Metabolism of Hydroanthracenones in Rabbits

By J. S. ROBERTSON and P. J. DUNSTAN*

Department of Pharmacy, University of Sydney,
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1. The metabolism of 1-oxo-octahydro- and 2- and 9-oxoperhydro-anthracenes was investigated in rabbits. All compounds increased the urinary glucuronide content. 2. The 1-oxo and 2-oxo compounds were reduced to the corresponding alcohols whereas the 9-oxo compound was hydroxylated. 3. The reduction *in vitro* of these compounds and related ketones was investigated with three different enzyme systems (liver alcohol dehydrogenase, hydroxy steroid dehydrogenase, aromatic aldehyde–ketone reductase) in an attempt to explain the results *in vivo*. 4. Successful reduction of many ketones with aromatic aldehyde–ketone reductase suggests that the kidney may be of importance in the reduction *in vivo* of certain cyclic carbonyl compounds.

The metabolic fate in rabbits of a number of alicyclic ketones has been reported, e.g. cyclohexanone (Elliott *et al.*, 1959), methylcyclohexanones (Elliott *et al.*, 1965), decalones (Elliott *et al.*, 1966), tetralones (Elliott & Hanam, 1968) and camphors (Robertson & Hussain, 1969). These studies indicated that relatively unhindered oxo groups were readily reduced, whereas a bridgehead carbon or a ring methyl group in the α -position to the oxo group inhibited reduction. In the present study, the hydroanthracenones exhibited this same pattern of reduction *in vivo*.

In attempts to understand the results *in vivo*, reduction studies of the methylcyclohexanones, decalones and camphors were done *in vitro* by the above authors (Elliott & Robertson, 1965; Elliott *et al.*, 1969; Robertson & Hussain, 1969), with the relatively non-specific oxoreductase liver alcohol dehydrogenase and NADH. Where reaction occurred it was found that the substrates did not occupy the 'forbidden regions' defined by the diamond lattice-section hypothesis of Prelog (1964), as modified by Graves *et al.* (1965). However, failure of this enzyme system to reduce some of the camphors that had been reduced *in vivo* (Robertson & Hussain, 1969) led to the conclusion that another enzyme system was responsible for reduction of these substrates *in vivo*.

In the present investigation, the reducing abilities of liver alcohol dehydrogenase, hydroxy steroid dehydrogenase (Talalay, 1963) and kidney aromatic aldehyde–ketone reductase (Culp & McMahon, 1968) were examined.

* Present address: Department of Zoology, University of Queensland, St. Lucia, Brisbane, Qld. 4067, Australia.

Materials

Melting points are uncorrected. Where a specific reference is not given, the literature melting points quoted were taken from Heilbron *et al.* (1965). Light petroleum (b.p. 60–80°C) was used for recrystallization and column chromatography.

($\pm$)-*sym*-Octahydroanthracen-1-one (compound I) (Fig. 1) was prepared by reacting tetralin with succinic anhydride in the presence of aluminium chloride to give β -(2-tetroyl)propionic acid (Krollpfeiffer & Schaefer, 1923) which on Clemmensen reduction gave α -(2-tetralyl)butyric acid. The latter was cyclized by the method of Rahman *et al.* (1963), with sulphuric acid as catalyst, to *sym*-octahydroanthracen-1-one, m.p. 44°C (literature value 49°C). The i.r. spectrum showed carbonyl absorption at 1690 nm and aromatic absorption at 1620 nm. The ketone formed a semicarbazone, m.p. 251°C (literature value 253°C). Clemmensen reduction of compound (I) gave *sym*-octahydroanthracene, m.p. 74°C (literature value 78°C), which confirmed that the butyric acid above had cyclized to the anthracene and not to the phenanthrene system. ($\pm$)-*sym*-Octahydroanthracen-1-ol (compound II) prepared by NaBH₄ reduction of the corresponding ketone (compound I) (Turner, 1954) had m.p. 96°C (literature value 92–93°C).

($\pm$)-*trans-cisoid-trans*-Perhydroanthracen-2-one (compound III) was prepared by oxidation of the corresponding alcohol (compound IV) (Dunstan & Robertson, 1971).

($\pm$)-*cis-trans*-Perhydroanthracen-9-one (compound V) was obtained by the method of Hill *et al.* (1961) (Robertson & Dunstan, 1971).

