they are membrane associated. The bacterial enzyme, however, has been extensively studied both structurally and mechanistically, since it is a small, water-soluble protein (*1*). Mechanistic interest in the bacterial enzyme has been stimulated by the fact that it has a very high turnover number—16×10^6.

Model systems which catalyze the isomerase reaction have been investigated. Catalysis by protons has been studied by Ringold's group (*2*). Jones and Wigfield (*3*) reported that hydroxide ion as well as phenols and heterocyclic nitrogen bases would also catalyze the isomerization. Jones and Wigfield further suggested that the Pseudomonas enzyme catalyzed the reaction by employing a tyrosine residue as a general acid to protonate the 3-keto oxygen and a histidine residue as a general base to abstract the C-4 proton to produce a $\Delta^{3,5}$-dienc-3-ol intermediate from the substrate. Reprotonation on C-6 and deprotonation of the 3-OH by these catalytic groups would yield the Δ^4-3-ketosteroid product. However, no direct evidence for the presence of these potentially catalytic functional groups in the catalytic site of any isomerase has yet been reported.

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We wish to report experiments suggesting that primary amines are capable of catalyzing the isomerase reaction via a mechanism involving imine intermediates. Since studies of the mechanisms of various isomerases will be based, in part, on precedents from organic chemistry, we wish to call attention to this previously unrecognized mechanistic possibility for isomerases.

MATERIALS AND METHODS

Glycylglycine, glycylglycine ethyl ester hydrochloride, glycylglycyl-glycine, L-alanine, and L-lysine were obtained from Mann Research Laboratories. Glycine and morpholine were purchased from Eastman Organic Chemicals. D-glucosamine hydrochloride and *N*-acetylglycosamine were products of Pfanstiehl Laboratories, Inc. Nitrilo-triethanol and Trishydroxymethylamino methane were products of Sigma Chemical Co.

3β-hydroxy-5-androstene-17-one was purchased from Mann Research Laboratories. 17β-hydroxy-4-androstene-3-one was obtained from Steraloids, Inc. 4-[^{14}C]-17β-hydroxy-4-androstene-3-one (50 mCi/mmole) was purchased from Amersham Searle.

5-androstene-3,17-dione was synthesized from 3β-hydroxy-5-androstene-17-one by oxidation with CrO_3 in acetone (*4*). After recrystallization from acetone the 5-androstene-3,17-dione had a melting point of 137–142°C.

17β-hydroxy-5-androstene-3-one (labeled and unlabeled with carbon 14) was synthesized from 17β-hydroxy-4-androstene-3-one using the method of Jones and Wigfield (*5*).

Δ^5-3-ketosteroid isomerase was purified from *P. testosteroni* by the method of Jarabak et al. (*6*).

Steroid Isomerization

Isomerization reaction mixtures typically were composed of the amine, the Δ^5-3-ketosteroid, and methanol or ethanol as a cosolvent with water. Reactions were initiated by addition of a freshly prepared solution of Δ^5-3-ketosteroid in methanol or ethanol to a solution of amine in water (adjusted to the appropriate pH with HCl or NaOH). All reactions were conducted at 30°C. In preliminary experiments, the rate of isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids was measured by following the increase in absorbance of reaction mixture at 248 nm, the wavelength of maximum absorption of the product. However, subsequent investigation of the spectral changes which occurred during the reaction showed that an ultraviolet absorbing intermediate appeared during the reaction (see Results) which absorbed maximally at 275 nm as well as possessing considerable absorbance at 248 nm. Thus, the absorbance changes at 248 nm reflected both intermediate and Δ^4-3-ketosteroid concentration changes.

The kinetics of the formation of the 275 nm-absorbing intermediate could be conveniently following by measuring the absorbance changes of reactions at the intermediate's absorption maximum, 275 nm, or with less interference from the final product, at 285 nm, where the final product possesses a very low molar extinction coefficient.

Trapping of Intermediate by Reduction with Sodium Borohydride

In order to convert the spectrophotometric intermediate to a chemically stable form suitable for isolation and characterization, reaction mixtures were treated at appropriate times with sodium borohydride. The reduced mixtures were qualitatively analyzed by thin layer chromatography on Silica Gel G plates using the solvent system *n*-butanol-glacial acetic acid-water (4:1:2 v/v/v). Steroidal compounds were located using methanol-sulfuric acid (*7*). Ninhydrin positive substances were visualized after spraying with 0.1 % ninhydrin in acetone and warming at 60–70°C for 30 min. Compounds containing N–H groups were identified by exposing the plates to chlorine gas generated by mixing equal volumes of saturated potassium permanganate and 3 N HCl. After allowing excess chlorine to dissipate from the plate for 10–20 min, it was then sprayed with aqueous 1 % soluble starch–1 % potassium iodide. The N–H containing substances appeared immediately as dark blue spots on a lighter blue background (*8*).

In order to obtain relatively large amounts (5–15 mg) of the borohydride-reduced intermediate and in order to eliminate complications arising from imine formation involving the 17-keto group of 5-androstene-3,17-dione, isomerization of 17β-hydroxy-5-androstene-3-one to 17β-hydroxy-4-androstene-3-one was conducted rather than the isomerization of 5-androstene-3,17-dione. The preparative reactions were carried out in 0.5 *M* glycylglycine, pH 8.0, containing 33 % ethanol and at a steroid concentration of 2 m*M*. When the concentration of intermediate had reached a maximal value (ca. 3 min), sodium borohydride was added to a final concentration of 10 m*M* borohydride. Addition of the borohydride resulted in immediate disappearance of the 275 nm-absorbing species. After 1 hr the pH of the reaction mixture was adjusted to 2.0 with concd HCl and the solution was extracted 3 times with 1 volume portions of redistilled ethyl acetate. Two different procedures for purification of the reduced intermediate from the ethyl acetate extract were followed. Method A was used when ^{14}C-labeled steroid was the substrate. Method B was used when nonradioactive steroid was employed as substrate.

