Deuterium Isotope Effects on Degradation of Hydrocortisone in Aqueous Solution

IAN H. PITMAN[A], T. HIGUCHI, M. ALTON, and R. WILEY

Abstract □ C-21-Dideuteriohydrocortisone (I) was synthesized and its stability against degradation in aqueous solution under both aerobic and anaerobic conditions was determined and compared with that of hydrocortisone (II). At pH values between 9 and 13, I was significantly more stable than II when oxygen and 0.1% disodium edetate were present but it appeared to degrade more rapidly than II when oxygen was excluded from the system. At pH 7 and 35°, both I and II were stable during 96 days in the presence of air and 0.1% disodium edetate but they did degrade slowly when the disodium edetate was excluded. Under these latter conditions, I was again more stable than II. A possible explanation for the enhanced stability of I over II under oxidative conditions is that the reaction path for these degradations involves a rate-determining enolization in the C-17-dihydroxyacetone side chain.

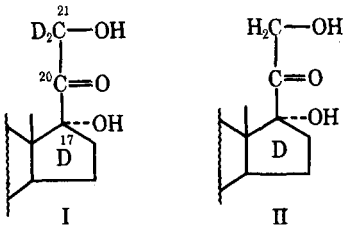
Keyphrases □ Hydrocortisone aqueous degradation—effect of C-21-dideuterio substitution □ Deuterium isotope effect—hydrocortisone aqueous degradation □ Steroids, C-17-dihydroxyacetone side chain—deuterium isotope effect on aqueous degradation of hydrocortisone

The aim of this study was to determine whether C-21-dideuteriohydrocortisone (I) was more stable against degradation in aqueous solution than hydrocortisone (II). If I was more stable than II, it is likely that substitution of deuterium atoms for hydrogen atoms at C-21 in all steroids with a C-17-dihydroxyacetone side chain would yield compounds with greater stability in aqueous solution than the original steroid. No studies have yet been undertaken to determine whether the pharmacological activities of I and II differ significantly. However, the structural differences between these two compounds are so small that it is reasonable to predict that they would have similar activities.

Previous studies indicated (1–3) that the degradation of steroids with a C-17-dihydroxyacetone side chain (*e.g.*, prednisolone) in water results primarily from reactions of the side chain. A variety of both oxidative and nonoxidative reactions have been postulated to occur, and some of these are shown in Scheme I.

The rationale for predicting that the C-21-dideuterium derivatives may degrade more slowly than the C-21-dihydrogen derivatives was as follows:

1. The dihydroxyacetone moiety contains an enolizable carbonyl group, and it is likely that enolization may precede all or some of the degradation reactions.



2. Base-catalyzed enolization reactions are known (4) to exhibit a deuterium isotope effect. For example, the rate of enolization of α-deuterio ketones is slower than that of α-protio ketones by a factor of about 5.

3. Consequently, if enolization of the C-20-carbonyl was the rate-determining step in any degradation reaction of a steroid, the deuterio derivative should degrade more slowly than the protio compound.

To test this prediction, the rates of degradation of I and II in aqueous solutions under a variety of conditions were measured and compared.

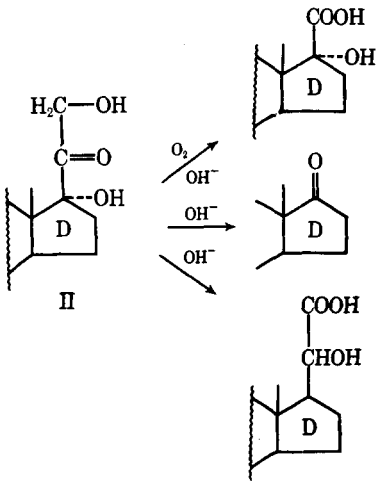
EXPERIMENTAL

Hydrocortisone (II)—Hydrocortisone[1] was recrystallized from methanol (m.p. 215–217°). Its elemental analysis was consistent with the theoretical value.

C-21-Dideuteriohydrocortisone (I)—Hydrocortisone[1] was refluxed for 6 hr. in methyl alcohol-*d*[2] containing sodium methoxide. Tartaric acid was then added to neutralize the sodium methoxide and, after evaporation of the excess methyl alcohol-*d*, the deuteriohydrocortisone was crystallized. The product was recrystallized from ordinary methyl alcohol to exchange the most labile deuterium atoms (*e.g.*, those on the 11-, 17-, and 21-OD groups) with hydrogen. The crystals had a melting point of 211°.

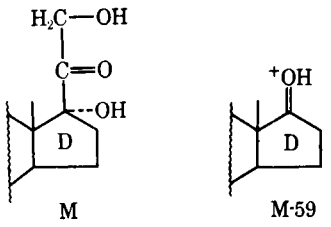
Anal.—Calc. for $C_{21}H_{28}D_2O_5$: C, 69.13; H + D, 8.78. Found: C, 68.99; H, 8.55.

