mmol) was treated with 250 mg (1.32 mmol) of tosyl chloride to yield 250 mg (61%) of tosylamide **20b**: mp 100 °C; $[\alpha]^{25}_D$ -49.0° (c 0.76, $CHCl_3$); mass spectrum, m/e 343 (1.5, M), 271 (6.3, M - $CONMe_2$), 172 (1.1, TsOH), 171 (5.4, TsO), 155 (15.7, Ts), 100 (26.1, $CONMe_2$ + 28), 91 (27.7, C_7H_7), 81 (100, furan=CH_2), 72 (25.5, $CONMe_2$). Anal. Calcd for $C_{15}H_{21}NO_6S$: C, 52.47; H, 6.17; N, 4.08. Found: C, 52.67; H, 6.08; N, 4.15.

D(-)-Allomuscarine Iodide (3). Compound **20b** (185 mg, 0.54 mmol) gave after recrystallization 60 mg (37% yield) of D(-)-allomuscarine iodide (**3**) as white crystals: mp 125-126 °C (acetone-petroleum ether); $[\alpha]^{25}_D$ -37.7° (c 0.65, H_2O); $[\alpha]^{25}_D$ -39.1° (c 0.38, EtOH) [lit.[17] $[\alpha]^{22}_D$ for natural alkaloid, -32° (c 1.1, H_2O)]. Anal. Calcd for $C_9H_{20}INO_2$: C, 35.89; H, 6.69; N, 4.64. Found: C, 36.03; H, 6.56; N, 4.48.

Acknowledgment. We are grateful to Dr. J. Igolen for his support and helpful discussions and acknowledge the assitance of Dr. S. Tran Dinh, CEA Saclay for ^1H NMR spectra at 250 Hz. We thank Professor M. M. Joullié, University of Pennsylvania, for helping to improve this manuscript. One of us (S.P.) also acknowledges the Délégation Générale à la Recherche Scientifique for a grant.

Registry No. 1, 24570-49-8; 2, 79827-61-5; 3, 35119-38-1; 4, 79827-62-6; 6a, 50908-41-3; 6b, 50908-40-2; 7a, 79827-63-7; 7b, 79827-64-8; 8a, 79827-65-9; 8b, 79827-66-0; 9a, 79827-67-1; 10a, 79827-68-2; 10b, 79827-69-3; 11a, 79827-70-6; 11b, 79827-71-7; 11b ditosylamide, 79827-82-0; 12, 5328-37-0; 13, 18546-37-7; 14a, 22837-37-2; 14b, 22837-36-1; 15, 3056-12-0; 16a, 79827-72-8; 16b, 79827-73-9; 17a, 79827-75-1; 18a, 79827-76-2; 18b, 79827-77-3; 19a, 79827-78-4; 19b, 79827-79-5; 20a, 79827-80-8; 20b, 79827-81-9; 2,5-dideoxy-1-[(dimethylamino)methyl]-β-D-erythropentofuranose, 79827-82-0.

Degradation of Cloprednol in Aqueous Solution. The Enolization Step

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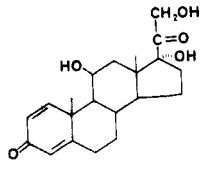
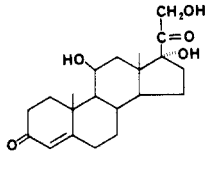
The degradation of the glucocorticoid cloprednol (**3a**) and its 21,21-dideuterio analogue (**3c**) was studied in aqueous solution at 60 °C. The kinetic deuterium isotope effect was found to vary from 5.5 under acidic conditions to 1.0 under alkaline conditions, indicating a change in rate-determining step as a function of pH. Incorporation of hydrogen into the C-21 position of cloprednol in partially degraded samples of **3c** occurred slowly or not at all under acidic conditions but occurred rapidly under alkaline conditions. These results are consistent with the formation of an obligatory enol intermediate in the degradation reaction of cloprednol and require a change in the rate-determining step from rate-determining enolization under acidic conditions to reversible enol formation under alkaline conditions. Prednisolone (**1**), hydrocortisone (**2**), and other glucocorticoids possessing the dihydroxyacetone group at C-17 are expected to behave similarly.