Method A. The combined ethyl acetate extracts were evaporated *in vacuo* to a white solid residue using a rotary evaporator. This residue, consisting mostly of glycylglycine but also containing the reduced intermediate, reduced 17β-hydroxy-5-androstene-3-one and reduced 17β-hydroxy-4-androstene-3-one, was suspended in 100–200 μl of methanol per ml of original reaction mixture and streaked onto a sheet of Whatman 3 MM paper which had been prewashed by elution with 30 % acetic acid overnight and then dried. The methanolic solution was loaded onto paper such that material extracted from 1 ml of original reaction mixture was deposited over 2 cm. The paper was developed by descending chromatography for 14–15 hr using *n*-butanol-acetic acid-water (7:1:2 v/v/v) as the eluting solvent. When 4-[^{14}C]-17β-hydroxy-5-androstene-3-one was used as the starting material, steroid-containing substances were located by scanning guide strips cut from the sheet using a Packard Model 7201 Radiochromatogram Scanner. The zone containing the reduced intermediate was excised and eluted first for 18 hr with 5 % (v/v) acetic acid and then for 24 hours with 30 % (v/v) acetic acid. The combined eluates were evaporated *in vacuo* to a colorless oil. This was taken up in 50 % acetic acid yielding a clear stock solution. When aliquots of this solution of the purified reduced intermediate was chromatographed on silica gel G tlc plates in butanol-acetic acid-

water (4:1:2 v/v/v) only a single component was detectable. It was $MeOH/H_2SO_4$ positive, Cl_2-starch/KI positive, ninhydrin positive (pink color) and was radioactive when ^{14}C-labeled steroid was used as substrate.

Method B. The combined ethyl acetate extracts were backextracted once with 0.50 volumes of water in order to remove coextracted glycylglycine. The ethyl acetate phase was concentrated *in vacuo* to 0.025 of its initial volume yielding an aqueous solution. This was extracted 5 times with equal volumes of *n*-heptane. The *n*-heptane extractions removed most of the borohydride reduced steroid while the reduced intermediate remained in the aqueous phase. The aqueous phase was evaporated to dryness using the rotary evaporator. The solid residue was extracted 3 times with 1 ml portions of ethyl acetate (redistilled) and the solid residue remaining was dried *in vacuo*. The purified reduced intermediate was crystallized from warm 95% ethanol. The crystalline product migrated as a single ninhydrin positive, methanol-sulfuric acid positive substance on silica gel G plates in the solvent systems benzene-95% ethanol (1:1) and *n*-butanol-acetic acid-water (4:1:2).

Treatment of Isomerase with Amino Group Reagents

Reaction of *P. testosteroni* Δ^5-3-ketosteroid isomerase with amino group reagents was performed under the following conditions. Acetimidation: isomerase was incubated in 0.2 *M* triethanolamine hydrochloride, pH 8.5, with 0.05 *M* methyl acetimidate for 2 hr. Trinitrophenylation: isomerase was incubated with 0.18 m*M* sodium 2,4,6-trinitrobenzene sulfonate in 0.05 *M* sodium borate, pH 9.0 containing 1 m*M* sodium sulfite for 10 min. Maleylation: the enzyme was incubated with 0.025 *M* maleic anhydride in 0.1 *M* sodium pyrophosphate buffer, pH 9.0. The maleic anhydride was added in dioxane solution in 5 equal portions. The pH was maintained at 9.0 by additions of 1 *M* NaOH.

Other Methods

Ultraviolet spectra were obtained using a Cary Model 16 spectrophotometer equipped with a Model 1610050 wavelength scanning accessory and Model G-2000 recorder. Radioactive substances on thin layer chromatography plates were located using a Varian Aerograph/Berthold Model LB2722 thin layer chromatogram scanner.

Quantitative measurements of radioactivity of ^{14}C-labeled reduced intermediate and ^{14}C-containing steroids were obtained using a Packard Model 3075 Liquid Scintillation Counter. Samples were added to polyethylene counting vials and 10 ml of Aquasol (New England Nuclear Corp.) was added. The measured count/min were corrected for a counting efficiency of 94–95% to provide the disintegrations per minute.

Proton magnetic resonance spectra were obtained using a Jeol Minimar 100 spectrometer or a Perkin Elmer Model 60 MHz spectrometer. Chemical shifts, δ, were measured relative to the internal standard tetramethylsilane.

Acid hydrolysis of the reduced intermediate was performed in sealed evacuated ignition tubes in 6 *N* HCl at 110°C for 24 hr according to Moore and Stein (9). Amino acid analyses of acid hydrolysates were performed using a Beckman Model 121 Amino Acid Analyzer.

Elemental analyses were performed by the Microchemical Analysis Laboratory, University of California, Berkeley, California.

