Metabolism of Perhydroanthracenes in the Rabbit

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(Received 19 April 1971)

1. The metabolism in rabbits of several perhydroanthracenes was investigated. All compounds were to different degrees excreted in the urine as glucuronides.
2. The aglycones of the glucuronides were found to be racemic secondary alcohols, having the hydroxyl group at a β -methylene carbon. Where determinable, the hydroxyl group was shown to have an equatorial configuration.
3. These results suggested that hydroxylation was enzyme mediated, and, when compared with the results from studies on other alicyclic hydrocarbons, that the same enzyme system participated generally in the hydroxylation of such hydrocarbons.
4. A model for the enzyme active site is proposed to accord with the pattern of hydroxylation observed.

In previous investigations of the metabolism in rabbits of saturated bicyclic hydrocarbons (Elliott, Robertson & Williams, 1966; Robertson & Champion, 1970) hydroxylation occurred at an unhindered methylene group β to the ring junction. The present investigation was carried out to ascertain whether this pattern of hydroxylation would obtain for a tricyclic hydrocarbon system. The finding that it did indicated that the hydroxylation was site specific.

From this consistent pattern of hydroxylation, and from the results of hydroxylation studies on other alicyclic hydrocarbons a model for the enzyme active site is proposed to accord with the pattern of hydroxylation observed.

MATERIALS

Melting points, which are uncorrected, were obtained on a Kofler block. Literature values were taken from Heilbron, Cook, Bunbury & Hey (1965) or from the original papers.

sym-Octahydroanthracene (V), *trans-cisoid-trans*-perhydroanthracene (I) and *trans-cisoid-trans*-perhydroanthracene-2-ol (II) were prepared as described by Dunstan & Robertson (1971). *cis-transoid-cis*-Perhydroanthracene (III) (m.p. 118-120°C) was prepared by the method of Crossley & Henbest (1960) who give m.p. 121°C. The i.r. and n.m.r. spectra were identical with those published by Clarke (1961) for the same compound. *cis-trans*-Perhydroanthracen-9-one (VII), m.p. 127°C, was prepared by the method of Hill, Martin & Stouch (1961), who give m.p. 128.5-131.5°C. The i.r. spectrum showed carbonyl absorption at 1715 nm.

METHODS

Animals and diet. These were as described by Robertson & Hussain (1969). Compounds were administered as

suspensions in starch mucilage (10%, w/v) followed by 20-40 ml of water.

Quantitative determination of glucuronide was carried out by the method of Hanson, Mills & Williams (1944) as modified by Paul (1951).

Infrared spectroscopy. This was carried out as described by Robertson & Hussain (1969).

Nuclear-magnetic-resonance and mass spectroscopy. Spectra were obtained as described by Robertson & Champion (1970).

Mass matching was performed by using perfluorokerosene (high-boiling fraction) and heptacosafuorotri-*n*-butylamine as internal standards. The accuracy was ± 0.001 atomic mass unit.

Gas-liquid and thin-layer chromatography. These were carried out as described by Robertson & Hussain (1969). The stationary phases were 10% UC-W98 (Hewlett-Packard) on Chromosorb P (100-200 mesh) (phase I) and 12½% Carbowax 20M on Chromosorb W (80-100 mesh) (phase II). Light petroleum (b.p. 60-80°C) was used for t.l.c. and column chromatography.

Treatment of urine. The 24 h, 48 h and 72 h urine samples were individually assayed for glucuronic acid content. The samples were then pooled and treated as follows.

(i) A 10 ml portion was extracted with ether to isolate any polar unconjugated metabolites, e.g. diols; in no case did g.l.c. or t.l.c. of the extracts reveal such a compound.

(ii) A second 10 ml portion was hydrolysed with β -glucuronidase (type II; Sigma Chemical Co., St Louis, Mo., U.S.A.) at pH 6.8 and 37°C for 48 h; the hydrolysate was extracted with ether.

(iii) A 25 ml portion was acidified to pH 1 with 10M-HCl and refluxed for 5 h. The hydrolysate was steam-distilled, then extracted with ether.

The ether extracts from (ii) and (iii) above were examined by g.l.c. and t.l.c.; where they gave the same result, the remainder of the urine was hydrolysed with acid. If they differed the urine was hydrolysed with β -glucuronidase.