Many of the synthetic glucocorticoids used clinically share a common structural feature, namely, the dihydroxyacetone group at C-17. The degradation of two glucocorticoids, prednisolone (**1**) and hydrocortisone (**2**), has been studied in aqueous solution, and the reactivity observed was derived exclusively from transformations of the dihydroxyacetone group.[1-3]



1



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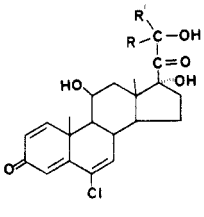

3a R=R'=H
3b R=H R'=D
3c R=R'=D

Kinetic and product studies of the degradation of both prednisolone (**1**) and hydrocortisone (**2**) have provided some insight into the reactivity of the dihydroxyacetone group, but elucidation of a detailed mechanism of decomposition has been frustrated by the complex nature of the reaction. Previous studies have shown that the degradation products are derived from both oxidative and solvolytic pathways. Both the type of degradation products formed and the rate of degradation have been shown to exhibit dependence on pH, trace metal ions, and oxygen.[1,4-11]

In every attempt to describe the mechanism of degradation of glucocorticoids an assumption has been made that enolization of the dihydroxyacetone group is the first step of the reaction.[4-6,8-10,12-14] There has been less agreement on the kinetic significance of enolization on the overall reaction rate. Pitman and co-workers presumed that enolization was the rate-determining step in the degradation of hydrocortisone in alkaline solution.[15] Recently, Hansen and Bundgaard have proposed a mechanistic scheme involving reversible enol formation and ionization to explain the observed pH-log k_{obsd} profile they obtained for hydrocortisone.[6] For an answer to some fundamental questions relating to the role enolization may play in the degradation of this class of steroids in aqueous solution,[16] kinetic isotope and exchange studies of deuterium at C-21 in a new systemic glucocorticoid, clopred-

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(3) K. J. Kripalani and D. L. Sorby, *J. Pharm. Sci.*, **56**, 687 (1967).
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(14) H. S. Wendler in "Molecular Rearrangements", P. Mayo, Ed., Interscience, New York, Part 2, p 1068.
(15) I. Pitman, T. Higuchi, M. Alton, and R. Wiley, *J. Pharm. Sci.*, **61**, 918 (1972).
(16) Cloprednol (**3**) differs from prednisolone (**1**) and hydrocortisone (**2**) in rings A and B only, far from the reactive site. The reactivity of the dihydroxyacetone side chain in these steroids is expected to be nearly identical.

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nol[17] (3a), were examined.

Experimental Section

Materials. Acetonitrile distilled in glass from Burdick and Jackson Laboratories Inc. and water purified through a Nanopure filtration system by Barnstead Co. were used. Deuterium oxide (99.7 atom % deuterium), methyl alcohol-*d* (99.5 atom % deuterium) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) were obtained from Aldrich Chemical Co. A 20% DCl/D_2O solution was obtained from Diaprep Inc. Cloprednol[17] (3a) was obtained from the Institute of Organic Chemistry, Syntex Corp. All other chemicals were of reagent grade and used without further purification.

HPLC. An analytical HPLC system consisting of an Altex Model 110A pump, a UV detector operated at 280 nm and 0.08 AUFS, and a Micromeritics Model 725 automatic injector with a 10-μL fixed loop were used. The method utilized a 25 cm × 4.6 mm (i.d.) Ultrasphere reverse-phase 5-μm C-8 column from Altex and a mobile phase consisting of water–acetonitrile (55/45) which developed a pressure of approximately 1000 psi at a flow rate of 0.8 mL/min. Detector response, as measured by peak height, was linear for 3 over the concentration range observed in the kinetic studies.