RESULTS

Product Identification

The identity of the final product of the reaction of 5-androstene-3,17-dione in the presence of 0.04 *M* glycylglycine pH 8.0 as catalyst was established by ultraviolet spectroscopy and thin layer chromatography. Figure 1 shows a comparison between the absorption spectrum of a final reaction mixture with that of an identical concentration of an authentic sample of 4-androstene-3,17-dione. It is seen that the two spectra are closely similar, suggesting that the final product of the reaction is the Δ^4-isomer.

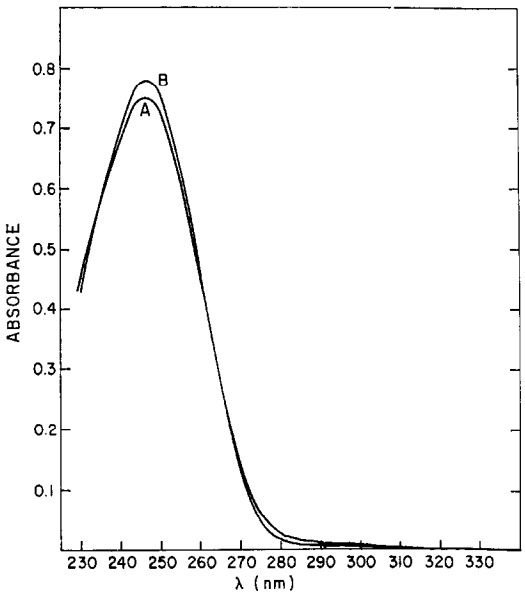


FIG. 1. Spectrum of the final product of the glycylglycine-catalyzed isomerization of 5-androstene-3,17-dione. Curve A: spectrum of the reaction mixture after 19 hr of reaction at 30°C. The 1.0 ml reaction mixture initially contained 0.04 *M* glycylglycine, pH 8.0, 0.0491 m*M*, 5-androstene-3,17-dione and 10% (v/v) methanol. Curve B: spectrum of 4-androstene-3,17-dione. The concentration of 4-androstene-3,17-dione was 0.0491 m*M*.

The final product also had chromatographic properties indistinguishable from that of 4-androstene-3,17-dione. Figure 2 shows a tracing of the thin layer chromatographic analyses of chloroform extracts obtained from 1.0 ml aliquots of a single reaction mixture containing initially 0.04 *M* glycylglycine, pH 8.0 and 0.184 m*M* 5-androstene-3,17-dione in 10% (v/v) aqueous methanol at 30°C. The chromatographic solvent employed was benzene:ethanol (9:1). It is seen that as time progresses, 5-androstene-3,17-dione disappears to be replaced by a substance with mobility close to that of authentic 4-androstene-3,17-dione. Chromatographic identity with 4-androstene-3,17-dione was also found using the solvent system chloroform–acetone (9:1).

Based on the chromatographic and spectral similarity of the final reaction product and 4-androstene-3,17-dione it was concluded that 4-androstene-3,17-dione was the only quantitatively significant final product of the reaction.

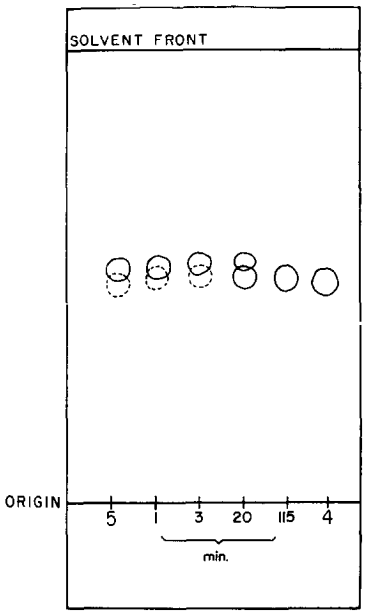


FIG. 2. Thin layer chromatographic analysis of isomerization of 5-androstene-3,17-dione by glycylglycine. The reaction mixture contained initially 0.04 *M* glycylglycine, pH 8.0, 0.184 m*M* 5-androstene-3,17-dione and 10% (v/v) methanol. At the indicated times during the reaction, 1.0 ml aliquots were extracted with chloroform and, after evaporation of the chloroform phase to dryness, spotted in ethanol solution onto the silica gel layer. The plate was developed with benzene-ethanol (9:1). In this solvent system 5-androstene-3,17-dione migrates just in front of 4-androstene-3,17-dione. The steroids were visualized as dark spots on a fluorescent background several hours after the developed plate was dried at room temperature. The lanes marked "5" and "4" contained 5-androstene-3,17-dione and 4-androstene-3,17-dione standards.

Spectral Changes During Glycylglycine-Catalyzed Isomerization

When the uv spectra of glycylglycine-catalyzed isomerization reaction mixtures were monitored at intervals during the reaction it was apparent that 4-androstene-3,17-dione was not the only uv absorbing species produced during the reaction, even though it was the sole final product. Instead, during the initial phases of the reaction a species was produced which absorbed maximally at approximately 275 nm. This species was observed in reactions conducted at pH values from 5 to 8. Absorption due to the presence of this species could be seen as a shoulder on the growing final product's absorption peak at 248 nm when the reaction was conducted at pH 8.0 and could be more clearly seen as a distinct absorption band when the reaction was performed in acetate buffer at pH 5.0. These spectral changes are shown in Figs. 3–4. The temporal relationship between the formation of the species absorbing at 275 nm and the final product, 4-androstene-3,17-dione, suggested that the species absorbing at 275 nm was an intermediate in the reaction.