RESULTS

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

Determination and characterization of metabolites in the urine

In all cases the urine gave a naphtharesorcinol reaction. The octahydro and oxoperhydro compounds were readily metabolized, most of the glucuronide conjugates being excreted over 48h; the other perhydro- compounds were conjugated to a smaller extent, and excreted during the 72h period (Table 1).

trans - cisoid - trans - Perhydroanthracene. This hydrocarbon (1g) was administered to each of six animals. The 24h urine contained the equivalent of 7.7%, the 48h urine 8.1% and the 72h urine 5.5% of the administered compound (Table 1). Steam-distillation of the acid-hydrolysed urine gave a white solid, which, after repeated recrystallization from hexane, had m.p. 159–160°C and was optically inactive. No other metabolite was found in the mother liquors, or in an ether extract of the hydrolysed urine after steam-volatilization.

The metabolite was found to have the same m.p., i.r., n.m.r. and mass-spectrometric characteristics as *trans - cisoid - trans - perhydroanthracen - 2 - ol* (Dunstan & Robertson, 1971).

cis-transoid-cis-Perhydroanthracene. This hydrocarbon (0.5g) was administered to each of eight rabbits. Excretion of conjugated metabolite was uniformly low over 3 days, and corresponded to 27.9% of the amount given. Steam-distillation of hydrolysed urine gave a small amount of white solid, which on recrystallization from *n*-heptane had m.p. 163–165°C. No other metabolite was detected in the mother liquors from recrystallization or in the steam-distilled urine. The metabolite, which was optically inactive, showed i.r. absorption at 3500nm, suggesting that the hydrocarbon had been hydroxylated to an alcohol. The mass spectrum showed a molecular ion at *m/e* 208, of weak relative intensity, and one at *m/e* 190 of high

intensity. High-resolution mass matching of the latter indicated a composition of $C_{14}H_{22}^+$, corresponding to loss of H_2O from the molecular ion. The metabolite was therefore an alcohol, $C_{14}H_{23}OH$. The 60MHz n.m.r. spectrum in perdeuteropyridine (the compound was not very soluble in deuteriochloroform) showed a multiplet at 5.2 δ (OH), a multiplet at 4.1 δ (HO-C-H) and a broad multiplet in the region 2.5–7 δ (alicyclic H) (Bhacca & Williams, 1964).

After exchange with D_2O , the 100MHz n.m.r. spectrum showed a multiplet at 3.8 δ (HO-C-H) that could not be resolved. Thus n.m.r. spectroscopy could not be used to determine the site of hydroxylation.

Oxidation of the metabolite with chromic acid yielded a solid, m.p. 131–133°C, which on mass spectrometry had a parent ion at the anticipated *m/e* 206. Significantly, the relative intensity of the *M*–44 peak was greater than that of the *M*–42 and *M*–43 peaks, whereas the intensity of the *M*–55 peak was similar to that of the *M*–56 and *M*–57 peaks.

It had been reported (Lund, Budzikiewicz, Wilson & Djerassi, 1963a,b) that in the related decalone system, *trans*-1- and *trans*-2-decalones could be distinguished by mass spectrometry. In *trans*-1-decalone the relative intensity of the *M*–43 peak was more intense than those of the *M*–42 and *M*–44 peaks, and the *M*–55 peak was more intense than the *M*–56 or *M*–57 peaks. (In the mass spectrum of *cis-trans*-perhydroanthracen-9-one, to which *trans*-1-decalone is closely related, the *M*–55 peak is very intense; it is the base peak.) In *trans*-2-decalone, the *M*–44 peak was found to be more intense than the *M*–42 and *M*–43 peaks, whereas the *M*–55, *M*–56 and *M*–57 peaks were of similar intensity.

The mass-spectral results suggested that the metabolite was *cis-transoid-cis*-perhydroanthracen-2-ol (IV). Further, the mass spectrum of this compound was nearly identical with that of *trans-cisoid-trans*-perhydroanthracen-2-ol. It was concluded that the metabolite was *cis-transoid-cis*-perhydroanthracen-2-ol.

Table 1. *Excretion of perhydroanthracenes as glucuronides*

Experimental details are given in the text. The values given are average values, with the ranges in parentheses; they are calculated from the increases in glucuronides in the urine 3 days after, compared with before, administration of the compounds.

