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The Relative Rates of Formation and Hydrolysis of the Thiosemicarbazones of Some Steroid Δ^4-3-Ketones*

James C. Orr, Priscilla Carter, and Lewis L. Engel†

ABSTRACT: The rates of formation and hydrolysis of the thiosemicarbazones of a number of Δ^4-3-keto steroids and one saturated 3-keto steroid have been measured. They are found to depend on structural features of the steroids at points remote from the site of the reaction (long-range effects).

The rate-determining step in the formation and hydrolysis of the derivative is considered to be that in which carbon atom 3 changes from trigonal to tetrahedral.

There have been many studies of the variations in the rates of enzymic reactions with a number of structurally related steroid substrates (Talalay and Marcus, 1956; Langer *et al.*, 1959; Joshi *et al.*, 1963). However, only recently has attention been directed to the possible differences in intrinsic reactivity, other than isotope effects, of the functional groups undergoing the reaction (Joshi *et al.*, 1963; Ringold *et al.*, 1964a; Ringold *et al.*, 1966).

The present study was designed to discover if the reactivity of the Δ^4-3-ketone grouping toward thiosemicarbazide is affected by variation in the structure of the steroid molecule. Such differences in reaction rates may serve for comparison with kinetic studies of substrate specificity of enzymic reactions at the 3-position.

Experimental Section

Materials and Methods. Ethanol (95%) was refluxed with *m*-phenylenediamine for 2 hr and distilled, collecting the fraction distilling at 78 ± 0.5°. To the distillate was added a small amount of thiosemicarbazide and *p*-toluenesulfonic acid, the solution was redistilled, and the fraction boiling at 78 ± 0.5° was collected

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TABLE I: Physical Constants of the Prepared Thiosemicarbazones.

Thiosemicarbazone of	Mp (°C)[a]	λ_{max}^{EtOH} (mμ)	ϵ_{max}	Calcd (%)				Found (%)			
				C	H	N	S	C	H	N	S
Testosterone ($C_{20}H_{31}N_3OS$)	158–161	302, 247.5	34,200, 10,900	65.95	9.12	11.42	8.96	66.45	8.64	11.63	8.85
19-Nortestosterone ($C_{19}H_{29}N_3OS \cdot H_2O$)	149–152	302	34,100	62.43	8.55	11.50	8.77	62.43	8.90	11.54	8.73
11α,17β-Dihydroxyandrost-4-en-3-one ($C_{20}H_{31}N_3O_2S$)	242 dec	302	35,400	63.62	8.28	11.13	8.49	63.56	8.40	10.74	8.06
11β,17β-Dihydroxyandrost-4-en-3-one ($C_{20}H_{31}N_3O_2S$)	197.5–200.5	302	33,600	63.62	8.28	11.13	8.49	63.12	8.63	11.33	8.71
17β-Hydroxyandrost-4-ene-3,11-dione ($C_{20}H_{29}N_3O_2S \cdot 0.5\ H_2O$)	238.5–240.5	302	34,600	62.51	7.86	10.94	8.35	62.14	8.29	10.21	8.13
17β-Hydroxyandrosta-4,9(11)-dien-3-one ($C_{20}H_{29}N_3OS \cdot 0.5\ H_2O$)	150–151	302	34,200	63.63	8.28	11.13	8.49	64.37	8.44	10.76	8.08
17β-Hydroxy-5α-androstan-3-one ($C_{20}H_{33}N_3OS$)	217–220	270	20,750	66.08	9.15	11.56	8.81	66.22	9.20	11.33	8.91

[a] The melting point of testosterone thiosemicarbazone was reported by Riondel *et al.* (1963) as 210–220°. Dr. M. Gut has informed us that this value is in error and that his product also has the melting point recorded above.

and stored at 5° until use. Analytical grade thiosemicarbazide was crystallized once from 95% ethanol. The steroids and those thiosemicarbazones made were crystallized, and the melting points, paper and thin layer chromatographic properties, ultraviolet spectra, and elemental analyses found to be satisfactory (Table I). 17β-Hydroxyandrosta-4,9(11)-dien-3-one thiosemicarbazone alone showed the presence of an impurity amounting to possibly 5%, as judged by paper chromatography.