Spectral Changes During Isomerization Catalyzed by Other Amines

Several different amines were tested at pH 8.0 as catalysts for the isomerization of 5-androstene-3,17-dione. All primary amines tested were catalysts and all primary

amine catalyzed reactions involved spectral changes similar to those seen when glycylglycine was the catalyst, indicating the involvement of a 270–280 nm absorbing intermediate. The following amines showed this behavior: glycine, glycylglycine ethyl ester,

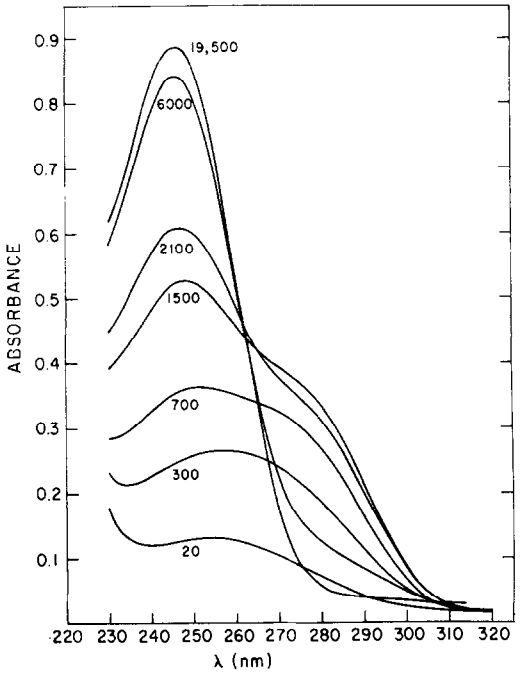


FIG. 3. Spectral changes during isomerization of 5-androstene-3,17-dione catalyzed by glycylglycine at pH 8. The 1.0 ml reaction mixture contained 0.04 *M* glycylglycine pH 8.0, 0.07 m*M* 5-androstene-3,17-dione and 10% v/v methanol. The temperature was 30°C. At the times indicated (sec) in the figure the spectrum scan was begun at 320 nm. The scan rate was 20 nm/min.

triglycine, L-lysine, D-glucosamine, and ethylenediamine. *N*-acetyl glucosamine and nitrilotriethanol were not effective as catalysts under these conditions. Interestingly, aminoacetonitrile was a very poor catalyst at pH 8.0 and a very good one at pH 5.0. Other amines exhibited the reverse behavior.

The isomerization catalyzed by glycylglycine was selected for more detailed study and characterization since it was typical.

Ultraviolet Absorption Spectrum of the Intermediate

In the particular case of glycylglycine-catalyzed isomerization, preliminary experiments showed that considerable quantities of the 275 nm-absorbing intermediate remained in the aqueous phase when a reaction mixture which contained this species was extracted with chloroform. In contrast, the 5-androstene-3,17-dione and 4-androstene-3,17-dione present in the reaction mixture partitioned almost totally in favor of the chloroform phase. Thus, the absorption spectrum of the 275 nm-absorbing intermediate could be obtained by rapidly extracting a reaction mixture which contained this intermediate and immediately determining the spectrum of the aqueous phase before appreciable further reaction. The results of this experiment are shown in Fig. 5.

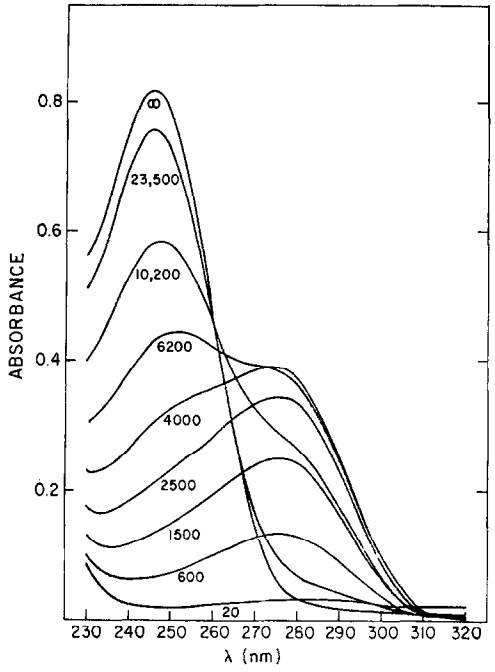


FIG. 4. Spectral changes during isomerization of 5-androstene-3,17-dione catalyzed by glycylglycine at pH 5. The 1.0 ml reaction mixture contained 0.04 *M* glycylglycine, 0.05 *M* sodium acetate-acetic acid buffer, pH 5.0, 0.0621 m*M* 5-androstene-3,17-dione and 10% (v/v) methanol. The temperature was 30°C. At the times indicated (sec) in the figure the spectrum scans were begun at 320 nm. The scan rate was 20 nm/min.

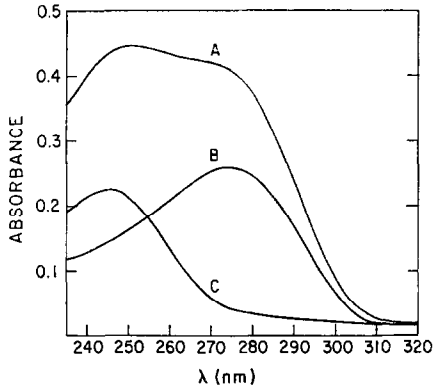


FIG. 5. Absorption spectrum of the intermediate in the glycylglycine-catalyzed isomerization of 5-androstene-3,17-dione. The reaction mixture contained initially 0.04 *M* glycylglycine, pH 8.0, 0.074 m*M* 5-androstene-3,17-dione and 10% v/v methanol. Spectrum A is the spectrum of the reaction mixture 2410 sec after the start of the reaction. Spectrum B is the spectrum of the same reaction mixture immediately after its extraction with chloroform. Spectrum C is the spectrum of the extracted reaction mixture 10000 sec after the extraction.

