Biochemistry of Metallocenes

IDENTIFICATION OF THE MAJOR METABOLITE OF ACETYLRUTHENOCENE

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1. After oral administration of acetylruthenocene to rats, metabolites were detected in bile and urine. 2. The major metabolite, which is present in both bile and urine, is a glucuronide with the structure:

3. The metabolite was identified by mass spectrometry of the permethylated glucuronide and mass spectrometry and n.m.r. of the aglycone. 4. The nature of the metabolite is discussed, and a comparison is made with the metabolism of the benzene analogue, acetophenone.

Previous studies in this laboratory (Wenzel *et al.*, 1976, 1977) showed that ruthenocene derivatives labelled with ^{103}Ru could be useful as radioactive diagnostic agents. One of these compounds, acetyl- ^{103}Ru ruthenocene (structure shown below), showed an affinity for adrenal glands. As part of a study to determine how and in what form the radioactivity accumulates in adrenals, the major metabolite of acetylruthenocene was isolated from urine and subsequently identified.

Acetylruthenocene

The metabolism of the benzene analogue of acetylruthenocene, acetophenone, has been extensively studied (see Kiese & Lenk, 1974, for a review) and both major and minor metabolites have been identified. The major metabolites formed are:

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(1) a glucuronide or sulphate conjugate produced after reduction of the keto group; (2) a hippuric acid conjugate formed after oxidation of the methyl group and decarboxylation (Thierfelder & Daiber, 1923; Thierfelder & Klenk, 1924). Traces of ring-hydroxylated *m*- and *p*-acetophenone were also detected, as well as small amounts of mandelic acid.

Since we showed that the parent compound ruthenocene is metabolized *in vivo* by the same pathway as is benzene (Taylor & Wenzel, 1977, 1978), it seemed likely that acetylruthenocene would follow the degradative scheme for acetophenone as far as the extra bulk of the cyclopentadiene metal 'sandwich' structure would allow.

Experimental and Results

Preparation of acetylruthenocene

Acetylruthenocene was prepared by Friedel-Crafts acylation of ruthenocene (Serva, Heidelberg, Germany). Ruthenocene (2.3 g, 0.01 mol) was dissolved in redistilled, dry methylene chloride and stirred while anhydrous AlCl_3 (2.7 g, 0.02 mol) was added. After 15 min at room temperature (20°C), the complex was refluxed (about 40°C) and redistilled acetyl chloride (0.8 g, 0.01 mol) was added in a dropwise manner. Reflux continued for 1.5 h. On cooling, the complex was destroyed with ice/water, and ascorbic acid was added until the layers cleared. They were then extracted with diethyl ether. The pooled ether extracts were washed, dried over anhydrous MgSO_4 and ether was removed *in vacuo*.

The reaction products were separated by applying portions of the crude product (dissolved in benzene)

to a column (25cm×2cm) of Kieselgel 60 (Merck, Darmstadt, Germany) and eluting it with ether/benzene (1:9, v/v) (approx. 100ml) and then with ether alone (approx. 120ml). Unchanged ruthenocene and monoacetylruthenocene were eluted, in that order, with ether/benzene, and diacetylruthenocene was eluted with ether. The u.v. absorption of the column eluate was monitored, and fractions corresponding to the three compounds were pooled. T.l.c. of portions of the pooled fractions on silica-gel plates (Merck) in benzene/ethyl acetate, (5:1, v/v) (solvent A) gave the following R_F values: ruthenocene 0.94; acetylruthenocene 0.55; diacetylruthenocene 0.16. The mass spectra of the three fractions on a Varian MAT CH-7A machine, operating at 70°C and 70eV, were also in agreement with the assigned structures. The yield of acetylruthenocene was 48% and the melting point was 110–112°C [uncorrected; in agreement with reported values (Rausch *et al.*, 1960)].