Rate Studies. The formation and hydrolysis of the thiosemicarbazones of the Δ^4-3-ketones were followed by measuring the changes in absorption at 302 mμ using a Beckman DU spectrophotometer with a Gilford sample changer and recorder. All reactions were carried out at 25 ± 0.2° in 3.0-ml cuvets. Each reaction vessel contained 95% ethanol (1.6 ml), aqueous hydrochloric acid (0.4 ml of 1.75 N), and thiosemicarbazide (1.0 ml of 0.01 M in 0.1 N HCl) giving a final hydrochloric acid concentration of 0.267 N in the cuvet. The steroid concentration in the reaction mixture was 0.4–3 × 10^{-5} M. This affords a thiosemicarbazide to steroid ratio of more than 100:1. The steroid in 30 μl of 95% ethanol was added from a microsyringe and the reaction mixture was stirred briefly with a footed Teflon stirring rod. Three such cuvets were prepared; one always contained the standard, testosterone. A fourth cuvet, used as a blank, contained all components but the steroid. The time required to mix the steroid in all three cuvets was less than 15 sec. In a few cases where the volume of ethanol required to dissolve the steroid was greater than 30 μl, a corresponding volume was deducted from the initial 1.6 ml of ethanol placed in the cuvet. The reactions were allowed to proceed to equilibrium.

The rate of formation of the thiosemicarbazone of the saturated 3-ketone, 17β-hydroxy-5α-androstan-3-one, was estimated by the rate of increase of the maximum at 270 mμ (ε 20,700). It could not conveniently be run with a testosterone standard because of the disparity in rates of reaction.

The kinetic data have been treated as pseudo-first-order reaction in which the reverse reaction is appreciable (Laidler, 1950). The form of the equation is

$$\frac{dx}{dt} = k_{+1}(a - x) - k_{-1}x$$

which upon integration yields

$$k_{+1} = \frac{X_e}{at} \ln \frac{X_e}{X_e - x}$$

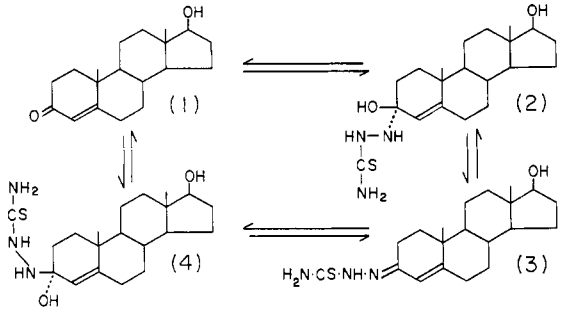
$$k_{-1} = \frac{k_{+1}}{X_e}(a - X_e)$$

where k_{+1} and k_{-1} are the first-order rate constants for formation and hydrolysis of steroid thiosemicarbazones at constant hydrogen ion concentration, a is initial steroid concentration, x is thiosemicarbazone

concentration at time t, and X_e is the equilibrium concentration of thiosemicarbazone.

In order to determine the extent of participation of carbinolamines in the equilibrium, testosterone thiosemicarbazone (Riondel *et al.*, 1963) was dissolved in the acidic aqueous ethanol used for the kinetic measurements. The disappearance of the 302-mμ band owing to testosterone thiosemicarbazone and the appearance of overlapping bands at about 243 mμ owing to free testosterone (ϵ 17,500) and thiosemicarbazide (ϵ 12,200) were followed by repeated scanning at 220–350 mμ in a Cary 15 spectrophotometer until equilibrium was attained. At equilibrium, 2% of the thiosemicarbazone remained: testosterone and thiosemicarbazide accounted for 96% of the original thiosemicarbazone. This leaves approximately 2% which may be due to carbinolamines or to experimental error.

