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The Specificity of a 7α-Hydroxy Steroid Dehydrogenase from *Escherichia coli*

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1. Thirty-eight steroids were tested as substrates for a 7α-hydroxy steroid dehydrogenase preparation from a strain of *Escherichia coli*; an improved method of making the crude enzyme is described. 2. Steroids having a 7α-hydroxyl group in the molecule were substrates except (*a*) when the 5β-cholan-24-oic acid side chain was shortened to less than four carbon atoms and (*b*) in certain cases when sulphate ester groups were present in the molecule. 3. For testing with the enzyme, a new specimen of 7α-hydroxy-3,12-dioxo-5β-cholan-24-oic acid was made, which had properties different from those previously described.

Aries & Hill (1970) described the isolation from micro-organisms of enzymic preparations that had specific 7α-hydroxy steroid dehydrogenase activity and required NAD^+ or $NADP^+$ as co-factors. Haslewood *et al.* (1973) also made such a preparation from *Escherichia coli* and applied it to the estimation of bile acids and their conjugates having 7α-hydroxyl groups; coincidentally similar and more extensive work on the use of a 7α-hydroxy steroid dehydrogenase was done by Macdonald *et al.* (1973).

We have now investigated the specificity of our enzyme preparation: a preliminary note of some of our tests has appeared (Haslewood & Haslewood, 1975).

Materials and Methods

Enzyme preparation

The method is modified from Haslewood *et al.* (1973). *Escherichia coli* strain O18 acK?H$^-$ (Haslewood *et al.*, 1973) was taken from freeze-dried stock and cultured on nutrient agar slopes contained in small rubber-stoppered bottles. A sub-culture was made from a slope on to a blood-agar plate and incubated at 37°C for 24h. This plate was kept in the refrigerator and used for enzyme preparation over 4–6 weeks.

To prepare the enzyme, up to 50 nutrient agar plates were cultured from the blood-agar plate at any one time and incubated at 37°C for 24h. Two L-shaped glass rods were used to scrape off the growth from the plates into 10ml of 0.02M-Tris buffer containing EDTA (1g/l) and adjusted to pH7.2 with 1M-HCl. The suspension was left at 4°C for 1h, with occasional stirring, and then treated with 50ml of cold acetone (−15°C), which had been redistilled over $KMnO_4$ crystals. The precipitate was collected on a Buchner funnel, washed twice with cold acetone and left on the bench to dry for about 30min. The dried powder was stored at −22°C and used to make up enzyme freshly for each day's work. If, for example, 20ml of enzyme solution was required, 200mg of dried enzyme powder was ground with alumina (Al_2O_3) in a pestle and mortar, 1ml of Tris buffer (see above) was added, with grinding, and a further 19ml of Tris buffer. The resulting suspension was centrifuged at about 30000*g* for 40min at 4°C and the supernatant used as the enzyme preparation.

Assay conditions

Standard solutions of bile salts (5mM) were stored in a mixture of methanol/water (1:1, v/v, adjusted to pH9.5 with 0.1M-NaOH). The latter solvent mixture was used as a blank. Standards used were 1–5mM, dilutions being made with methanol/water (see above). The following mixtures were incubated in covered tubes in a water bath at 37°C for 1h: 100μl of test, standard or blank solution; 1.0ml of sodium pyrophosphate buffer (0.1M, adjusted to pH8.5 with 1M-HCl); 1.0ml of NAD^+ solution (2.5M, in water, freshly prepared); 0.1ml of hydrazine hydrate solution (99–100% hydrazine hydrate made up to 0.1M in water and adjusted to pH9.5 with 0.5M-H_2SO_4); 0.3ml of enzyme preparation.

Absorption at 340nm was then measured for duplicate mixtures in each case; the average blank value was subtracted from each average test and standard reading and the remaining extinction values were plotted on the abscissae against millimolar concentrations.

7α-Hydroxy-3,12-dioxo-5β-cholan-24-oic acid

Methyl 3α,7α-diacetoxy-12-oxo-5β-cholan-24-oate (0.2g) was solvolysed at C-3 by boiling for 30min

Table 1. *Substances tested as substrates for a 7α-hydroxy steroid dehydrogenase preparation from Escherichia coli*