($\pm$)-17 β -Hydroxy-5 α -oestrane-3-one was prepared, by the method of Bowers *et al.* (1958), by lithium-

Fig. 1. Structures of hydroanthracenones

(I) *sym*-Octahydroanthracen-1-one; (II) *sym*-octahydroanthracen-1-ol; (III) *trans-cisoid-trans*-perhydroanthracen-2-one; (IV) *trans-cisoid-trans*-perhydroanthracen-2-ol; (V) *cis-trans*-perhydroanthracen-9-one; (VI) partial diamond lattice section (Graves *et al.*, 1965); X denotes a forbidden position; (VII) partial diamond lattice section viewed from above; ● denotes atoms above the plane of the cyclohexanone ring.

ammonia reduction of 17β -hydroxy- 5α -oestra-4-en-3-one (19-nortestosterone) and had m.p. 128°C [Bowers *et al.* (1958) gave 130 – 131°C]. ($\pm$)- 17β -Hydroxy- 5α -oestra-4-en-3-one was kindly supplied by Dr. R. Gall, Department of Organic Chemistry, University of Sydney.

Methods

Animals and diet

White New Zealand $\times$ albino cross-bred rabbits weighing 1.8–3.5 kg were used. Their daily diet consisted of 100 g of Pelleted Rabbit Food (Drug Houses of Australia, Sydney, N.S.W., Australia) and unlimited water. Compounds were administered, as starch-mucilage suspension, by stomach tube followed by about 30 ml of water.

Determination of urinary glucuronides

Quantitative determination of glucuronic acid, by

the method of Hanson *et al.* (1944), as modified by Paul (1951), was done on urine samples before and after administration of compounds.

Characterization of aglycones

The glucuronides contained in the urine were hydrolysed to establish the identity of the aglycones. The 24, 48 and 72 h urine samples were pooled and treated in the following ways: (a) a small unhydrolysed portion was extracted with ether to detect the presence of any polar unconjugated metabolite such as a diol, but such a metabolite was never found; (b) a second portion was hydrolysed at pH 6.8 and 37°C with β -glucuronidase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) for 48 h; the hydrolysate was extracted with ether; (c) a third portion was acidified to pH 1 with HCl and refluxed for 5 h. The ether extracts of the hydrolysates of (b) and (c) were compared on t.l.c. and g.l.c. Where they were identical, the main urine sample was acid-hydrolysed.

Infrared spectroscopy

Measurements were made with Perkin-Elmer model 21 and Unicam SP.200G spectrophotometers. Compounds were examined as liquid films, KBr discs or solutions in chloroform.

Thin-layer chromatography

This was done as described by Robertson & Hussain (1969). Aglycones were subjected to chromatography in a solvent system consisting of *n*-hexane-ethyl acetate (3:1, v/v) (system 1) or (9:1, v/v) (system 2). Compounds were located with phosphomolybdic acid reagent (Kritchevsky & Kirk, 1952).

Gas chromatography

A Hewlett-Packard F & M 5750 gas chromatograph equipped with a flame-ionization detector was used. The stationary phases were 12% Carbowax 20M on Chromosorb W (HMDS 80-100 mesh) and 10% UC-W98 (Hewlett-Packard) on Chromosorb P (100-120 mesh); N₂ was used as carrier gas.

Nuclear-magnetic-resonance spectroscopy

Spectra were obtained by using Varian model A-40 (Varian Associates, Palo Alto, Calif., U.S.A.) and Perkin-Elmer model R12 (Perkin-Elmer Ltd., Beaconsfield, Bucks., U.K.) n.m.r. spectrometers. Compounds were dissolved in deuteriochloroform; tetramethylsilane was used as internal reference.

Experiments in vitro

With liver alcohol dehydrogenase. Equine liver alcohol dehydrogenase and NADH (Sigma Chemical Co.) were used without further purification.

Reduction rates with liver alcohol dehydrogenase were compared with that of cyclohexanone at substrate concentrations of 0.17mM; NADH, 0.10mM; alcohol dehydrogenase 210 μ g; 0.3M-potassium phosphate buffer, pH7.0, containing 0.15ml of dioxan. The total volume was 10 or 12ml and the substrate was added in dioxan (0.05ml). The results are shown in Table 1.