Curve A shows the absorption spectrum of a reaction mixture 2410 sec after addition of the steroid and just prior to extraction of the solution with chloroform. Curve B shows the absorption spectrum of the aqueous phase immediately after chloroform extraction. This spectrum closely resembles that of the 1500 sec spectrum of the reaction conducted at pH 5.0 shown in Fig. 5. Curve C shows the absorption spectrum of the

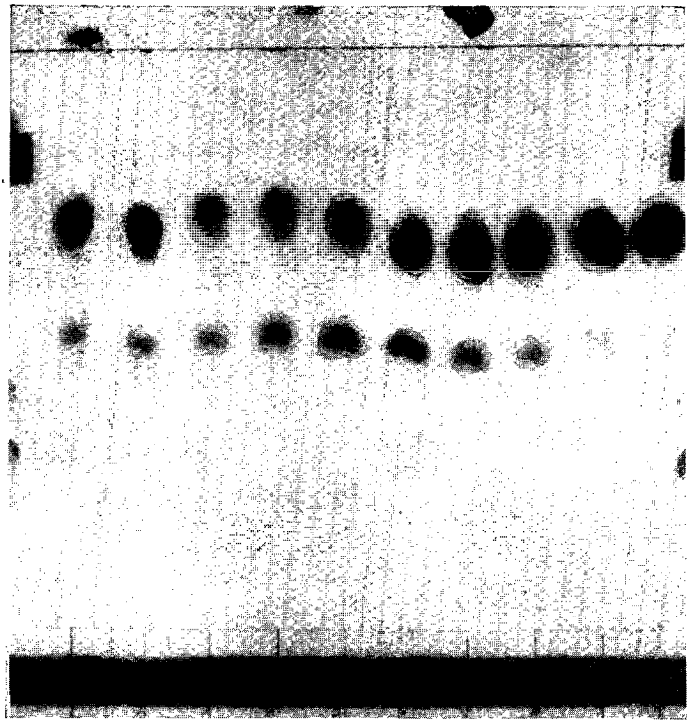


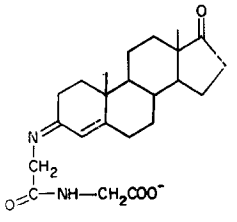
FIG. 6. Thin layer chromatographic analysis of glycylglycine-catalyzed isomerization of 4-[^{14}C]-17β-hydroxy-5-androstene-3-one after sodium borohydride reduction. The reaction mixture contained 0.1 *M* glycylglycine, pH 8.0, 1.91 m*M* 4-[^{14}C]-17β-hydroxy-5-androstene-3-one (specific activity = 49 400 dpm/μmole), 35% v/v ethanol at 30°C. The reaction was initiated by the addition of steroid. Immediately 75 μl of the resulting mixture was added to 2.925 ml of 0.1 *M* glycylglycine, pH 8.0 in 35% (v/v) ethanol maintained at 30°C in a 1 cm path length quartz cuvette. The absorbance of the diluted reaction mixture was monitored at 285 nm as a spectrophotometric assay of intermediate concentration. In order to assay the intermediate in its borohydride reduced form, 1.0 ml aliquots from the remaining undiluted reaction mixture were withdrawn at intervals and quickly mixed with 10 μl portions of 1 *M* $NaBH_4$ in dimethylformamide. Each of the reduced aliquots was then acidified to pH 3 with 6 *N* HCl and extracted with 3 × 1 ml redistilled ethyl acetate. The combined extracts were evaporated to dryness and the residues taken up in 100 μl portions of 50% (v/v) aqueous acetic acid. 20 μl aliquots were spotted on a single silica gel-G plate and the plate was developed using the solvent system *n*-butanol–acetic acid–water (4:1:2). After development and drying, the lanes were scanned in duplicate for radioactive material using the thin layer chromatogram scanner. The total radioactivity of the reduced intermediate in each lane was determined by integration of the corresponding peak produced by the scanner and was taken as a measure of the amount of reduced intermediate present in the original reaction mixture at the time of borohydride reduction. After all lanes had been scanned twice the steroidal compounds were visualized using the methanol–sulfuric acid spray reagent. The resulting chromatogram was photographed immediately. In the solvent system employed steroid not conjugated to glycylglycine runs with the highest R_f. The reduced intermediate migrates at about $\frac{1}{3}$ of this R_f.

extracted aqueous phase 10 000 seconds after the extraction was performed. Curve C is the same as the absorption spectrum of 4-androstene-3,17-dione.

Using the molar extinction coefficient of 4-androstene-3,17-dione at 248 nm and the absorbance at this wavelength in curve C of Fig. 6 one could calculate the amount of intermediate in moles which gave rise to that amount of the final product. Then, using the spectrum of the intermediate, curve B, Fig. 6, the molar extinction coefficient of the intermediate at any wavelength could be calculated. At its λ_{max} of 275 nm the extinction coefficient of the intermediate was calculated by this method to be 19 400 M^{-1} cm^{-1}.