Structure of Deuteriohydrocortisone—A characteristic fragmentation of hydrocortisone in a mass spectrometer has been shown (5) to be due to the loss of the C-17-dihydroxyacetone side chain in the reaction illustrated in Scheme II. Consequently, in the mass spectrum of hydrocortisone (II), there was a peak for the parent molecular ion, M (at *m/e* 362), and a peak for the fragment without the C-17-dihydroxyacetone side chain, M-59 (at *m/e* 303). The mass spectrum



Scheme I—Some suggested (1) routes of degradation in water of steroids with the 17-dihydroxyacetone side chain

[1] Steraloids, Inc., Pawling, N. Y.
[2] Diaprep, Inc., Atlanta, Ga.



Scheme II

of the deuterated hydrocortisone had seven peaks which corresponded to parent molecular ions at *m/e* values between 362 and 368. The peak at *m/e* 362 corresponds to the molecular ion of II, and each of the other peaks corresponds to molecular ions of hydrocortisone containing from one to six deuterium atoms.

The relative amounts of molecules containing two or more deuterium atoms were estimated to be greater than 96% by comparing the sum of the intensities of peaks with *m/e* values between 364 and 368 to the sum of the intensity of peaks with *m/e* values between 362 and 368. Each peak for molecular ions containing two or more deuterium atoms had a corresponding fragment ion peak at M-61. Hence, it appears certain that two of the deuterium atoms in each case were on the dihydroxyacetone side chain on C-21. The position of extra deuterium atoms was not determined, but their presence or absence from various positions in the nucleus of the steroid molecule was not expected to affect significantly chemical reactions of the C-17 side chain.

NMR Spectrum of C-21-Dideuteriohydrocortisone—The fact that dideuteration of hydrocortisone had occurred at C-21 was confirmed by the absence of the C-21-methylene protons from the NMR spectrum of the deuterated material. This evidence could not be obtained unequivocably from observation of the NMR spectra of the two compounds in dimethyl sulfoxide-d_6 because of an overlap of the C-21-methylene proton peak (δ = 4.28 p.p.m.) with that of the C-11-hydroxy group (δ = 4.25 p.p.m.) (6). However, when trichloroacetylisocyanate[3] was added to a $CDCl_3$ suspension of II, it reacted with the hydroxy group on C-11 (7) to produce a soluble carbamate, and it displaced the chemical shift for the methylene protons of hydrocortisone to an isolated peak at δ = 5.1 p.p.m. This peak was absent from the spectrum of I when it was treated similarly.

Rate of Degradation of I and II—Unless otherwise stated, reactions were carried out in 0.1% solutions of disodium edetate in aqueous 5×10^{-2} *M* sodium hydroxide or borate buffers in black-painted glass containers. Disodium edetate was included to sequester metal ions and eliminate metal-ion-catalyzed reactions (2). Light was excluded to inhibit any possible photolytic reactions. Although either or both of these reaction paths may be important in the degradation of real formulations, their sensitivity to deuterium isotope effects was not examined in the present study. Reactions were carried out with varying amounts of oxygen by the following procedures.

Anaerobic Experiments—The reaction solutions were prepared in freshly boiled and cooled glass-distilled water through which oxygen- and carbon dioxide-free nitrogen (washed with aqueous pyrogallol and sodium hydroxide) had been bubbled for at least 1 hr. At the commencement of the experiment (t = 0 min.), 1 ml. of dimethylacetamide containing a known amount of I or II was pipeted into a volumetric flask containing 200 ml. of the reaction solution which had equilibrated at the reaction temperature. The flask was then flushed with nitrogen, stoppered, shaken vigorously, and clamped in a water bath. At appropriate time intervals the flask was opened, 10- or 20-ml. samples were removed for assay, and the flask was flushed again with nitrogen and stoppered.

Aerobic Experiments—The reaction solutions used in the aerobic experiments were not degassed with nitrogen but were either equilibrated with air at atmospheric pressure or saturated with oxygen. Equilibration with air was achieved by vigorously agitating the reaction solution in an open beaker for at least 1 hr. before the experiment. Saturation with oxygen was achieved in the same manner as saturation with nitrogen in the anaerobic experiments. In each case the equilibration was achieved at the experimental temperatures. Some experiments were carried out in open beakers under atmospheric conditions. In other cases, the reaction solution was kept in a volumetric flask under an atmosphere of oxygen, or it was divided into 10- or 20-ml. aliquots as soon as the dimethylacetamide solutions of I or II had been introduced and these aliquots were sealed in black vials with aluminum-lined caps and stored in a water bath. Oxygen was kept bubbling through the solutions used in those experiments where it was desired to study reactions under high oxygen tensions. In all cases, aliquots were removed at appropriate times and assayed for undegraded I or II.

Assay Procedure—The assay procedure described by Guttman and Meister (1) was followed very closely. Values of the absorbance of the product of the reaction between I and II and blue tetrazolium were converted to concentrations of I and II by using previously constructed calibration curves.

[3] Eastman Organic Chemicals, Rochester, N. Y.

Table I—Rate Data for Decomposition of I and II in Aqueous Solution[a]

10^2 [NaOH], *M*	Temperature	10^4 [O_2][b], m*M* ml.$^{-1}$	10^4 [I], m*M* ml.$^{-1}$	10^4 [II], m*M* ml.$^{-1}$	$t_{1/2}^{I}$, min.	$t_{1/2}^{II}$, min.	$t_{1/2}^{I}/t_{1/2}^{II}$
5	20.5°	13.4[c]	2.96	—	172	—	3.0
5	20.5°	13.4[c]	—	3.46	—	57	
5	50°	0.5[d]	3.47	—	29	—	1.3
5	50°	0.5[d]	—	3.44	—	22	
5	50°	2.6[e]	3.29	—	15	—	2.5
5	50°	2.6[e]	—	3.44	—	6	
5	50°	2.6[e]	—	6.90	—	7	
5	50°	2.6[e]	—	13.90	—	4	
5	50°	0[f]	3.85	—	59	—	0.7
5	50°	0[f]	—	6.39	—	83	
10	50°	0[f]	3.48	—	29	—	0.9
10	50°	0[f]	—	3.61	—	33	

[a] Each solution contained 0.1% disodium edetate and was protected from light. [b] Estimated as described in the text. [c] Oxygen was bubbled through the reaction mixture during the experiment. [d] In open beakers with carbon dioxide-free air circulating over the surface. [e] In sealed volumetric flasks whose contents had previously been saturated with oxygen at 50°. [f] In sealed volumetric flasks whose contents had previously been saturated with nitrogen at 50°.

RESULTS AND DISCUSSION

Mass spectral and NMR evidence indicated that the deuterated hydrocortisone (I) had two deuterium atoms at C-21 and from one to four deuterium atoms at other sites in the molecule. Because the assay procedure used in the degradation studies only distinguished between molecules of steroid with and without the dihydroxyacetone side chain, it has been assumed that the deuterium atoms which

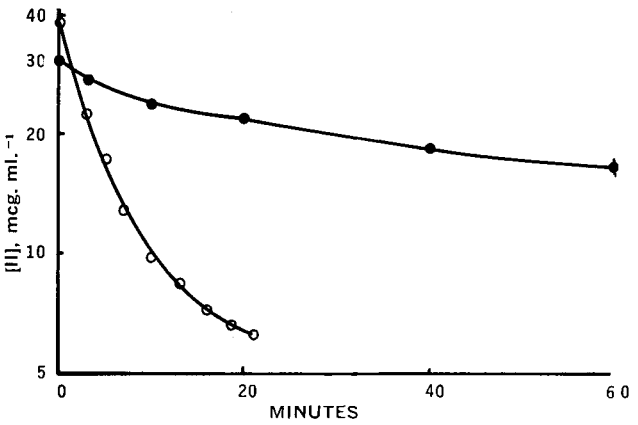


Figure 1—*Plots against time of logarithms of residual concentrations of hydrocortisone during its degradation at 50° in 0.05* M *NaOH (containing 0.1% disodium edetate) under* (○) *aerobic conditions with an initial oxygen concentration of 2.6 m*M *ml.*$^{-1}$ *and* (●) *anaerobic conditions.*

Table II—Course of Degradation of I and II[a] in Sealed Vials at 35° Containing 0.05 *M* NaOH and 0.1% Disodium Edetate

Time after Degradation Commenced, min.	Percent Degradation of I (1)	Percent Degradation of II (2)	Ratio (2)/(1)
11	10	15	1.5
21	12	23	1.9
46	34	40	1.2
121	61	56	0.9

[a] $[I]_{initial} = [II]_{initial} = 6.9 \times 10^{-4}$ m*M* ml.$^{-1}$. $[O_2]_{initial} = 1.58 \times 10^{-4}$ m*M* ml.$^{-1}$.

are not associated with the side chain had little or no effect on the reactions studied.

The assay procedures used in the degradation studies yielded values of residual concentrations of I and II. As can be seen from the typical examples in Fig. 1, plots against time of the logarithms of these residual concentrations were not linear for the reactions carried out under either aerobic or anaerobic conditions. Hence, in contrast to the inferences drawn in other studies (1, 2), it was not possible to describe the degradation of I or II by simple first-order rate laws. Interpretation of the kinetics in absolute terms was further complicated by the fact that the extent of the oxidative reactions varied with both the steroid and the oxygen concentration. This point is discussed later. Because of these complexities of the kinetics, no attempts were made in the present study to calculate absolute rate constants. Rather, relative rates of degradation of I and II were obtained by comparing $t_{1/2}$ values or the extent of degradation after particular time intervals in experiments in which approximately equimolar amounts of starting material were used and in which identical experimental procedures were followed.

The initial concentration of dissolved oxygen in solutions that had equilibrated with air was estimated using the method suggested by Schroeter (8). When equilibration had been carried out in an atmosphere of pure oxygen, the concentration estimated for equilibration with air was multiplied by 5 to account for the increased partial pressure of oxygen.

The results in Table I show that the deuterated compound (I) degraded less rapidly than II in alkaline solutions containing disodium edetate under aerobic conditions. For example, in the presence of a high constant concentration of dissolved oxygen (maintained by constantly bubbling oxygen through the solution at 20.5°), the value of the ratio $t_{1/2}^{I}/t_{1/2}^{II}$ was 3.0.

At higher temperatures, II still degraded more rapidly than I as long as oxygen was present but the ratio of their half-lives was considerably less than 3. This reduction in the ratio could be caused by the reduction in the concentration of dissolved oxygen which results at higher temperatures or by mechanistic changes brought about by increasing the temperature or by both factors. The present study was not aimed at delineating these factors but simply at observing the relative stabilities of the species under a variety of conditions. If the mechanism of the oxidation reaction is similar to that postulated (9) for the alkaline autoxidation of the α-ketal, benzoin, it is likely that the reaction rate is independent of the oxygen concentration as long as sufficient oxygen is present to react with any enolized form of the steroid. Such a mechanism would be expected to exhibit a deuterium isotope effect in the observed direction (4), because the enolization reaction would be rate determining. When all available oxygen had been consumed, the enolization reaction would not necessarily lead to degradation.

Similar results were also obtained at pH 9 in a borate buffer.

At pH 7 under aerobic conditions, neither I nor II degraded more than 10% during 96 days in the presence of 0.1% disodium edetate. However, when the latter metal-ion-sequestering agent was not included, slow degradation of I and II did occur. After 300 hr. at 35° and pH 7.3 in the absence of disodium edetate, the ratio of degraded II to I was 2.9. Hence, at pH 7.3 it appears that a metal-ion-catalyzed reaction predominates and that this reaction is significantly slowed up by replacing the hydrogen atoms at C-21 by deuterium atoms.

When oxygen was excluded from the reaction system (anaerobic conditions), the degradation of I appeared to proceed slightly faster than that of II. This was an unanticipated result and its explanation is not immediately obvious. However, the fact that this reversal in reactivity occurs becomes important when considering the relative stabilities of solutions of I and II which are sealed in airtight containers together with a small amount of air. Under these conditions, it would be predicted that II would degrade faster than I until all the molecular oxygen in the system had been consumed but that I would then degrade faster than II as long as anaerobic conditions were maintained. Evidence that this sequence of events occurs is provided by the results in Table II. Here it can be seen that the ratio of percentage degradation of II to that of I fell from a value of >1 after 10 min. (when a significant amount of oxygen was still present) to <1 after 120 min. (when all the oxygen had been consumed). Hence, these results suggest that I is appreciably more stable than II if the major degradation route is oxidative. Under anaerobic conditions, it appears that I would not be more stable than II but that it may even degrade more rapidly.

REFERENCES

(1) D. E. Guttman and P. D. Meister, *J. Amer. Pharm. Ass., Sci. Ed.*, **47**, 773(1958).

(2) T. O. Oesterling and D. E. Guttman, *J. Pharm. Sci.*, **53**, 1189 (1964).

(3) References in *References 1* and *2*.

(4) T. Riley and F. A. Long, *J. Amer. Chem. Soc.*, **84**, 522(1962).

(5) M. Spiteller-Friedman and G. Spiteller, *Org. Mass Spectrom.*, **2**, 901(1969).

(6) B. Hampel and J. M. Krämer, *Chem. Ber.*, **98**, 3255(1965).

(7) R. Trehan, C. Monder, and A. K. Base, *Tetrahedron Lett.*, **1968**, 67.

(8) L. C. Schroeter, in "Sulfur Dioxide," Pergamon, New York, N. Y., 1966.

(9) W. Weissberger, *Biol. Antioxidants, Trans. 3rd Conf.*, **1948**, 33.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 27, 1971, from the *Department of Analytical Pharmaceutical Chemistry and Pharmaceutics, University of Kansas, Lawrence, KS 66044*

Accepted for publication March 2, 1972.

Presented in part to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, San Francisco meeting, March 1971.

Supported in part by a grant from Alza Corp., Palo Alto, Calif.

▲ To whom inquiries should be directed.