With aromatic aldehyde-ketone reductase. Aromatic aldehyde-ketone reductase (Culp & McMahon, 1968) was prepared from rabbit kidney. An adult rabbit was killed by cervical dislocation and the kidneys removed and placed on crushed ice. After removing all fatty tissue and dissecting out the medulla, the kidney cortex was weighed, minced and homogenized in an equal weight of ice-cold 0.1M-Sorensen phosphate buffer, pH7.4, in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700g for 5min in a refrigerated centrifuge

Table 1. *Relative rates of reduction of ketones with NADH and liver alcohol dehydrogenase*

Experimental details are given in text. The number of determinations is given in parentheses.

Compound	Relative rate
Cyclohexanone	100 (12)
<i>sym</i> -Octahydroanthracen-1-one	0* (10)
<i>trans-cisoid-trans</i> -Perhydroanthracen-2-one	24 (12)
<i>cis-trans</i> -Perhydroanthracen-9-one	0* (10)

* The relative rates were negligible and within experimental error.

to remove gross tissue debris. The supernatant was then recentrifuged at 30000g for 30min (MSE High Speed 18) and the supernatant was used in incubation experiments. The reduction rates of several substrates (adamantanone, cyclohexanone, *cis*-2-decalone) by using this enzyme preparation were very similar to the results obtained by Culp & McMahon (1968) with their more purified aromatic aldehyde-ketone reductase. Reduction rates were compared with that of *p*-nitrobenzaldehyde at substrate concentrations of 0.3mM; NADPH, 0.1mM; 0.03M-potassium phosphate buffer, pH7.0, containing 0.15ml of methanol and crude aromatic aldehyde-ketone reductase (0.05ml). The total volume was 10ml and the substrate was added in methanol (0.05ml). The results are shown in Table 2.

With hydroxy steroid dehydrogenase. A mixed 3 α and 3 β , 17 β -hydroxy steroid dehydrogenase was prepared from *Pseudomonas testosteroni*. Dried cells of *Ps. testosteroni* (A.T.C. 11996) (50mg) (Sigma Chemical Co.) were sonically disrupted (MSE Ultrasonic Disintegrator, 60W) in a 0.03M-potassium phosphate buffer, pH7.0 (20ml), containing EDTA (1mM) for 15min. The suspension was centrifuged at 9000g (MSE High Speed 18) for 20min at 2°C. After removal of the supernatant, the disrupted cells were washed with further buffer (10ml) and recentrifuged at 9000g. The first and second supernatants were combined and used in incubation experiments. Reduction rates were compared with that of 17 β -hydroxy-5 α -androstan-3-one (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) at a substrate concentration of 38 μ M, NADH 0.1mM, hydroxy steroid dehydrogenase solution (0.4ml), 0.03M-phosphate buffer, pH7.0, containing dimethylformamide (0.25ml). The total volume was 10ml and the substrate was added in dimethylformamide (0.05ml). Results are shown in Table 3.

All reductions were followed by measuring the disappearance of NADH at 340nm with a Unicam SP.700 or Perkin-Elmer 402 UV spectrophotometer.

Table 2. *Relative rates of reduction of ketones with kidney aromatic aldehyde-ketone reductase*

Experimental details are given in text. The number of determinations is given in parentheses. The relative rates obtained by Culp & McMahon (1968) with the more purified enzyme are also given.

Compound	Relative rate	
	This work	Culp & McMahon
<i>p</i> -Nitrobenzaldehyde	100 (30)	100
Cyclohexanone	37 (5)	35
Benzaldehyde	19 (3)	34
<i>sym</i> -Octahydroanthracen-1-one	10 (6)	—
<i>trans-cisoid-trans</i> -Perhydroanthracen-2-one	15 (6)	—
<i>cis-trans</i> -Perhydroanthracen-9-one	0* (6)	—

* The relative reduction rate was negligible and within experimental error.

Table 3. *Relative rates of reduction of ketones with mixed hydroxy steroid dehydrogenase of P. testosteroneii*

Experimental details are given in text, and the number of determinations is given in parentheses.

Compound	Relative rate
17 β -Hydroxy-5 α -androstan-3-one	100 (12)
17 β -Hydroxy-5 α -oestra-3-one	73 (6)
<i>sym</i> -Octahydroanthracen-1-one	0* (10)
<i>trans-cisoid-trans</i> -Perhydroanthracen-2-one	18 (6)
<i>cis-trans</i> -Perhydroanthracen-9-one	0* (10)

* These reduction rates were negligible and within experimental error.

All reactions were done at 25°C in silica cuvettes (4 or 5 cm light-path length). A blank containing all components except the substrate was run simultaneously.