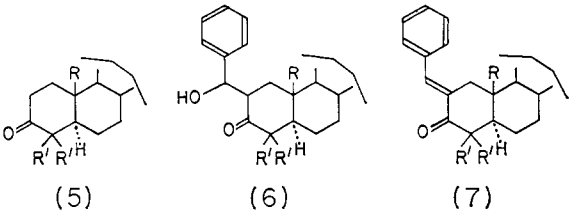
In order to check more directly the number of molecular species present at equilibrium, a reaction was run under the conditions employed for kinetic measurements. After equilibrium had been reached, the products were extracted with ethyl acetate. The residue obtained upon evaporation was chromatographed on Whatman No. 2 paper in the system ligroin–toluene (67:33) and methanol–water (70:30) at 37°. Upon spraying with alkaline *m*-dinitrobenzene only two spots were observed; they corresponded in mobility to testosterone (**1**) and its thiosemicarbazone (**3**).



Chloroform extraction of a run in which testosterone was in equilibrium with the thiosemicarbazone in the presence of 160-fold excess thiosemicarbazide yielded a solid which on fiberglass thin layer chromatography (tlc)–paper partition chromatography in the system 99% carbon tetrachloride–1% methanol showed only two blue spots on spraying with sulfuric acid–ethanol (1:1). These corresponded in R_F to testosterone and its thiosemicarbazone. Thiosemicarbazide does not move in this system and gives a yellow color.

Discussion

Long-range effects in the reactions of functional groups in rigid fused-ring systems were clearly demonstrated by Barton and his co-workers (Barton and Head, 1956; Barton *et al.*, 1957, 1960) in their study of the rates of base-catalyzed condensation of benzaldehyde with steroid (**5**, R′ = H) and triterpenoid 3-ketones (**5**, R′ = CH_3). The



rate-determining step in this reaction was shown (Noyce and Reed, 1959; Stiles *et al.*, 1959) to be the conversion of the anion of the intermediate hydroxy ketone (**6**) to the conjugated system (**7**). The rates of dehydration were found to be markedly dependent on structural features remote from the site of reaction. From comparison of the rates of a large number of triterpenes and steroids of widely different character, Barton *et al.* (1957) concluded that the variation in reactivity was due neither to electrostatic charge or polarity of the remote substituent, nor to steric interactions of axial substituents (axial buttressing), but was attributable to deformation of bond angles through the fused-ring system. The strain energies produced by a number of substituents were approximately additive, each substituent having a characteristic "group-rate factor." The direction and extent of the deformations were considered at greater length by Robinson and Whalley (1963), Bucourt (1964), and Norton (1965). In another study, altering the strain in rings A and B was not found to have any appreciable effect on the position of equilibrium between 20-ketopregnanes and their 17α epimers (Rubin and Blossey, 1964).

Although Barton had shown that considerable variation in reaction rate could be expected, the reaction he employed was not directly comparable to any common enzymic reaction of steroids. A reaction of the 3-keto group which appeared to merit investigation was the conversion to the thiosemicarbazone.

In the case of the Δ^4-3-keto steroids, the formation of the derivative could readily be followed by the appearance of the band at 302 mμ (ϵ 34,300). The only saturated 3-ketone tested, 17β-hydroxy-5α-androstan-3-one, had maximal absorption of its thiosemicarbazone at 270 mμ (ϵ 20,750); in this region free thiosemicarbazide shows negligible absorption.

The greater speed of reaction of the saturated 17β-hydroxyandrostan-3-one than that of the conjugated enones both in the direction of thiosemicarbazone formation and hydrolysis suggests that the rate-determining step in the conjugated enone case is in passing from the conjugated system to a nonconjugated intermediate, rather than the dehydration of the carbinolamine. This is in agreement with the similar semicarbazone formation reaction where the rate-determining step is considered (Royals, 1954) to be the attack of the nitrogen atom on the carbon of the protonated ketone. A similar conclusion has been reached for

TABLE II: Relative Rates of Formation and Hydrolysis of Thiosemicarbazones of Testosterone Derivatives.