Preparation of acetyl[^{103}Ru]ruthenocene

Labelled acetylruthenocene was prepared by heating acetylferrocene (approx. 10mg; Alfa Europe Products, Rotterdam, The Netherlands) with 20–50 μCi of $^{103}\text{RuCl}_3$ (approx. 100mCi/mmol; Isotopendienst West, Sprendlingen, Germany) in sealed evacuated ampoules (Langheim *et al.*, 1975). The reaction products were dissolved in benzene, and labelled acetylruthenocene was separated from other radioactive products on thin-layer silica-gel plates in solvent A as described by Langheim *et al.* (1975).

Radiochromatography

^{103}Ru -labelled compounds were located and determined by measuring the β - and Auger-electrons of ^{103}Ru (Wenzel & Abdul-Wahid, 1975) on a thin-layer scanner (Berthold, Wildbad, Germany).

Preparation of a solution of acetylruthenocene

Unlabelled acetylruthenocene (100–150mg) was mixed with the radioactive compound (approx. 1.5 μCi) and dissolved in propylene glycol (2ml).

Metabolism experiments

Three female adult Wistar rats (200–250g; Hagemann, 4923 Extertal, Germany) received orally 30–50mg of the radioactive acetylruthenocene mixture in propylene glycol (max. vol. 1ml). They were placed in metabolism cages, and urine and faeces were collected. A layer of toluene was placed in the urine-collecting vessels. Samples were collected daily for 4 days after administration of acetylruthenocene, and the amount of radioactivity in toluene, urine and faeces was determined by counting the samples in the well of a NaI-crystal γ -counter (Berthold, Wildbad,

Germany). At 90h after administration, urine contained 54% of the administered radioactivity, faeces 28% and the toluene extract of urine 2% (values obtained from pooled samples).

Extraction and identification of the major metabolite (glucuronide)

The urine obtained over a 4-day period after oral administration of acetylruthenocene was stored frozen at -20°C and thawed when required. T.l.c. of a portion in butan-1-ol/acetic acid/water (4:1:1, by vol.) (solvent B) showed two radioactive components, a major one at R_F 0.48 and a minor one at R_F 0.72. Separation and analysis of the metabolites was as follows.

(1) Portions (10ml) of urine were chromatographed on a column (10cm×1cm) of Amberlite XAD-2 (Serva, Heidelberg, Germany) by the method of Bradlow (1968). The methanol eluate contained approx. 70% of the applied radioactivity, and t.l.c. of a portion showed two peaks in solvent B on silica-gel plates [typical R_F values 0.44 (major) and 0.68 (minor); the major component accounted for 91% of applied radioactivity, the minor, 9%].

(2) A portion of the methanol extract was divided into three equal samples that were evaporated to dryness and then dissolved in 0.5ml of 0.1M-sodium acetate buffer, pH4.5. Penicillin (500i.u./ml; Hoechst A.G., Frankfurt, Germany) was added to each to prevent bacterial growth during the 24-h incubation period. The test incubation contained β -glucuronidase [0.09 unit ($\mu\text{mol}/\text{min}$)/ml; ox liver; Serva] and the control incubation mixtures contained either boiled enzyme or no enzyme. After 24h at 37°C , portions of the incubates were chromatographed on thin-layer plates in solvent B and radioautograms prepared. The major metabolite was completely converted by β -glucuronidase into a component of R_F value 0.9–1.0. The minor component was only partially converted. With the control incubates there was no conversion of the major or minor components into secondary products. The test incubation mixture was extracted with toluene, the extract was concentrated *in vacuo* and chromatographed on thin-layer plates in solvent A, which produced a single radioactive peak at R_F 0.4. Further t.l.c. proved that the aglycone formed by hydrolysis with β -glucuronidase was identical with the component extracted with toluene from urine.