Semipreparative HPLC was performed with an Altex Model 110A pump, a Cecil CE 212 variable-wavelength detector operated at 350 nm and 2.0 AUFS, and a Rheodyne 70-10 fixed-volume 100-μL loop injector. A 25 cm × 10 mm (i.d.) Altex 5-μm Lichrosorb column and a mobile phase of methylene chloride-methanol (94/6) were used. A 2.0-mL/min flow rate developed a pressure of 250 psi, and 3 gave a retention time of approximately 16 min.

Instrumentation. All ^1H NMR spectra were determined at 300 MHz on a Bruker WM 300 FT NMR. The samples were run at a concentration of 25 mg/mL in Me_2SO-d_6 containing D_2O to completely exchange the three hydroxyl protons in 3. Mass spectra were obtained in the direct-inlet mode on a Varian MAT 1125 mass spectrometer equipped with an SS-200 data system. CI spectra were obtained at 95 eV with isobutane as the reagent gas.

Synthesis of 21,21-Dideuteriocloprednol (3c). A solution of 1.72 g of cloprednol (3a) dissolved in 10 mL of CH_3OD was added to a solution consisting of 3.6 g of tris(hydroxymethyl)-aminomethane buffer and 90 mL each of D_2O and CH_3OD. The resulting solution was stored in a stoppered flask at 60 °C for 23 h, cooled to room temperature, and acidified with a 20% DCl solution. Most of the methanol was removed by evaporation under reduced pressure, and the resulting mixture was extracted with ethyl acetate (2 × 50 mL). The ethyl acetate washings were combined and dried over sodium sulfate, and the solvent was removed under reduced pressure to give 1.56 g of a clear glass. Approximately 40 μg of the crude steroid residue was applied onto silica gel GF plates and developed with ethyl acetate–methylene chloride (3:2). Visualization under UV light showed a small amount of impurity at the origin of the plate. Purification by column chromatography on 300 g of silica gel with 1:1 acetone-/ethyl acetate as the eluant gave two fractions that were pure by TLC. The combined fractions were evaporated under reduced pressure, and the clear glass residue was redissolved in 10 mL of ethyl acetate. This ethyl acetate solution was then added to 150 mL of hexane with stirring, and the resulting precipitate was filtered and dried to give 1.09 g of a white solid.

The electron-impact mass spectrum of this material showed a weak molecular ion peak at *m/e* 394; but like 3a itself, the relative size of the peaks near the M^+ peak varied with the sample temperature and time of heating, making the quantitative assignment of isotope abundance impossible. A chemical-ionization mass spectrum with isobutane showed a strong MH^+ of *m/e* 395, but difficulties similar to that described above were encountered in obtaining repeatable spectra. The ^1H NMR spectrum was identical with that of 3a except for the absence of the resonance signals due to the two protons at C-21 (see the section on NMR Analysis of Exchange for a more detailed analysis of the C-21 proton region).

(17) Cloprednol is the generic name for 6-chloro-11β,17α,21-trihydroxypregna-1,4,6-triene-3,20-dione.

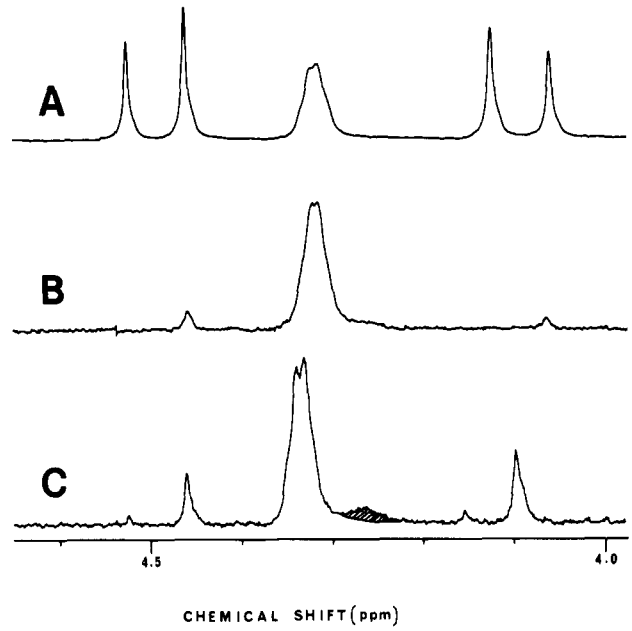


Figure 1. ^1H NMR spectra of 3a (A), 3c (B), and a mixture of 3a–c (C). The shaded area in spectrum C is an impurity peak.