Trapping of Intermediate by Sodium Borohydride Reduction

Characterization of the 275 nm-absorbing intermediate by means other than spectrophotometry was not feasible since the intermediate was unstable in aqueous solution, undergoing conversion to 4-androstene-3,17-dione. It was taken as a working hypothesis that the intermediate was the Δ^4-3-imine derived from glycylglycine and the steroid (Structure A). This was reasonable since Johnson et al. (*10*) had synthesized several



A

steroidal Δ^4-3-imines of 3-ketosteroids. Typical of these was that derived from pyrollidine and 4-androstene-3,17-dione which had a λ_{max} of 276 nm and $\varepsilon_{276} = 18.9 \times 10^3$ M^{-1} cm^{-1}, values closely similar to the corresponding spectral parameters of the intermediate spectrally observed in the isomerization reaction.

If the Δ^4-3-imine were the 275 nm absorbing intermediate, it would be converted to a stable species by reduction of the carbon-nitrogen double bond with sodium borohydride. This was attempted successfully in trapping experiments using 17β-hydroxy-5-androstene-3-one as the substrate rather than 5-androstene-3,17-dione in order to avoid complications involving 17-imine formation.

It was possible to qualitatively observe the appearance and disappearance of the borohydride reduction product of the 275 nm-absorbing intermediate formed during glycylglycine catalyzed isomerization of 17β-hydroxy-5-androstene-3-one. To accomplish this, timed aliquots from an isomerization reaction mixture were treated at intervals with sodium borohydride and the steroidal substances extracted from each with ethyl acetate. The ethyl acetate extracts from each timed aliquot were analyzed for steroidal components by thin layer chromatography. The steroids were located using methanol-sulfuric acid reagent. Figure 6 is a photograph of the developed chromatogram. As can be seen, a steroidal substance with an R_f somewhat less than that of the substrate or final product appears and disappears during the course of the isomerization. Also noteworthy is the fact that the total amount of substrate and final product steroid—these are not resolved from each other by this chromatographic system—*decreases* and

then *increases* during isomerization in inverse kinetic relation to the *increase* and *decrease* of the reduced intermediate.

That the reduction product was derived from the spectrophotometrically detected 275 nm absorbing intermediate was supported by an experiment in which the kinetics of formation and disappearance of the 275 nm-absorbing intermediate was compared to the kinetics of the formation and disappearance of the intermediate-like substance detected chromatographically after borohydride reduction. In this experiment the appearance and disappearance of the spectral intermediate was followed by continuous monitoring of the absorbance at 285 nm (where there is no interference from the final product, 17β-hydroxy-4-androstene-3-one). The amount of borohydride-reduced intermediate was measurable by employing 4-[^{14}C]-17β-hydroxy-5-androstene-3-one as the substrate. The amount of reduced intermediate could then be quantitated by scanning of a thin layer chromatogram of an extract of a borohydride-treated aliquot of the reaction mixture. The total count/min in the reduced intermediate spot was taken as a relative measure of the amount of reduced intermediate present. Figure 7 shows the

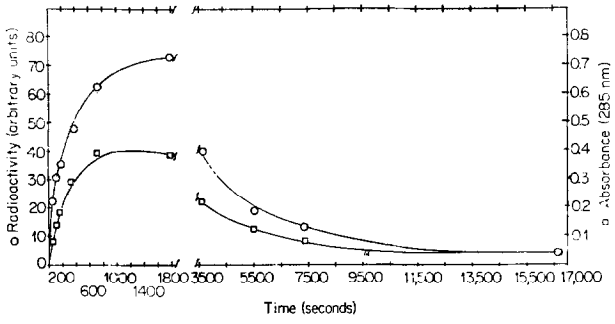


FIG. 7. Kinetics of intermediate formation and decomposition as determined by simultaneous absorbance and chromatographic techniques. See legend to Fig. 6 for experimental details.

results of such an experiment in which 4-[^{14}C]-17β-hydroxy-5-androstene-3-one was isomerized by 0.1 *M* glycylglycine, pH 8.0. As shown in the figure, the time course for appearance and disappearance of the spectrophotometric intermediate (lower curve) parallels the time course for appearance and disappearance of the chromatographically-observed reduced intermediate. This result provides support for the conclusion that the reduced material is derived from the spectrophotometric intermediate.

Characterization of Borohydride-Reduced Intermediate

The structure of the reduced intermediate was established by several analytical techniques. These included amino acid analysis, quantitative radioactivity measurement, proton magnetic resonance spectroscopy, elemental analysis, and specific color tests on thin layer chromatograms.

The purified reduced intermediate (see Materials and Methods) was found to give certain specific color tests on thin layer plates. That it was ninhydrin positive (pink color) suggested the presence of a primary or secondary amino group. It gave a purple color after spraying with methanol-sulfuric acid (1 : 1) (7) suggesting the presence of a steroid moiety and gave a deep blue color with the chlorine/starch-KI reagent. The latter

result is characteristic of compounds containing N–H groups (*8*, *11*). These tests suggest that the reduced intermediate contains both steroidal and glycylglycine moieties.

The molar ratio of acid-releasable glycine to steroid moiety was established using [^{14}C]-labeled reduced intermediate prepared from 4-[^{14}C]-17β-hydroxy-5-androstene-3-one of known specific activity. A solution of the chromatographically pure reduced intermediate was prepared (Method A in Materials and Methods). One aliquot was analyzed for steroid moiety content by ^{14}C analysis using the scintillation counter (counting efficiency was 94–95%). Another aliquot was hydrolyzed with 6 *N* HCl by the method of Moore and Stein (*9*). Table 1 shows the results of these analyses. These data show that the molar ratio of acid-releasable glycine to steroid is 1.05, suggesting

TABLE 1

Analysis	Amount per ml of stock solution	
	Before hydrolysis	After hydrolysis
^{14}C	301 500 dpm	—
"steroid"[a]	6.12 μmoles	—
glycine	N.D.[b]	6.45 μmoles
glycylglycine	N.D.[b]	N.D.[b]

[a] Calculated from ^{14}C content and a substrate specific activity of 49 400 dpm/μmole.