(3) The remaining methanol extracts from the Amberlite XAD-2 column were pooled, concentrated and chromatographed on thin-layer plates in solvent B by using prewashed plates and redistilled solvents. After three purifications on t.l.c., 8mg of the major metabolite (R_F 0.44–0.48) were obtained. The repeated chromatography of the minor metabolite caused degradation, and the amounts recovered were insufficient for analysis. The purified major meta-

Fig. 1. (a) Mass spectrum (200°C , 70 eV) of the permethylated glucuronide of acetylruthenocene and (b) mass spectrum (70°C , 70 eV) of the metabolite aglycone showing the typical ruthenium 'fingerprint' (Ru isotopes 96–104, main isotope 102).

(a) Ions at m/e 101, 149, 201, 231 are characteristic of the permethylated glucuronide moiety (Thompson *et al.*, 1973). The ions at m/e 550, 536 and 522 (Ru = 102) are the molecular ion plus the artefacts produced during permethylation (see the text for details). The ions at m/e 318, 304 and 290 are derived from 550, 538 and 520 by loss of permethylated glucuronide, and the relatively strong ion at m/e 259 is indicative that the carbonyl group is intact ($\text{C}_5\text{H}_3\text{RuC}_5\text{H}_4\text{C}\equiv\text{O}^+=259$). (b) The molecular ion at m/e 290 fragments to give $\text{C}_5\text{H}_3\text{RuC}_5\text{H}_4\text{C}\equiv\text{O}^+$ (259) and $\text{C}_5\text{H}_3\text{RuC}_5\text{H}_4^+$ (231), which is characteristic of acylmetallocene compounds (see Mandelbaum & Cais, 1964).

bolite was divided into two portions and analysed as follows.

(4) Approx. 4mg of the metabolite was permethylated by the method of Leclercq & Desiderio (1971). The sample was dissolved in 800 μ l of redistilled dry dimethyl sulphoxide, flushed with N₂, treated with the sodium salt of dimethyl sulphoxide for 15 min and then 150 μ l of methyl iodide was added. After 1.5 h at room temperature, 1.5 ml of water was added, the mixture was extracted with 4 $\times$ 1 ml of chloroform, the extracts were pooled, washed with water, dried over anhydrous MgSO₄, and chloroform was removed *in vacuo*. Mass spectrometry of the sample (with a Varian MAT CH-7A instrument with an EI/FD ion source operating at 200°C and 70 eV) gave the spectrum in Fig. 1(a). The interpretation of the spectrum is discussed below.

(5) Another portion of the purified metabolite was hydrolysed with β -glucuronidase under the same conditions as described in paragraph (2). After 24 h, the solution was extracted with redistilled toluene, then another 0.09 unit of enzyme was added/ml and at 48 h the solution was again extracted with toluene. Toluene was removed *in vacuo* and the material remaining was chromatographed twice in solvent A on pre-washed silica-gel plates to yield a single

component (R_f 0.32–0.37). P.m.r. of the sample in [2H]chloroform on a Bruker WP-60 instrument gave the spectrum in Fig. 2(a) and mass spectrometry at 70°C and 70 eV produced the spectrum in Fig. 1(b). The spectra are discussed in the next section.

Biliary excretion of metabolites

Two rats were anaesthetized and fitted with jugular-vein and bile-duct cannulae. Anaesthesia was maintained by administering Nembutal (Deutsche Abbott, Ingelheim am Rhein, Germany) through the jugular-vein cannulae. A solution of acetylruthenocene (150 μ l; 11 mg/kg) in propylene glycol was also administered via the jugular vein, and bile samples were collected every hour for 6 h. In the 6-h period, between 43 and 70% of the dose was excreted in bile. T.l.c. of the bile in solvent B, and of a chloroform extract of bile in solvent A, showed that the bile contained a major metabolite that was chromatographically identical with the major metabolite in urine. The chloroform extract contained small amounts of the aglycone and smaller amounts of unchanged acetylruthenocene. Hydrolysis of the major metabolite in bile with β -glucuronidase and

Fig. 2. P.m.r. of the metabolite aglycone (a) and acetylruthenocene (b)

The strong signal at 2.29 p.p.m., corresponding to the methyl protons of acetylruthenocene, is missing from the aglycone spectrum, showing that the molecule is hydroxylated on the methyl group and not in the rings.

comparison by t.l.c. of the aglycones in bile and urine showed that they were identical.