Kinetic Isotope Effect. Buffer solutions were prepared at the appropriate buffer concentration and pH. These solutions contained 1 × 10^{-4} M EDTA as well as potassium chloride when needed to maintain an ionic strength of 0.20. The CAPS buffer solution was filtered through a 1-μm filter membrane to remove some insoluble impurities present in the buffer. All pH measurements were made at 60 °C by using a Radiometer Model 26 pH meter equipped with a Corning Model 476056 combination electrode.

In a typical experiment 300 μL of a 25 mg/mL methanol stock solution of 3a was added to 150 mL of buffer and stirred vigorously to assure dissolution.[18] The solution was then transfered to 10-mL amber glass ampules and sealed under air. The ampules were placed into a controlled-temperature water bath at 60.0 ± 0.05 °C for the appropriate period of time, removed, and then kept at 4 °C until assayed by HPLC. The pH 0.82 and 5.06 solutions were injected directly onto the HPLC column, but the more basic solutions required adjustment to acidic pH with HCl prior to injection. The percent of 3 remaining in degraded samples was determined by comparison of peak height with that of a standard.

Isotope-Exchange Studies. Partial Degradation and Isolation of 3. The example isotope-exchange study described below was carried out at pH 0.82 but is general for all pH's studied except where noted. Buffer solutions for these studies were prepared as described above.

The reaction was initiated by adding 2 mL of a 53 mg/mL methanol solution of 3c to 500 mL of the buffer solution at 60 °C. The resulting solution (212 μg/mL) was slightly turbid and after 2 weeks at 60 °C was cooled and filtered through a 1-μm filter membrane. An aliquot of the solution diluted tenfold with mobile phase and analyzed by reverse-phase HPLC showed approximately 57% of 3 remaining. The pH of the reaction solution was then adjusted to pH 6.5 and extracted with methylene chloride (2 × 100 mL). The combined methylene chloride extract was dried over sodium sulfate and the solvent removed to give a glassy residue. This residue (approximately 100 mg) was dissolved in 5 mL of mobile phase and injected in 1-mL increments onto the semipreparative HPLC system.[19] The eluant fraction corresponding to 3 was collected. The mobile phase was then evaporated from the pooled collections under reduced pressure to give 42 mg of recovered 3.

NMR Analysis of Exchange. In Figure 1A a portion of the

(18) The equilibrium solubility of cloprednol in water was measured to be 300 μg/mL at 25 °C.

(19) For the reaction at pH 9.05, almost no degradation had occurred under the reaction conditions, and no purification of the residue was needed.

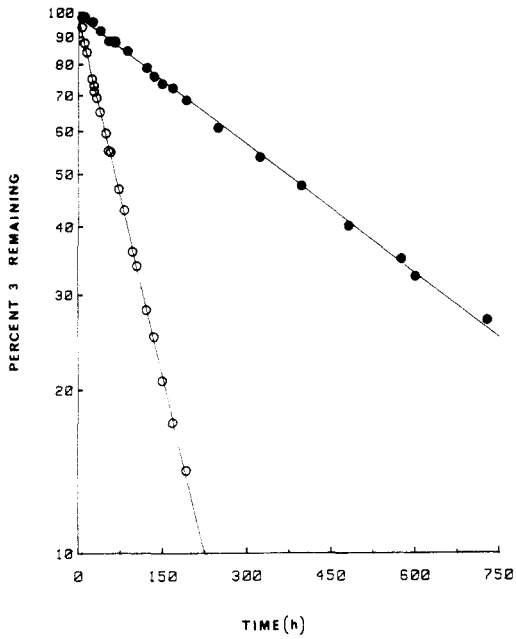


Figure 2. Plot of log (percent remaining) vs. time for the degradation of **3a** (open circles) and **3c** (solid circles) in aqueous solution at pH 0.82.