The following substances were used as standards for substrates as shown: J (taurocholate), for A–I and K–M; Q (cholate), for N, O, R, V, X and Y; R (chenodeoxycholate), for P, S, T, U and W. Sources of bile salts etc. were as follows: A, purified from toad *Bufo bufo formosus*; B and C, from *Catostomus commersoni* and *Ctenopharyngodon idella* respectively (Anderson & Haslewood, 1970); D, E, F and H, from *Rana esculenta*, *Rana temporaria*, *Rana pipiens* and *Discoglossus pictus* respectively (Anderson *et al.*, 1974); G, from a shark (Bridgwater *et al.*, 1962); I, J, Q and R, from Weddel's Pharmaceuticals, London E.C.1, U.K.; K, from the lizard *Anolis richardi*, whose bile salts consist largely of this substance; L, M, N, S, T, U, W, X and Y, were synthesized in this laboratory; O, given by Dr. A. R. Tammar; P, isolated from pig bile; V, see the text. AA, from *Latimeria chalumnae* (Anderson & Haslewood, 1964; a small amount of activity was attributed to a little 5α-cyprinol sulphate known to be present in these bile salts); BB, from hagfish, *Myxine glutinosa*; CC, DD, MM and NN, were synthesized in this laboratory; EE, from boid snakes; FF, GG and HH, were prepared as described by Haslewood & Haslewood (1976); JJ, from BDH, Poole, Dorset, U.K.; KK, isolated from pig bile; LL, a gift from the late Professor T. Shimizu. All substances, except as mentioned above or in the Discussion, were highly purified as judged by g.l.c. and/or t.l.c.

	Substrates	Extinction as % of standard at 5mM		Non-substrates
A	5β-Bufol sulphate [5β,25ξ-cholestane-3α,7α,12α,25,26-pentol (probably) 26-sulphate]	100	AA	Latimerol sulphate [5α-cholestane-3β,7α,12α-26,27-pentol 26 (or 27)-sulphate]
B	5α-Chimaerol sulphate [5α,25ξ-cholestane-3α,7α,12α,24(+),26-pentol 26-sulphate]	97	BB	Myxinol disulphate (5α,25ξ-cholestane-3β,7α,16α,26-tetrol 3,26-disulphate)
C	5α-Cyprinol sulphate [5α-cholestane-3α,7α,12α,26,27-pentol 26 (or 27)-sulphate]	93	CC	Glycodeoxycholate (glycine-conjugated 3α,12α-dihydroxy-5β-cholan-24-oic acid)
D	5β-Cyprinol sulphate (the 5β-epimer of C)	80	DD	Taurodeoxycholate (the taurine analogue of CC)
E	5α-Ranol sulphate (27-nor-5α-cholestane-3α,7α,12α,24ξ,26-pentol 24-sulphate)	90	EE	Tauropythocholate (taurine-conjugated 3α,12α,16α-trihydroxy-5β-cholan-24-oic acid)
F	5β-Ranol sulphate (the 5β-epimer of E)	82	FF	Cholic acid 3-sulphate
G	Scymol sulphate [5β-cholestane-3α,7α,12α,24ξ,26,27-hexol 26 (or 27)-sulphate]	100	GG	Chenodeoxycholic acid 3-sulphate
H	Taurotrihydroxycoprostanate [taurine-conjugated 3α,7α,12α-trihydroxy-5β-cholestan-26(or 27)-oic acid]	94	HH	Deoxycholic acid 3-sulphate
I	Glycocholate (glycine-conjugated 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid)	100		
J	Taurocholate (the taurine analogue of I)	100	JJ	Deoxycholate
K	Tauroallocholate (the 5α-epimer of J)	78	KK	Hyodeoxycholate (3α,6α-dihydroxy-5β-cholan-24-oate)
L	Glycochenodeoxycholate (glycine-conjugated 3α,7α-dihydroxy-5β-cholan-24-oic acid)	100	LL	Ursodeoxycholate (3α,7β-dihydroxy-5β-cholan-24-oate)
M	Taurochenodeoxycholate (the taurine analogue of L)	100	MM	3α,7β,12α-Trihydroxy-5α-cholan-24-oate
The following 5β-cholan-24-oic acids (as anions):				
N	2β,3α,7α,12α-tetrahydroxy-	73	NN	23,24-Bisnorcholate (3α,7α,12α-trihydroxy-5β,23,24-bisnorcholan-22-oate)
O	2β,3β,7α,12α-tetrahydroxy-	94		
P	3α,6α,7α,-trihydroxy-(hyocholate)	100		
Q	3α,7α,12α-trihydroxy-(cholate)	100		
R	3α,7α-dihydroxy-(chenodeoxycholate)	100		
S	3β,7α-dihydroxy-	100		
T	3α,7α-dihydroxy-12-oxo-	100		
U	7α,12α-dihydroxy-3-oxo-	100		
V	7α-hydroxy-3,12-dioxo-	80		
W	7α-hydroxy-3-oxo-	100		
X	25-Homocholate (3α,7α,12α-trihydroxy-5β-25-homocholan-25-oate)	96		
Y	24-Norcholate (3α,7α,12α-trihydroxy-5β-24-norcholan-23-oate)	86		