Structural Change	Compound	Relative Rates[a]			
		Formation		Hydrolysis	
		Rate	Std Dev[b]	Rate	Std Dev
4-Methyl	17β-Hydroxy-4-methylandrost-4-en-3-one	0.16	0.005	1.09[c]	0.052
2α-F	2α-Fluoro-17β-hydroxyandrost-4-en-3-one	0.28	0.030	0.25	0.010
6β-Methyl	17β-Hydroxy-6β-methylandrost-4-en-3-one	0.69	0.026	0.51	0.013
17α-OH	17α-Hydroxyandrost-4-en-3-one	0.93[c]	0.030	1.05[c]	0.033
$\Delta^{9(11)}$	17β-Hydroxyandrosta-4,9(11)-dien-3-one	0.93[c]	0.056	0.98[c]	0.028
16α-Methyl	17β-Hydroxy-16α-methylandrost-4-en-3-one	0.93	0.026	1.06[c]	0.097
17α-Methyl	17β-Hydroxy-17α-methylandrost-4-en-3-one	0.98[c]	0.052	1.02[c]	0.022
	Testosterone	1.00		1.00	
16β-F	16β-Fluoro-17β-hydroxyandrost-4-en-3-one	1.01[c]	0.050	1.11	0.027
16β-Cl	16β-Chloro-17β-hydroxyandrost-4-en-3-one	1.06[c]	0.052	1.05[c]	0.046
6α-F	6α-Fluoro-17β-hydroxyandrost-4-en-3-one	1.19	0.048	0.58	0.032
19-Nor	17β-Hydroxyestr-4-en-3-one	1.25	0.008	1.69	0.077
6α-Methyl	17β-Hydroxy-6α-methylandrost-4-en-3-one	1.28	0.040	1.05[c]	0.041
11β-OH	11β,17β-Dihydroxyandrost-4-en-3-one	1.31	0.006	1.62	0.058
11α-OH	11α,17β-Dihydroxyandrost-4-en-3-one	1.36	0.024	1.11	0.023
6β-F	6β-Fluoro-17β-hydroxyandrost-4-en-3-one	1.41	0.041	0.69	0.034
11-C=O	17β-Hydroxyandrost-4-ene-3,11-dione	1.42	0.074	1.20	0.056
4,5α-H_2	17β-Hydroxy-5α-androstan-3-one	8.4	0.47	20.9	0.1

[a] Testosterone = 1.00. Under the experimental conditions employed k_{+1} and k_{-1} are approximately 3.6 and 4.4 $\times 10^{-4}$ sec^{-1}. [b] Standard deviation. [c] Not significantly different ($p > 0.05$) from testosterone.

Schiff base formation (Cordes and Jencks, 1962; do Amaral *et al.*, 1966) where, under acid conditions, attack of the amine on the protonated aldehyde is the rate-determining step. Hydrolysis also appears to occur with rate-determining addition of water to the protonated imine (Reeves, 1965).

The addition of nitrogen base to the protonated steroid 3-ketone (**1**) and of water of the thiosemicarbazone (**3**) is likely to take place by preferential α attack. The preferred carbinolamine intermediates in the thiosemicarbazone formation (**2**) and hydrolysis (**4**) are diastereoisomers epimeric at C-3 and are not of equal energy. The amount of each carbinolamine present at equilibrium is not known, but must be small compared to the conjugated systems, which account for 98 ± 5% of the observed ultraviolet spectrum of the equilibrium mixture. This is in accord with the observation that the conversion of carbinolamines to the conjugated system is relatively fast. No carbinolamines were detected by chromatography; however, they may have been reconverted to the parent enone during the extraction.