spectrum of **3a** is shown which contains the diastereotopic C-21 protons. The two protons are magnetically nonequivalent and have significantly different chemical shifts (4.50 and 4.08 ppm). Each of the two protons exhibit a gem coupling constant of 21 Hz resulting in a pair of doublets on either side of the C-11 proton multiplet. The spectrum of 21,21-dideuteriocloprednol (**3c**) in Figure 1B shows the complete absence of **3a** but approximately 4% **3b** as evidenced by the two singlets (no gem proton to couple with) whose chemical shifts are upfield approximately 0.03 ppm compared to the diprotio analogue.[20] The mixture shown in Figure 1C was obtained by reacting **3c** at pH 9.05 for 1.5 h and recovering the remaining **3**. The C-21 protons in this mixture appear as a pair of doublets, with the upfield peak of each apparent doublet predominating due to the large amount of **3b** present. The chemical shifts of the C-11 and upfield C-21 protons in Figure 1C are not superimposable with those in Figure 1A and 1B. This may be a consequence of the different temperature (47 °C for **1c** and 17 °C for **1a** and **1b**) and amount of D_2O present in each sample.

Quantitation of the extent of hydrogen incorporation at C-21 in partially degraded samples of **3c** was accomplished by integration of the combined C-21 proton signals for **3a** and **3b** (e.g., the pair of apparent doublets in Figure 1C) compared to the C-11 proton signal that acted as an internal standard representing one proton. The percent incorporation of *both* hydrogens (or percent exchange of both deuterons) was then calculated from eq 1.

$$\text{percent deuterium exchange} = \frac{\text{C-21 proton area}}{\text{C-11 proton area} \times 2} \times 100 \quad (1)$$

Results

The kinetics of degradation of both **3a** and **3c** were studied in aqueous solution at 60 °C and ionic strength 0.20. All solutions contained 1×10^{-4} M EDTA to prevent trace metal ion catalysis of degradation. The major products of the reaction throughout the pH region studied are similar to those described for hydrocortisone (**2**)[4] and are due exclusively to degradation of the C-17 side chain of the glucocorticoid.[21] Quantitation of the amount of drug remaining in degraded solutions was provided by reverse-phase HPLC. As Figure 2 shows, excellent adherence of the observed kinetics to the first-order rate law was found for both **3a** and **3c**. The observed rate constants for the degradation of **3a** (k_H) and **3c** (k_D) are shown in Table I as well as the calculated deuterium isotope effect (k_H/k_D) values.

Table I. Observed Rate Constants for the Degradation of 3a (k_H) and 3c (k_D) in Aqueous Solution at 60 °C

pH	buffer (concn, M)	$10^7 k_H$, s^{-1}	$10^7 k_D$, s^{-1}	k_H/k_D
0.82	HCl (0.20)	28.0	5.08	5.5
5.06	acetate (0.10)	6.99	2.10	3.3
5.06	acetate (0.05)	4.70	1.23	3.8
5.06	acetate (0.020)	2.86	0.727	3.9
6.80	phosphate (0.025)	10.9	4.85	2.3
9.06	CAPS (0.025)	34.5	33.2	1.0
9.07	CAPS (0.080)	35.9		

Contributions to the observed rate constants k_H and k_D made by buffer species present in solution could alter the kinetic isotope effect and possibly help to obscure the effect pH alone may have on k_H/k_D values. Examination of Table I shows that, in fact, catalysis of the k_H and k_D reactions by acetate at pH 5.06 is occuring but not to the same extent.[22] The result is that some dependence of k_H/k_D on buffer concentration is evident. This dependence appears slight at low buffer concentration, however, and $k_H/k_D = 3.9$ can be taken as the kinetic isotope effect at pH 5.06 in the absence of buffer catalysis. The k_H/k_D values obtained at pH 6.80 and 9.06 are likewise representative of the kinetic isotope effect in the absence of buffer catalysis since CAPS is not significantly catalytic (Table I) and phosphate is not expected to exhibit catalysis.[22] It is apparent, then, that in the absence of any buffer effect there exists a significant isotope effect dependence on pH.