[b] N.D.: none detected.

TABLE 2

NMR Spectrum of Reduced Intermediate (Hydrochloride)[c]

δ (ppm)	100 MHz in d_6-DMSO	
	Multiplicity	Assignment[a]
0.67	singlet	(a)
1.04	singlet	(b)
3.44	triplet, J = 7 Hz	(c)
3.77	singlet	(d)
3.87	doublet, J = 6 Hz	(e)
5.34	singlet[b]	(f)
8.90	triplet, J = 6 Hz	(g)

[a] See structure B.

[b] The dehedral angle between the C-3α proton and the vinyl proton on C-4 is close to 90°. Thus, no coupling is observed.

[c] The characteristic "methylene envelope" of steroidal pmr spectra was present but is not tabulated since single methylene resonances were not resolved.

that one of the two glycyl residues of glycylglycine is covalently linked to the steroid by an acid stable bond.

A large amount of the reduced intermediate was prepared and purified by Method B (Materials and Methods). This preparation was characterized by proton magnetic resonance spectroscopy and elemental analysis.

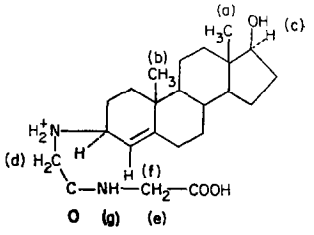
Table 2 summarizes the 100 MHz proton magnetic resonance spectrum of the intermediate (hydrochloride) and for comparison, in Table 3, the 60 MHz spectrum glycyl-

TABLE 3

NMR SPECTRUM OF GLYCYLGLYCINE HYDROCHLORIDE

	60 MHz in d_6 DMSO	
δ (ppm)	Multiplicity	Assignment
3.57	singlet (broad)	N-terminal-CH_2-
3.81	doublet ($J = 6$ Hz)	C-terminal-CH_2-
8.79	triplet ($J = 6$ Hz)	amide hydrogen

glycine hydrochloride, both in d_6-DMSO as the solvent. Assignments are made in terms of the proposed structure for the reduced intermediate, B.



B

Difficulty was experienced in obtaining a satisfactory elemental analysis of the reduced intermediate hydrochloride since it was found to be very hygroscopic. The following analysis was obtained on a sample dried at 56°C *in vacuo* over P_2O_5 for 16 hr before being sent for analysis.

C 58.5 H 8.45 N 5.95

C:N ratio = 9.83

Theory for $C_{23}H_{38}N_2O_4Cl \cdot 2H_2O$

C 57.8 H 8.85 N 5.86

C:N ratio = 9.86

The C:N weight ratio, which would be independent of the state of hydration, was in satisfactory agreement with the theoretical value for structure B, 9.86.

In order to determine if the *P. testosteroni* isomerase utilized any of its amino groups

in a catalytic mechanism involving imine intermediates the enzyme was treated with certain amino group specific reagents and the effect of such treatment on enzyme activity determined. Control experiments consisting of enzyme incubations in buffer in the absence of reagents showed no loss of enzyme activity. It was found that treatment with trinitrobenzene sulfonate or maleic anhydride rapidly inactivated isomerase suggesting some role for the enzyme's amino groups in the expression of enzyme activity. The failure of methyl acetimidate to inactivate the enzyme suggests that no amino group participates in the catalytic mechanism by forming an imine linkage with the steroid substrate since this reagent converts amino groups to amidines. Assay of methyl acetimidate-treated isomerase for unmodified amino groups by the method of Habeeb (15) showed that of all of the enzyme's five amino groups had reacted with methyl acetimidate. Thus the failure of methyl acetimidate to inactivate the enzyme could not be attributed to a failure to react with some critical amino group.

DISCUSSION

The isomerization of 5-androstene-3-one derivatives to 4-androstene-3-one derivatives is catalyzed glycylglycine via a mechanism involving the intermediacy of a 275 nm-absorbing intermediate. This is supported by the spectral changes during the course of the reaction (Figs. 3–4) which show the formation of this substance before the final product and in the extraction experiment of Fig. 5 which shows the conversion of this component to the final product.

The concentration changes of this substance during the course of the reaction (Fig. 7, lower curve) are characteristic of those of an intermediate which is formed in a fast step and converted to product in a slower step.

The intermediate could be converted to a stable form by treatment with sodium borohydride *in situ*. Structural characterization of this reduced intermediate provided evidence supporting its structural formulation as B. The fact that the reduced intermediate gave a positive test with methanol-sulfuric acid, was radioactive when ^{14}C-steroid was isomerized, and had the nmr resonances characteristic of C-18 and C-19 angular methyl groups (0.67 and 1.04 ppm) in its pmr spectrum strongly indicates that the intermediate is steroidal in nature. The fact that the reduced intermediate is ninhydrin positive, chlorine–starch/KI positive, liberates glycine on acid hydrolysis, possesses methylene and amide hydrogen resonances similar to those of glycylglycine (see Tables 2 and 3), and by elemental analysis possesses a C/N weight ratio of 9.83 supports the conclusion that the reduced intermediate contains a moiety of glycylglycine.

