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Studies on the Functional Specificity of Cortisone Reductase

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Crystalline preparations of cortisone reductase (EC 1.1.1.53) have certain 3- and 20-hydroxy steroid-NAD⁺ oxidoreductase activities that may possibly depend on one active centre with complex steroid-binding properties (Gibb & Jeffery, 1971). Studies are now described in which a series of test substrates was used to elucidate certain aspects of the specificity of these reactions. It was shown that there is no absolute requirement for the hydrogen added to or removed from C-3 to be α , β , axial or equatorial, or for the chirality at C-3 to be *R* or *S*. In the 5 α -androstan-3-one compounds studied, the hydrogen added to or removed from C-3 was β and equatorial, but C-3 had *R* or *S* chirality. The 5 β -androstan-3-one compounds studied were very poor substrates, and among these compounds preference was found for utilization of 3 β -hydrogen, which was axial and C-3 *R*, rather than 3 α -hydrogen, which was equatorial and C-3 *S*, though the latter was observed. These poor substrates were utilized so inefficiently that it was not possible to establish kinetically the relationship between the slight 3 β -hydroxy steroid dehydrogenase activity and either the 3 α - or the 20-hydroxy steroid dehydrogenase activity.

The non-steroid adamantanone was a substrate, undergoing a reaction clearly analogous to the 3-hydroxy steroid dehydrogenase activity towards

steroids, but achiral, and equally related to 3 α , 3 β , 3-axial and 3-equatorial activity. This activity was not kinetically independent of the 20-hydroxy steroid dehydrogenase activity (Gibb & Jeffery, 1972).

The hydrogen added to or removed from C-20 was *R* or *S*, but was consistently 20 α in the compounds so far investigated.

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Studies on the Active Centre for the 3-Hydroxy Steroid-Nicotinamide-Adenine Dinucleotide Oxidoreductase Activity of Cortisone Reductase

By WILLIAM GIBB and JONATHAN JEFFERY (*Department of Obstetrics and Gynaecology and Department of Chemical Pathology, University of Aberdeen, Aberdeen AB9 2ZD, U.K.*)

Kinetic studies were carried out with 17 β -hydroxy-5 α -androstan-3-one (I), a series of related steroids differing in structure only in ring A, and the analogous non-steroid adamantanone. The 1 α -methyl and 2 α -methyl derivatives of compound (I) had apparent *K_m* and apparent *V* values similar to those of compound (I). The 1 β -methyl and 4 α -methyl derivatives of compound (I) were not substrates, but were moderately effective inhibitors. The 1 α -hydroxy derivative of compound (I) was a very poor substrate with high apparent *K_m* and low apparent *V*. These findings would allow the view that the binding site is hydrophobic in the region around the A-ring; that it is possibly of somewhat more restricted dimensions (or lower flexibility) in the direction across the A-ring (1 β –4 α), and somewhat bigger than a steroid (or highly flexible) in the region out of the plane of the A-ring (i.e. from above the β -side to below the α -side). This would lead to an estimate of roughly 0.60×0.52nm for the minimum dimensions of the site where the A-ring binds. Such a view could accommodate in a general way the further findings that 17 β -hydroxy-5 β -androstan-3-one and the non-steroidal but analogous adamantanone were poor substrates; and even that 17 β -hydroxy-1 α ,2 α -methylene-5 α -androstan-3-one and the corresponding 1 β ,2 β -methylene compound were moderately effective inhibitors, though it is less clear why these two compounds were not substrates. Certain other

relationships and various matters of detail are similarly difficult to account for, and are the subject of further studies.

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Studies on the Reaction of Glutamate Dehydrogenase with Ethoxyformic Anhydride

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The use of ethoxyformic anhydride for studying the acylation of histidine residues has considerable advantages over other similar reagents. The extent of the reaction can be followed spectrophotometrically by measuring the increase in absorption at 242 nm due to the formation of ethoxyformylhistidine residues (Ovadi *et al.*, 1967). The reagent is, however, not specific for histidine residues, but is susceptible to nucleophilic attack from tyrosine, cysteine, lysine and tryptophan residues (Rosen & Fedorcsak, 1966; Múhlrad *et al.*, 1967), although at pH 6.1 it reacts preferentially with histidine residues. The reagent has proved particularly useful in the present studies on glutamate dehydrogenase.

Reaction of the reagent at pH 6.1 in 0.1 M-sodium acetate-acetic acid buffer containing 0.1 M-NaCl produced a rapid loss of about 40% of the initial activity as a result of the acylation of a single histidine residue. Over a longer period of time two further residues could be modified without any further loss of activity. When the enzyme was treated with the reagent at pH 7.5 in 0.1 M-tris-HCl buffer containing 0.1 M-NaCl complete loss of enzymic activity occurred after modification of only two histidine residues. When the enzyme was first allowed to react at pH 6.1 and the pH then adjusted to 7.5 only a single residue reacted at the latter pH, although this resulted in the complete loss of the enzymic activity. If the excess of ethoxyformic anhydride was removed after the pH 6.1 treatment by gel filtration loss of activity was not observed on adjusting the pH to 7.5 unless further anhydride was added.

The reagent appeared to react only with histidine residues at both pH 6.1 and 7.5. Analysis of the difference spectrum produced on modification of the enzyme showed none of the characteristics associated with ethoxyformylation of the aromatic

residues. In addition, no decrease in the number of cysteine residues present was observed. It was not possible to exclude reaction with all the lysine residues present in the molecule, although lysine-97, which reacts specifically with pyridoxal phosphate (Piszkiwicz *et al.*, 1970) and *N*-(*N*'-acetyl-4-sulphamoylphenyl)maleimide (Holbrook & Jeckel, 1969), was not acylated.

Both the loss of activity at pH 7.5 and the extent of ethoxyformylation were decreased by the presence of glutamate. This protection was not observed at pH 6.1, suggesting that the residue protected was important for activity but was not available for acylation at pH 6.1. At pH 7.5 NAD⁺ did not affect the loss of activity but it decreased the extent of modification of the enzyme.

The implications of these results will be discussed.

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The Reduced Nicotinamide-Adenine Dinucleotide Phosphate- and Oxygen-Dependent *N*-Oxygenation of Trimethylamine by *Pseudomonas aminovorans*

By P. J. LARGE, C. A. BOULTON and M. J. C. CRABBE (*Department of Biochemistry, University of Hull, Hull, HU6 7RX, U.K.*)

Extracts of *Pseudomonas aminovorans* grown on trimethylamine contain an enzyme catalysing the NADPH- and oxygen-dependent conversion of trimethylamine into trimethylamine *N*-oxide. The specific activity of the enzyme in cells grown on other methylamines or succinate was only about 2-3% of the activity in trimethylamine-grown cells.

The enzyme was assayed spectrophotometrically by measuring the rate of trimethylamine-dependent oxidation of NADPH spectrophotometrically at 340 nm. A number of other tertiary amines were active substrates, including ethyldimethylamine, diethylmethylamine, *NN*-dimethylethylenediamine, *NN*-dimethylethanolamine, triethylamine, *NN*-dimethylpropylenediamine, 3-dimethylaminopropan-1-ol, 3-dimethylaminopropan-2-ol and *NN*-dimethyl-2-chloroethylamine. All substrates showed inhibition at concentrations above about 1 mM. NADPH was