Compound	No. of animals used	Dose (mmol/kg body wt.)	% of dose excreted as glucuronide
<i>trans-cisoid-trans</i> -Perhydroanthracene	6	1.62–2.32	21 $\pm$ 5 (17–29)
<i>cis-transoid-cis</i> -Perhydroanthracene	8	0.71–1.17	28 $\pm$ 17 (7–57)
<i>sym</i> -Octahydroanthracene	8	1.54–2.15	47 $\pm$ 17 (28–69)
<i>cis-trans</i> -Perhydroanthracen-9-one	4	0.71–1.05	65 $\pm$ 25 (33–90)

sym-Octahydroanthracene. A 1g portion was administered to each of eight rabbits. The increase in glucuronic acid for 3 days after administration corresponded to 47% of the compound being conjugated (Table 1). Excretion occurred mainly on the first 2 days with little excretion on the third day. Steam-distillation of the acid-hydrolysed urine gave a very small quantity of material, which could not be characterized. Material obtained by ether extraction of the hydrolysed urine consisted almost entirely of one compound, which was purified by column chromatography on alumina. Recrystallization from cyclohexane-*n*-hexane gave a solid of m.p. 127°C, which was optically inactive. This compound showed i.r. absorption at 3330nm, and the mass spectrum contained a molecular ion at m/e 202, the identity of which was shown to be $C_{14}H_{18}O^+$ by high-resolution mass matching. Together, these results indicated that mono-hydroxylation to an alcohol had occurred.

The 60MHz n.m.r. spectrum showed among other features, a septet at 4.08 δ (HO-C-H). The 100MHz n.m.r. spectrum showed the splitting of the septet to be 5Hz. Thus the alcohol group was β to the ring junction (Bhacca & Williams, 1964; Robertson & Champion, 1970; Dunstan & Robertson, 1971). The n.m.r. spectrum of *sym*-octahydroanthracen-1-ol showed resonance at 4.78 δ , and the spectrum was clearly different from that of the metabolite. The metabolite was thus *sym*-octahydroanthracen-2-ol (VI).

($\pm$) - *cis-trans-Perhydroanthracen-9-one*. This ketone (0.5g) was administered to each of four rabbits. The increase in glucuronic acid excretion during the 3 days after administration corresponded to 65% of the amount given (Table 1). Excretion occurred mainly in the first 24h.

Steam-distillation of the acid-hydrolysed urine gave a small quantity of a pale-brown oil. This oil was shown by g.l.c. to contain several components, which were not identified. The non-steam-volatile ether extract was chromatographed on alumina to yield a white solid. After recrystallization from cyclohexane-*n*-hexane this metabolite, which was optically inactive, had m.p. 138–140°C. The i.r. spectrum showed absorption at 3270nm and 1715nm, indicating the presence of hydroxyl and ketonic functions respectively. The mass spectrum contained a significant molecular ion at m/e 222, which was shown to be $C_{14}H_{22}O_2^+$ by high-resolution mass matching. The results suggested that hydroxylation of the compound had occurred without reduction of the ketone.

The 60MHz n.m.r. spectrum showed a septet at 3.53 δ (HO-C-H); splitting was found to be 5Hz, indicating that the hydroxyl group was equatorial and β to the ring junction. However, the β carbon atoms in the compound are non-equivalent because

of the different ring fusion of the two outside rings to the central ring. Neither n.m.r. nor mass spectroscopy proved helpful in elucidating the specific locus of hydroxylation. The problem is similar to that encountered by Robertson & Champion (1970) with the methyldecalins, where the methyl group makes the two rings no longer equivalent.

The metabolite was thus a *cis-trans*-perhydroanthracen- β -ol-9-one (VIII).

DISCUSSION

It was found that in all four compounds administered, hydroxylation had occurred at a methylene carbon β to the ring junction. The secondary alcohols so produced were optically inactive.

For the *trans-cisoid-trans*-perhydroanthracene and *cis-trans*-perhydroanthracen-9-one metabolites the hydroxyl group was shown to be in the equatorial configuration.

Fig. 1. Hydroxylation of hydroanthracenes in the rabbit. (I) *trans-cisoid-trans*-Perhydroanthracene; (II) *trans-cisoid-trans*-perhydroanthracen-2-ol; (III) *cis-transoid-cis*-perhydroanthracene; (IV) *cis-transoid-cis*-perhydroanthracen-2-ol; (V) *sym*-octahydroanthracene; (VI) *sym*-octahydroanthracen-2-ol; (VII) *cis-cisoid-trans*-perhydroanthracen-9-one; (VIII) *cis-cisoid-trans*-perhydroanthracen- β -ol-9-one.