Possible exchange of hydrogen for deuterium at C-21 in **3c** was studied in large-scale versions of the pH 0.82, 5.06 (0.020 M), and 9.05 buffer solutions described above for which k_H/k_D values were obtained. After partial degradation (monitored by reverse-phase HPLC) at 60 °C, the isotopic cloprednol mixture (**3a–c**) was isolated and purified by normal-phase semipreparative HPLC. ^1H NMR analysis of the C-21 region was then used to determine the extent of hydrogen incorporation into the C-21 position of the steroid.

The percent exchange (eq 1) and extent of degradation of **3c** are shown in Table II. It is apparent that the degree of exchange of hydrogen into the C-21 position is, like the isotope effect values, strongly dependent on pH. At pH 0.82, even after 43% degradation, no hydrogen incorporation at C-21 was observed, whereas at pH 5.06 some hydrogen incorporation was found after only 11% degradation. In basic solution at pH 9.05 the reaction was stopped before significant degradation occurred, yet incorporation of hydrogen at C-21 was evident.

If deuterium exchange in **3c** is a first-order rate process, crude rate constants for exchange (k_{exch}) can be calculated

(20) A similar deuterium isotope effect on the chemical shift of the proton in acetone-d_5 has been reported by H. S. Gutowsky, *J. Chem. Phys.*, 31, 1683 (1959).

(21) D. M. Johnson, unpublished results.

(22) Catalysis of hydrocortisone degradation by acetate ion but not by phosphate (pH 5.9–8.0) was reported by Hansen and Bundgaard.[6]

Table II. Deuterium Exchange at C-21 of 3c in Aqueous Solution at 60 °C

pH	buffer (concn, M)	time, h	% deuterium exchanged[a]	% degradation by HPLC
0.82	HCl (0.20)	335	0	43
5.06	acetate (0.020)	618	5	11
9.05	CAPS (0.025)	0.17	9	<1
9.05	CAPS (0.025)	1.5	34	2

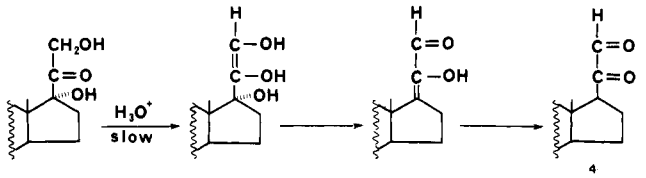
[a] Calculated from eq 1.

Table III. Deuterium-Exchange Kinetics and Isotope Effect as a Function of pH

pH	$10^7 k_{exch}$,[a] s^{-1}	k_{exch}/k_D	k_H/k_D
0.82	<0.08	<0.02	5.5
5.06[b]	~0.5	~0.7	3.9
6.80[c]			2.3
9.05	~1500	~45	1.0

[a] See footnote 23. [b] 0.020 M acetate buffer. [c] 0.025 M phosphate buffer.

Scheme I



from the data in Table II.[23] These values are shown in Table III as well as the ratio k_{exch}/k_D. As was found for k_H/k_D values (also shown in Table III), the rate of deuterium exchange relative to that for degradation is strongly pH dependent.