The rates of formation and hydrolysis of the thiosemicarbazones of a series of testosterone derivatives are given in Table II. They are listed relative to the corresponding rates for testosterone run simultaneously. Each value tabulated is the mean of at least three such comparisons. Student's t test was used to estimate the probability that each of the ratios differed from unity.

The low relative rate (0.16) produced by the introduction of a 4-methyl group is probably due to steric interaction with the approaching thiosemicarbazide molecule. Surprisingly, there is no reduction in the rate (1.09) of hydrolysis of the thiosemicarbazone. This may, however, be due to the smaller size of the approaching water molecule or to relief of steric strain between the thiosemicarbazide residue and the 4-methyl group on changing C-3 from trigonal to tetrahedral. The stereochemistry of the C=N bond of the thiosemicarbazone is not known.

A 2α-fluoro substituent causes strong depression of the rates of both the thiosemicarbazone formation (0.28) and hydrolysis (0.25). This is in marked contrast to the enzymic reduction of a Δ^4-3-keto steroid to the corresponding 3α-allylic alcohol (Ringold *et al.*, 1964b) where 2α-fluorotestosterone was reduced at almost double the rate of testosterone. This cannot be due to the dipole interactions of the 2α-fluoro group with the 3-ketone as this has been shown to make the ketone behave more like the saturated case.

The reduction in rate could be due to stabilization of the protonated heteroatoms by hydrogen bonding to the fluorine. Fluorine has been shown to form intermolecular hydrogen bonds more readily than

the other halogen atoms (West *et al.*, 1962). We have found no study of intramolecular hydrogen bonding in saturated fluorohydrin or protonated fluoro ketones. The boiling points of the *o*-, *m*-, and *p*-fluorophenols are respectively 151, 178, and 186°, suggestive of intramolecular hydrogen bonding in the *ortho* isomer.

Introduction of the axial 6β-methyl group also causes reduction in the reaction rates (formation 0.69, and hydrolysis 0.51). The radical change in optical rotatory dispersion pattern of the enone chromophore on introduction of a 6β-methyl substituent has been shown (Villotti *et al.*, 1959) to be due to steric repulsion of the angular 10-methyl group. From inspection of models, this repulsion appears to cause a movement of C-3 toward the β side of the steroid, the enone chromophore becoming more nearly planar than in testosterone which would cause increased resonance stabilization.

The 6α-methyl group, on the other hand, causes little conformation change as shown by optical rotatory dispersion. The reactivity of the 6α-methylenone to thiosemicarbazide is, however, significantly increased (1.28) while the hydrolysis rate (1.05) is that of unsubstituted testosterone. In both cases, decreased rates due to the inductive effect would have been expected. Substitution of the molecule at positions 16 or 17 causes little change in the reactivity of the Δ^4-3-ketone or of the thiosemicarbazone.

The 9(11)-unsaturated compound has reaction rates (0.93 and 0.98) which are not significantly different from those of testosterone. The benzaldehyde condensation rate is increased (1.24) by the presence of a 9(11) double bond (Barton *et al.*, 1960).

Removal of the angular 10-methyl group of testosterone causes a change in rate (to 1.25) in the same direction as that caused by removal of the similarly situated 6β-methyl ((1.00/0.69) = 1.45). The same pattern is seen in the hydrolysis (1.69 *vs.* (1.00/0.51) = 1.96).

Introduction of a 6α- or a 6β-fluorine causes an increase in the rate of formation of the thiosemicarbazone (1.19 and 1.41, respectively). It is probable that the effect of the fluorine is inductive, causing the enone to behave more like a saturated 3-ketone (Ringold *et al.*, 1964a). The reason for the increased stability (rates 0.58 and 0.69, respectively) of the thiosemicarbazones of the 6-fluoro compounds is not obvious. Models show that no direct spatial interaction is likely.