In *sym*-octahydroanthracene, the planar central ring forces the two outer rings into flexible half-chair conformations. It might then be anticipated that there could be no preferred configuration of the hydroxyl group. However, the n.m.r. spectrum suggested that a quasi-equatorial configuration was favoured.

As discussed above, though it was not possible to show definitely that *cis-transoid-cis*-perhydroanthracene had been hydroxylated in the β position, strong evidence was obtained in favour of this site.

The site of ring hydroxylation and configuration of the resulting hydroxyl group in the present series of compounds (Fig. 1) is completely consistent with those found for the decalins (Elliott *et al.* 1966) and the methyldecalins (Robertson & Champion, 1970). It seems a reasonable conclusion that such site-specificity of hydroxylation is enzyme-mediated.

It would be no surprise if the enzyme system effecting hydroxylation of the perhydroanthracenes was the same as that hydroxylating the decalins and the methyldecalins. Indeed it may well be asked if this enzyme system is concerned in the hydroxylation of alicyclic hydrocarbons generally? With this question in mind, the results of metabolic hydroxylation of a number of alicyclic hydrocarbons were reviewed.

For cyclohexane, hydroxylation yielded mainly cyclohexanol (Elliott, Parke & Williams, 1959). Methylcyclohexane (Elliott, Tao & Williams, 1965) gave a mixture of racemic 2-, 3- and 4-methylcyclohexanols in the proportion 1:22:17 and no 1-methylcyclohexanol.

Tetralin was hydroxylated at the α and β positions in the ratio of approx. 2:1 (Elliott & Hanam, 1968). However, it was noted that in this case, the α carbon is chemically more reactive, a situation not obtaining in the more saturated compounds.

The requirements of this enzyme system (which may consist of one or several enzymes) are that it so accommodates these substrates that hydroxylation occurs at a relatively unhindered carbon atom to give optically inactive alcohols with an equatorial configuration.

It is possible to construct a composite molecule of these substrates and products with hydroxylation occurring from a fixed site; a surface view of such a molecule is shown in Fig. 2(a).

The only products detected after administration of decalins and perhydroanthracenes contained a β -hydroxyl group, never an α -hydroxyl group. This suggested that there was limited space in the vicinity of the active centre of the enzyme for the accommodation of substrates. The reason for this limitation is not known. However, as a hypothesis, it is suggested that the active centre is located in a symmetrical depression in the enzyme surface, the sides of the depression acting as a lateral barrier. For simplicity, the depression is visualized as being cone-shaped (Fig. 2a).

To account for the favoured equatorial configuration of the hydroxyl substituent, the substrate molecules must be so arranged that the equatorial C-H bond is directed to the hydroxylating site while at the same time the axial C-H bond is protected. Such an arrangement of substrate and enzyme active centre is shown in Fig. 2(b).

Since the stereochemistry of the bridgehead does not appear to affect the site of hydroxylation, i.e. *cis* and *trans* isomers are both hydroxylated at a β carbon, the active site need only be large enough to accommodate and anchor a cyclohexane molecule or moiety. The remainder of the molecule may be attached to non-specific sites.

The anchoring site should also be able to accommodate the smaller cyclopentane molecule. The metabolites of bornane, which may be regarded as a bicyclopentane, were found to be borneol and

Fig. 2. Suggested framework for the β -hydroxylation of alicyclic hydrocarbons; (a) surface view; (b) profile.

Fig. 3. Sitings of bornane in the proposed framework for hydroxylation, leading to the favoured production of epiborneol (b) over borneol (a).

epiborneol (Robertson & Hussain, 1969); suitable alignments of bornane in the postulated hydroxylating framework are shown in Fig. 3.

In surface view, it can be seen that the methyl group attached to C-1 would tend to inhibit the production of borneol, but be less hindering to the production of epiborneol (cf. 2- and 3-methylcyclohexanol). Indeed epiborneol production is greater than that of borneol (Robertson & Hussain, 1969).

The utility of the proposed framework lies in suggesting the design of compounds with which it can be tested.

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