Discussion

The primary kinetic deuterium isotope effect of 5.5 observed for the degradation of cloprednol at pH 0.82 (Table III) demands that proton transfer occur in the rate-determining step at this pH. The magnitude of this deuterium isotope effect is similar to those found for acid-catalyzed enolization of ketones[24] and provides strong evidence that the degradation mechanism involves rate-determining enol formation. The absence of hydrogen incorporation into the C-21 position of **3c** even after extensive degradation (Table II) at pH 0.82 is also consistent with a mechanism in which the enolization step is rate determining. Enol formation also provides a logical route to the major degradation product of glucocorticoids at low pH, the 17-deoxyglyoxal steroid **4**.[4-6,9,10,13] Rate-determining acid-catalyzed enolization followed by dehydration and rearrangement shown in Scheme I thus provides a mechanistically reasonable pathway for degradation of these steroids at low pH.[25]

From an examination of the pH dependence of the deuterium isotope effect in Table III it is evident that a change in mechanism is occurring as the pH increases. The gradual decrease in deuterium isotope effect observed as the pH increases indicates that enolization is only partly rate determining in the neutral pH region and not at all under alkaline pH conditions. Enol formation under neutral to basic conditions is required, however, to explain the exchange of hydrogen for deuterium at C-21 in **3c** (Table II). Examination of the ratio k_{exch}/k_D in Table III shows that at low pH (pH 0.82) the hydrogen–deuterium exchange is slow or nonexistent compared to degradation of **3c**, whereas at higher pH, hydrogen–deuterium exchange is competitive with (pH 5.06) or much faster than degradation (pH 9.05).

A general mechanism demonstrating the role enolization plays in degradation reactions of glucocorticoids[25] is shown in Scheme II where the simplifying assumption that the enol is a common intermediate from which all products are formed has been made.

Scheme II

$$\text{ketone} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{enol} \xrightarrow{k_2} \text{product}$$

In this scheme, enol formation *must* precede product formation at low pH due to the large deuterium isotope effect observed. At neutral or basic pH, enolization is *required* by the hydrogen incorporation observed at C-21.[26] The pH dependence found for both k_H/k_D and k_{exch}/k_D means that while enolization is irreversible ($k_2 > k_{-1}$) and rate-determining ($k_1 < k_2$) at low pH, it gradually becomes reversible ($k_{-1} > k_2$) and nonrate-determining ($k_1 > k_2$) as the pH increases.[27] Elaboration of Scheme II to account for the kinetics of degradation of glucocorticoids as a function of pH is currently under investigation.

Acknowledgment. I thank Dr. M. Maddox and Ms. J. Nelson for obtaining the ^1H NMR spectra and Dr. I. Massey for obtaining the mass spectra.

Registry No. 3a, 5251-34-3; **3c**, 79817-27-9.

(23) The rate constant obtained from the data in Table II was doubled to account for the two exchangable deuterium atoms in **3c**. k_{exch} in Table III thus represents the rate constant for exchange of one deuterium at C-21.

(24) C. G. Swain, E. C. Stievers, J. F. Renwer, Jr., and L. J. Schaad, *J. Am. Chem. Soc.*, **80**, 5885 (1958).

(25) The intermediacy of an enol in dehydration reactions of dihydroxyacetone and reducing sugars containing the dihydroxyacetone moiety has been assumed for some time. G. L. Lookhart and M. S. Feather, *Carbohydr. Res.*, **60**, 259 (1978); J. C. Speck, Jr., *Adv. Carbohydr. Chem.*, **13**, 63 (1958). The enolization step was characterized as a reversible equilibrium reaction which now seems unlikely under acidic conditions.

(26) At neutral to basic pH, product formation directly from the ketone in Scheme II cannot be ruled out. In this case enolization would be a dead-end equilibrium step.

(27) Pitman and co-workers presumed that enolization was the rate-determining step in the degradation of hydrocortisone in alkaline solution.[15] Their claim that 21,21-dideuteriohydrocortisone was significantly more stable than its 21,21-diprotio analogue ($k_H/k_D > 1$) in 0.05 M NaOH containing 0.1% EDTA and oxygen is not supported by our findings and is viewed with suspicion since pseudo-first-order kinetics were not observed. Hansen and Bundgaard[6] did observe pseudo-first-order kinetics for hydrocortisone degradation under conditions similar to those used by Pitman and co-workers.