under reflux with methanol (4ml) and 11.8M-HCl (0.2ml). Dilution of the cooled reaction mixture with water gave flat colourless needles (0.17g) of methyl 7α-acetoxy-3α-hydroxy-12-oxo-5β-cholan-24-oate, m.p. 179–183°C [Wieland & Kapitel (1932) give 184°C]. This substance (0.1g) dissolved in acetic acid (1ml) was treated with CrO_3 (0.1ml, 200g/litre in acetic acid). After 1h the mixture was diluted with water and extracted twice with diethyl ether. The combined extracts were washed with water, aq. $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated. The residue dissolved in methanol (8ml) was heated under reflux with 1M-NaOH (1ml) for 1h. Solvent was evaporated. The residue, dissolved in water, was treated with excess of 2M-HCl and the precipitate collected, washed with water and dried by evaporation with ethanol. The residue was crystallized from diethyl ether and then from aq. ethanol from which 7α-hydroxy-3,12-dioxo-5β-cholan-24-oic acid gave stout colourless prisms (40mg), which had m.p. 124–126°C, $[\alpha]_D^{22}$ +70±2° (*c* 0.9 in ethanol) [Found (by Dr. F. B. Strauss, Oxford, U.K., on a sample dried to constant wt. at 100°C): C, 69.1; H, 9.3. Calc. for $C_{24}H_{36}O_5,H_2O$; C, 68.3; H, 9.0%]. Wieland & Kapitel (1932) give m.p. 196°C for this substance. Dr. L. Tökes (Syntex Research, Palo Alto, CA, U.S.A.) kindly examined the nuclear-magnetic-resonance and mass spectra of our substance and reported that these fully supported the proposed structure. On t.l.c. in the system 2,2,4-trimethylpentane/ethyl acetate/acetic acid (7:12:3, by vol.), the acid had an R_F almost the same as that of chenodeoxycholic acid, and on g.l.c. as described by Haslewood (1971) its methyl ester had a retention time of 4.65 relative to methyl deoxycholate. The corresponding ethyl ester described by Haslewood (1946) proved, on fresh examination, to be ethyl 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oate.

Results

The results of enzyme specificity tests are shown in Table 1, in which substances that did not react measurably with the enzyme are listed as 'non-substrates'. Substrates were tested as described against the 'standards' listed in the legend to Table 1. Not all supposedly pure standards gave the same extinction values at 1–5mM, but the (1–5mM) values lay almost on a straight line in every case. Duplicate readings (extinctions) did not differ by more than 0.03 in any case.

Discussion

The enzyme specificity is clearly very wide: with 5α or 5β steroids having hydroxyl groups at C-3, C-7 and C-12, the side chains can be extended to at least eight carbon atoms and can have hydroxyl and sulphate ester groups in various positions without loss of activity. Bile acid anions or those of the corresponding glycine or taurine conjugates are effective as substrates. However, shortening the 5β-cholanoic acid side chain beyond C-23 does result in inactivity and so, apparently, does esterification with sulphate at C-3. The failure of latimerol sulphate to react is rather surprising, in view of the activity of 3β,7α-dihydroxy-5β-cholan-24-oate. We conclude that it cannot be assumed that any sulphate ester will necessarily react with the enzyme, even if a free hydroxyl group at C-7α is present in the molecule.

The activity of substrates listed in Table 1 does not seem to be quantitatively the same. We have reason to think that the assay conditions do bring about complete oxidation for cholic and chenodeoxycholic acid, and their glycine and taurine conjugates (Haslewood *et al.*, 1973), but somewhat lower extinctions at 1–5mM were found for some other substrates. In some cases, e.g. substances D, E, F, H and K (Table 1), this might be explained by impurities present in the bile salts, but for other supposedly pure substances, e.g. N, V and Y (Table 1), the explanation may be that oxidation was incomplete in our assays. It seems clear, therefore, that if the 7α-hydroxysteroid dehydrogenase is to be used for quantitative work, conditions of assay appropriate to the expected substrates must be worked out in each case. For semi-quantitative or qualitative work, the enzyme might be useful, e.g. for the detection of norcholic acid in a preparation of bisnorcholic acid or of 5α-cyprinol sulphate in *Latimeria* bile salts.

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