Discussion

The results show that after oral administration of acetylruthenocene to rats, urine contains a glucuronide as the major metabolite, another metabolite that appears unstable under the purification procedure, and small amounts of the aglycone of the major metabolite.

Identification of the major metabolite as a glucuronide allowed us to formulate possible structures for the compound. If acetylruthenocene follows the acetophenone pattern, the keto group should be reduced and then conjugated to give a compound with mol.wt. 508 after permethylation. Alternatively, if the methyl group is oxidized and conjugated, or if one of the rings is hydroxylated and conjugated (three isomers possible), the molecular weight after permethylation should be 522. (Natural ruthenium consists of isotopes with atomic weights 96–104. The isotope with at.wt. 102 is the most abundant and therefore forms the most intense ions. All mass numbers given in the text are based on $Ru=102$.) The permethylated metabolite gave ions showing the characteristic isotopic distribution of ruthenium at m/e 550, 536, 522 and 508 and 318, 304 and 290 (Fig. 1). The second group of ions represents a loss of permethylated glucuronide from the molecule and it is significant that there is no ion at m/e 508 – 232 i.e. 276. This rules out the possibility of a glucuronide with mol.wt. 508.

When acetylruthenocene was subjected to the permethylation procedure, and analysed by mass spectrometry, extra ions at M^++14 and M^++28 were observed. These were attributed to the replacement of two methyl hydrogens by methyl groups. Owing to the electron-withdrawing effect of the aromatic cyclopentadiene ring and the keto group, the methyl protons become slightly acidic and are attacked by the extremely powerful reagents. If this is the case with acetylruthenocene, then the true molecular weight of the permethylated metabolite is 550–28 or 536–14 i.e. 522. It was not possible to differentiate between the possible isomeric forms with mol.wt. 522 by analysis of the fragmentation pattern.

Mass spectrometry of the aglycone gave a simple spectrum with the expected M^+ ion at 290 corresponding to $C_{12}H_{12}O_2Ru$, but could not provide positive proof as to which isomer was present. P.m.r. of acetylruthenocene and the aglycone however, proved conclusively that the aglycone had the formula $C_5H_5-Ru-C_5H_4-CO-CH_2-OH$. The methyl protons of acetylruthenocene show a singlet at 2.29 p.p.m. (Fig. 2) that is missing from the metabolite spectrum. Moreover, the singlet at 4.59 p.p.m. representing

the five equivalent hydrogen atoms in the unsubstituted ring is present in both spectra, thus ruling out the possibility of a 1-acetyl-1'-hydroxyruthenocene molecule. Similarly, the triplet signals at 4.8 and 5.1 p.p.m. occur in both spectra, which discounts the possibility of a 1-acetyl-2-hydroxyruthenocene or 1-acetyl-3-hydroxyruthenocene aglycone. The major metabolite of acetylruthenocene is thus formed by oxidation of the methyl group, followed by conjugation of the primary alcohol with glucuronic acid. If $Rc = C_5H_5-Ru-C_5H_4-$, then the reaction can be written as follows:

Acetophenone, however, is metabolized mainly by reduction of the keto group and conjugation of the secondary alcohol so formed with glucuronic acid or sulphate. A secondary pathway of acetophenone metabolism involves degradation of the side chain to give benzoic acid, which is excreted as its glycine conjugate, hippuric acid (for a review, see Kiese & Lenk, 1974).

Therefore acetylruthenocene does not produce the pattern of metabolism observed with acetophenone. It seems that the extra bulk of the aromatic cyclopentadiene metal sandwich structure prevents reduction of the keto group to the extent seen with acetophenone. Instead, the methyl group of acetylruthenocene is oxidized to a primary alcohol that is conjugated with glucuronic acid. Whether or not the unidentified minor metabolite is a glycine conjugate of a carboxylic acid arising from further metabolism of this primary alcohol remains to be determined.

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