Results and Discussion

None of the compounds showed any pharmacological action at the doses administered.

($\pm$)-*sym*-Octahydroanthracen-1-one (500 mg, 0.7–1.0 mmol/kg body wt.) was given to each of six rabbits. After 24 h 49.6% by weight was excreted as glucuronide and a further 6.0% after 48 h. G.l.c. of the ether extract of the β -glucuronidase-hydrolysed urine revealed one major metabolite, which had the same retention time as synthetic *sym*-octahydroanthracen-1-ol. The remaining urine was hydrolysed enzymically and the ether extract chromatographed on a column of alumina (30 g). Recrystallization of the fraction eluted with ethyl acetate–toluene (1:50, v/v) gave a solid of m.p. 92°C, which had the same i.r. spectrum as synthetic *sym*-octahydroanthracen-1-ol,

($\pm$)-*trans-cisoid-trans*-Perhydroanthracen-2-one (45 mg) was administered to each of two rabbits.

Steam-distillation of the acid-hydrolysed urine yielded, after recrystallization, pure *trans-cisoid-trans*-perhydroanthracen-2-ol, m.p. 159–160°C, further identified by i.r. spectroscopy and mass spectrometry.

($\pm$)-*cis-trans*-Perhydroanthracen-9-one (500 mg) was administered to each of four rabbits (0.7–1.0 mmol/kg body wt.); 65 $\pm$ 25% of the dose was excreted as glucuronide. Acid hydrolysis and ether extraction of the urine gave a single metabolite, shown by i.r. analysis to have ketonic and alcoholic functional groups. The nature of this metabolite has been discussed elsewhere (Robertson & Dunstan, 1971).

The oxo groups of *sym*-octahydroanthracen-1-one and *trans-cisoid-trans*-perhydroanthracen-2-one were reduced *in vivo* to give secondary alcohols whereas that of the 9-oxo compound was not.

An explanation of these results was sought by attempting to reduce the ketones *in vitro*; the enzymes used were liver alcohol dehydrogenase, hydroxy steroid dehydrogenase and kidney aromatic aldehyde-ketone reductase.

A large number of alicyclic ketones have been found to be reduced by NADH in the presence of liver alcohol dehydrogenase (Merritt & Tomkins, 1959; Prelog, 1964; Robertson & Elliott, 1965; Graves *et al.*, 1965). From Table 2 it can be seen that only the 2-oxo compound was reduced by this system. These results are explicable if the stereo-specific requirements for reduction as specified by Graves *et al.* (1965) are considered (Fig. 1, compounds VI–VII). In the case of the 9-oxo compound, it can be seen that the two outer rings are occupying 'forbidden positions', so reduction cannot occur. Also in the case of the 1-oxo compound reduction is not expected and did not occur as the substrate would again be occupying forbidden positions.

With the 2-oxo compound the forbidden positions are not occupied by groups larger than hydrogen, so reduction could and did occur.

The relative rates of reduction given by the mixed hydroxy steroid dehydrogenase from *Ps. testosteroni* are given in Table 3. Again, only the 2-oxo compound was reduced. Extensive studies on the steric requirements of this enzyme have been made with a variety of substrates ranging in complexity from cyclohexanone to steroidal ketones. A composite model of substrate stereochemical requirements has been proposed (Ringold *et al.*, 1966, 1967*a,b*) which is sufficiently similar to that suggested for liver alcohol dehydrogenase to predict that the 2-oxo compound would be reduced and that the 1- and 9-oxo compounds would not.

The inability of the foregoing dehydrogenases to reduce the 1-oxo compound, which was reduced *in vivo*, prompted investigation with kidney aromatic aldehyde-ketone reductase, which had been shown to reduce a wide variety of substrates (Culp & McMahon, 1968).

Table 3 shows that both the 1-oxo and 2-oxo compounds were reduced, at comparable rates, as well as a number of other substrates that had been found previously not to be reduced with liver alcohol dehydrogenase (Robertson & Hussain, 1969). It would seem that liver alcohol dehydrogenase and hydroxy steroid dehydrogenase are capable of reducing a β -located oxy group but not an α -oxo group, whereas oxo groups in either location can be reduced by aromatic aldehyde-ketone reductase; however none of the enzymes reduce an oxo group located between two rings, as in the 9-oxo compound.

Possession of aromatic aldehyde-ketone reductase by the kidney suggests that the latter is a possible site of metabolism for certain ketones.

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