An oxygen atom at the 11 position, whether α (1.36), β (1.31), or ketonic (1.42), increases the rate of thiosemicarbazone formation and hydrolysis (1.11, 1.62, and 1.20, respectively). These are the only clear instances of long-range effects observed in this study. Oxygen substitution at 11 has a pronounced effect on the position of maximal ultraviolet absorption of ring-A chromophores (Orr *et al.*, 1964).

There is no apparent correlation between the rate of formation and of hydrolysis of the thiosemicarbazones of the different 3-ketones studied. Neither the forward nor the reverse reaction shows any significant correlation with the nuclear magnetic resonance chemical shift of the C-19 methyl hydrogens, nor with the intensity of the ultraviolet absorption maximum of the Δ^4-3-ketone chromophore.

In the enzymic conversion of a substrate to a product, three steps can be distinguished. These are: association of the substrate(s) with the enzyme in the proper orientation, reaction, and desorption of the product(s). The present study has demonstrated that differences in the reaction rate (step 2) can be expected with a number of related substrates. Judging from these data and the group rate factors reported by Barton, it may be anticipated that the conformational effects of remote substituents will have little more than a two- to threefold influence upon reaction rates. Differences in reaction rate greater than this can reasonably be attributed, at least in part, to the degree of fit of enzyme and substrate or product (enzyme recognition). Where a number of related substrates show only slight differences in reaction rate, caution should be exercised in drawing conclusions as to the geometry of the active site; the variable factor may be the intrinsic reactivity of the substrate molecule.

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The Isolation and Purification of Solubilized Hexokinase from Bovine Brain*

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ABSTRACT: Bovine brain hexokinase was purified 1129-fold by a two-phase procedure. The specific activity of the solubilized enzyme was 80 μmoles of glucose 6-phosphate formed/min per mg of protein. The first phase involved purification of particulate hexokinase by successive treatment of the "mitochondrial sediment" with chymotrypsin, deoxycholate, and Triton X-100; the second phase involved solubilization of the particulate hexokinase with Triton X-100 followed by chromatography of the solubilized enzyme on DEAE-cellulose. Analysis by ultracentrifugation and cellulose acetate electrophoresis indicated that the enzyme was homogeneous. The uncorrected sedimentation constant of the purified enzyme was calculated to be 4.44 S.

The absorption spectrum of the enzyme was typical of a protein except that fine structure was observed below 280 mμ. The amino acid analysis indicated that the enzyme had a high content of acidic amino acids, and a low content of tyrosine. Some preliminary kinetic properties of the solubilized enzyme are reported.

Enzyme studies utilizing the technique of differential centrifugation have indicated that brain hexokinase is associated with the mitochondrial fraction of brain homogenates (Utter *et al.*, 1945; Crane and Sols, 1953; Johnson, 1960; Beattie *et al.*, 1963). Only the particulate form of the enzyme had been described (Crane and Sols, 1953, 1955) when this investigation was initiated. Subsequently, Moore and Strecker (1963) and Jagannathan (1963) reported methods for the solubilization and purification of brain hexokinase; however, low yields were obtained and no information regarding purity was reported. This paper describes isolation and purification of solubilized hexokinase from bovine brain and documents some of its physical and chemical properties.

Materials

The following materials were obtained commercially: glucose 6-phosphate dehydrogenase (type 5), ATP,[1] ADP, TPN^+, EDTA, and Tris from Sigma Chemical Co., St. Louis, Mo.; three-times-crystallized α-chymotrypsin (salt free) and purified pancreatic lipase from Worthington Biochemical Corp., Freehold, N. J.; sodium deoxycholate from Matheson Coleman and Bell, Rutherford, N. J.; ammonium sulfate, special enzyme grade, from Mann Research Laboratories, N. Y.; Sephadex gels from Pharmacia, Uppsala, Sweden; DEAE-cellulose (Cellex D) from Bio-Rad Laboratories, Richmond, Calif.; L-histidine monohydrochloride from

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides.