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THE STEREOCHEMISTRY OF ENZYMIC REDUCTION OF 21-DEHYDROCORTISOL TO CORTISOL

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SUMMARY

A 21-hydroxysteroid dehydrogenase of sheep liver that causes reduction of 21-dehydrocortisol to cortisol does so with transfer of hydrogen from the 4-pro-S-position of NADH to the 21-pro-S-position of cortisol.

A soluble dehydrogenase present in sheep liver catalyzes reduction by NADH of the aldehyde, 21-dehydrocortisol (I), to cortisol (II, X = βOH) [1]. Other enzymes catalyze oxidation of cortisol at position 21 to carboxylic acids [2, 3]. Examination of the fate of the 21-hydrogens *in vivo* required corticosteroids labeled at the 21-R- and 21-S positions. The present study provides two lines of evidence that establish the stereochemistry of reduction by the 21-hydroxysteroid dehydrogenase [4].

Nuclear magnetic resonance allows distinction to be made between the prochiral 21-hydrogen atoms of cortisol. By formation of a silicon derivative of cortisol, the 17 and 21-hydroxyl groups were linked in a cyclic ether that prevented rotation of the steroid side chain. By varying substitution at the 11-position on the steroid nucleus, one 21-hydrogen of this derivative, which appears relatively downfield in n.m.r., was found to be more influenced by the nature of the substituent at the 11-position than was the other 21-hydrogen. This downfield hydrogen was found by deuterium labeling to correspond to that introduced enzymically from NADH. From consideration of models, it appeared likely that this is the 21-pro-S-hydrogen of cortisol.

This assignment was confirmed by using the 21-hydroxysteroid dehydrogenase to introduce tritium at the 21-position of cortisol. Oxidative cleavage of the side chain gave the labeled two-carbon fragment, glycollic acid; the stereochemistry of the label was determined by incubation with glycollate oxidase, an enzyme known to remove the glycollate 2-pro-R hydrogen. Both lines of evidence point to the transfer of hydrogen from NADH to the 21-pro-S position of cortisol by the 21-hydroxysteroid dehydrogenase.

The n.m.r. spectrum of cortisol in [$^2H_5$]-pyridine contains a quartet due to the geminal hydrogens of the 21-position, J 19 Hz [5], to which we assign δpro-S 5·24 ppm downfield from tetramethylsilane, δpro-R 4·80 ppm. The farthest upfield line of the quartet is partially obscured by the broad peak of the 11α-hydrogen. Addition of a drop of diacetoxydimethylsilane [6, 7] to the solution at 31°C in the n.m.r. tube causes rapid (<1 min) conversion to the 21-dimethylsilylacetoxy derivative (III, X = βOH) with a marked downfield shift of the 21-H_2 quartet (J 19 Hz, δ21-pro-S 5·456 ppm, δ21-pro-R 5·019 ppm). All four lines are then clearly visible. Slower conversion (virtually complete in 40 min) to the six membered ring silicon ether (IV, X = βOH), in which the 21-H_2 quartet is shifted considerably upfield (J 16·3 Hz, δpro-S 4·66 ppm, δpro-R 4·401 ppm), then takes place. The use of the steroids stereospecifically deuterated at the 21-position established that the downfield proton remained the downfield proton during the series of transformations (II)→(III)→(IV).

A similar two-step sequence was observed with cortisone (II, X = 0=) and with cortexolone (17,21-dihydroxypregn-4-ene-3,20-dione) (II, X = H_2), in both of which the farthest upfield line of the original 21-H_2 quartet is not obscured by the broad singlet of the 11α-H, as it is in free cortisol. Although the 21-H_2 quartet of the cyclic derivative (IV, X = βOH) of cortisol was so closely spaced as to appear to be a singlet in many runs, the corresponding quartets derived from cortisone and cortexolone were clearly visible. Calculation of the chemical shifts of the two hydrogens of the cyclic silicon ethers (IV) showed that in all three steroids, the upfield proton is little affected by the nature of the substituent at the 11-position, while the downfield proton of the spiro derivative of cortisol (IV, X = βOH) is farther upfield than that of the spiro derivative from cortisone (IV, X = 0 =) or 17α,21-dihydroxypregn-4-en-3-one (IV, X = H_2). Repetition of the study in two other solvent systems [$^2H_8$]-tetrahydrofuran (containing 4% [$^2H_5$] pyridine as a catalyst for the reaction) and [$^2H_7$] dimethylformamide, which needed no additional catalyst, confirmed the greater variability of the downfield proton (Table 1).

4-S-[4-^{2}H]-NADH was prepared by reduction of [4-^{2}H]-NAD with ethanol and yeast alcohol dehydrogenase. This cofactor with the 21-hydroxysteroid dehydrogenase and 21-dehydrocortisol gives cortisol (42 and 56% d_1 in two experiments), in which the remaining 21-hydrogen of those molecules containing

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Table 1. The chemical shifts (ppm from internal tetramethylsilane) of the downfield and upfield 21-hydrogens of the corticosteroid 17,21-cyclic dimethylsilyl ethers in three solvent systems

	Cortexolone		Cortisol		Cortisone	
	Downfield	Upfield	Downfield	Upfield	Downfield	Upfield
[$^2H_5$]-Pyridine	4·524	4·425	4·466	4·401	4·528	4·396
[$^2H_8$]-Tetrahydrofuran	4·355	4·221	4·309	4·212	4·388	4·213
[$^2H_7$]-Dimethylformamide	4·434	4·261	4·380	4·241	4·514	4·246

deuterium appears in the n.m.r. spectrum as a narrow triangle. The position of this signal (4·83 ppm) corresponds to a remaining upfield proton, shifted 0·03 ppm farther upfield by an isotope effect. Similar upfield shifts caused by geminal deuterium have been observed [8].

The epimeric [21-^{2}H]-cortisol was synthesized as follows. 21-Dehydrocortisol (I) in pyridine was converted with one equivalent of sodium borodeuteride to [21-^{2}H]-cortisol with little stereoselectivity (54% 21-S; 46% 21-R). The reaction was followed by the marked change in position of the 18-H_3 from 1·492 ppm (21-dehydrocortisol) to 1·275 ppm (cortisol).* Reoxidation of the [21-^{2}H]-cortisol with cupric acetate gave the [21-^{2}H] aldehyde, which on enzymic reduction with unlabeled NADH gave 21-R-[21-^{2}H]-cortisol (79 and 60% d_1 in two experiments). The remaining 21-hydrogen attached to those molecules bearing deuterium gives the characteristic triangular peak shifted upfield by 0·03 ppm from the calculated line position of the downfield 21-hydrogen. It is concluded that the downfield hydrogen of cortisol corresponds to that introduced from NADH by the 21-hydroxysteroid dehydrogenase. A parallel study of the epimeric [21-^{2}H]-cortisones is entirely consistent with that of cortisol.

From models of the corticosteroid cyclic 17,21-dimethylsilyl ethers (IV) it appears that they are likely to exist in the conformationally mobile boat form [11, 12] in which the 21-pro-S hydrogen is considerably closer in space to C-11 than is the 21-pro-R hydrogen. Since there are six bonds interposed between C-11 and the 21-hydrogens, no simple through-bond influence of the 11-substituent on the 21-hydrogens would be expected, and it can reasonably be argued that the downfield 21-hydrogen, the one more influenced in its chemical shift, would be 21-pro-S.

The assignment of 21-pro-S stereochemistry to the more variable, downfield doublet of the quartet was not entirely unequivocal. Conformational transmission might cause the silicon-containing ring to take up different conformations dependent on the nature of the 11-substituent, and might in turn cause greater change in the chemical shift of the more remote 21-pro-R-hydrogen than of the 21-pro-S hydrogen. Independent evidence for the stereochemistry of enzymically 21-labeled cortisol was therefore obtained.

Enzymic reduction of 21-dehydrocortisol with 4-S-[4-^{3}H]-NADH gave [21-^{3}H]-cortisol. This was acetylated at the 21-position and the steroid 17,20-bond cleaved by sodium bismuthate to give, from the side chain, [2-^{3}H]-glycollic acid acetate. Hydrolysis of the acetate was effected with refluxing aqueous sodium carbonate, and the reagent removed by addition of Dowex 50 in the acid form. Chromatography of the neutralized aqueous solution on Dowex-1 chloride gave [2-^{3}H]-glycollic acid. Oxidation of the glycollic acid so prepared with glycollate oxidase from spinach leaves [10] in the presence of flavin adenine dinucleotide and catalase gave the corresponding aldehyde, glyoxylic acid, with retention of tritium. Spinach glycollate oxidase is known [13] to transfer the 2-pro-R-hydrogen of glycollate to water, leaving the originally 2-pro-S hydrogen attached to the glyoxylate. The 2-pro-S-hydrogen of glycollate, which corresponds to the 21-pro-S hydrogen of cortisol, must therefore be that introduced from NADH by the 21-hydroxysteroid dehydrogenase.

A parallel series of transformations was carried out on the epimeric [21-^{3}H]-cortisol derived by reduction of [21-^{3}H]-21-dehydrocortisol with unlabeled NADH. In this case, glycollate oxidase caused transfer of the tritium to water, leaving the glyoxylate unlabeled. The tritium had therefore occupied the position of the 2-pro-R hydrogen of glycollate, and consequently the 21-pro-R position of cortisol. Both glycollate oxidase incubations therefore indicated that the hydrogen transferred to 21-dehydrocortisol from NADH by the 21-hydroxysteroid dehydrogenase takes up the 21-pro-S position of cortisol; a result in accord with the stereochemistry of the dehydrogenase reaction deduced from the n.m.r. studies.

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* Surprisingly, in [^{2}H] chloroform there is apparently little difference in chemical shift between the 18-H_3 of cortexolone and of the corresponding 21-aldehyde [9].

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