The presence of a carbon-carbon double bond in the reduced intermediate is supported by the presence of the vinyl proton resonance at 5.34 ppm. This double bond is most reasonably placed in the Δ^4 position rather than the Δ^5 position since, in the case of the Δ^5 isomer, a vinyl hydrogen on C-6 would exhibit either triplet or quartet spin-spin splitting due to the C-7 protons. In the case of Δ^4 isomer, the vinyl hydrogen on C-4 would appear as a singlet or doublet depending upon the dihedral angle between the C-3 proton and the C-4 vinyl proton. Construction of molecular models of the 3α and 3β isomers of B shows that the 3α isomer would have a dihedral angle close to 30° predicting

a coupling constant near 7 Hz, whereas in the 3β isomer, this dihedral angle would be close to 90° and thus a coupling constant close to 0 Hz (*12*). Since a singlet vinyl proton was observed, the 3β isomer of B is the more likely structure. Since B is proposed to be derived by reduction of A, it is reasonable that the 3β isomer be the principal reduction product since in the reduction of Δ^4-3-ketosteroids by sodium borohydride, the Δ^4-3β-ol is the predominant product isomer (*13*).

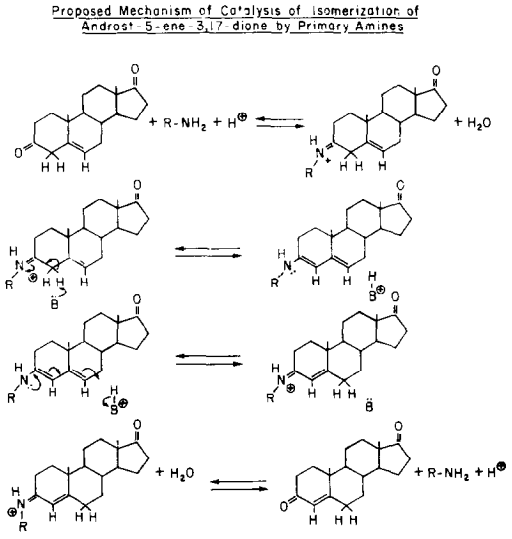
B is derived from the 275 nm absorbing intermediate since its time course for appearance and disappearance parallels the time course for appearance and disappearance of the 275 nm absorbing intermediate (Fig. 7).

Formulation of the 275 nm absorbing intermediate as A is reasonable on two grounds. First, Johnson et al. (*10*) found that several steroidal 4-ene-3-imines absorbed maximally near this wavelength. Second, formation of B by borohydride reduction is quite reasonable if A were the species reduced.

These experiments strongly suggest that primary amines such as glycylglycine catalyze the isomerization via Δ^4-3-imine intermediates. Although we have no experimental results to support the following speculations on the mechanism, they are sufficiently reasonable that they deserve mention.

First, one may ask what species is the precursor of the Δ^4-3-imine. It is highly unlikely that the Δ^4-3-one is the precursor since the kinetics of formation of the Δ^4-3-imine (Figs. 3, 4 and 7) do not parallel product formation, but exhibit rise and fall prior to Δ^4-3-one formation characteristic of a *precursor* to the Δ^4-3-one. In a control experiment to check the possibility that the Δ^4-3-imine arose by reaction of the Δ^4-3-one with glycylglycine, no evidence for imine formation was seen in the ultraviolet spectrum of 4-androstene-3,17-dione in the presence of 0.5 *M* glycylglycine, pH 8.0, the highest amine concentration employed in this study. The spectrum observed was indistinguishable from that obtained in water in the absence of glycylglycine.

A reasonable precursor of the Δ^4-3-imine is the Δ^5-3-imine since a Δ^5-3-ketone is the starting material of the reaction. The Δ^5-3-imine would be expected to isomerize



readily to the Δ^4-3-imine due to the acidifying effect of the protonated 3-imine on the C-4 hydrogens. In their study of the amine catalyzed enolization of acetone, Bender and Williams (*14*) estimated that the rate of proton exchange of neutral acetone with water is 10^8 times slower than the exchange rate of the corresponding protonated ketimine. Since at neutral pH a substantial fraction of an imine exists as the iminium ion while only an insignificant fraction of a ketone is protonated, rate enhancement of the exchange is observed when amines are present. The same process by which amines stimulate enolization can be invoked to account for the catalytic properties reported in this paper. A proposed mechanism for isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids involving imine intermediates as shown in Scheme I.

Since isomerases possess primary amino groups by virtue of their N-terminal and lysine residues, it is possible that imine intermediates are involved in enzymatic isomerization. Although acetimidation of the amino groups of the *P. testosteroni* isomerase does not result in loss of activity (Table 4) suggesting that imine intermediates are not

TABLE 4

EFFECT OF AMINO GROUP REAGENTS ON ACTIVITY OF KETOSTEROID ISOMERASE

Reagent[a]	Effect
Methyl acetimidate	None
Sodium trinitrobenzene sulfonate	inactivation
Maleic anhydride	inactivation

[a] For conditions of reactions see Materials and Methods. Control experiments showed that incubation of the enzyme in the buffers in which the reagents were employed had no effect on enzyme activity.

part of that isomerase's mechanism, the yet unstudied mammalian isomerases may employ an imine mechanism. It is hoped that the research on the model system reported herein will stimulate investigations of the importance of the amino groups of other isomerases.

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