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Center of Toxicology, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

CHRISTOPHER P. CHENGELIS
ROBERT A. NEAL

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The metabolism of alphaxalone by isolated rat hepatocytes

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Alphaxalone (3α-hydroxy-5α-pregane-11,20 dione) is a steroid anaesthetic agent which is used clinically both for the induction and maintenance of anaesthesia. Indirect evidence obtained from *in vivo* studies on rats [1] and rabbits [2] suggests that this drug is metabolized in the liver. No data relating to the rates of metabolism of alphaxalone by the liver *in vivo* or by liver preparations *in vitro* have been reported. Such data are of importance in assessing the ability of the liver to convert the drug to an inactive form under normal or pathological conditions.

Isolated hepatocytes in suspension have been used as a convenient model system to study many aspects of liver metabolism [3] including drug detoxification [4,5]. In this communication, a method is described for the measurement of the degradation of alphaxolone by isolated rat hepatocytes and the kinetic parameters of this process are reported.

Collagenase for the preparation of isolated hepatocytes was purchased from C.F. Boehringer und Soehne, Mannheim. Alphaxalone and 3β-hydroxy-5α-pregn-16-ene 11,20 dione were kindly donated by Glaxo–Allenbury's Research Ltd, Greenford, Middlesex, U.K. Bovine serum albumin was purchased from the Sigma Chemical Co., St Louis, MO, U.S.A.

Hepatocytes were prepared from normally fed male Wistar albino rats of weight 250–300 g by the method of Berry and Friend [6], as modified by Krebs *et al.* [7]. The viability of the cells was > 90%, as assessed by Trypan Blue exclusion. Cells were suspended at the appropriate concentration in Krebs–Henseleit bicarbonate buffer [8] at pH 7.4 and 37° under a gas phase of 95% O_2/5% CO_2. The incubation medium also contained 2% w/v dialysed bovine serum albumin (fraction V). The reaction was begun by the addition of the appropriate concentration of alphaxalone. Samples were withdrawn at various times and deproteinised by the addition of perchloric acid (5% w/v final concentration). Denatured protein was removed by centrifugation at 10,000 *g* for 30 sec in a bench centrifuge (Eppendorf Zentrifuge Model 3200). The supernatant was stored at −20° until assayed. Cell protein was determined by a biuret method [9] using bovine serum albumin as a standard. Alphaxalone was added to the cells as a concentrated solution in methanol. Control experiments showed that methanol at the concentrations used had no cytotoxic effects.

Alphaxalone was assayed by a gas chromatographic technique based on the method of Chambaz and Horning [10], as modified by Sear and Prys-Roberts [11] using 3β-hydroxy-5α-pregn-16-ene 11,20 dione as the internal standard. Extraction and derivatisation of alphaxalone from suspensions of isolated hepatocytes gave a recovery of 91 ± 5% (mean ± S.D. of five observations), and repeated assays of a single sample gave a coefficient of variance of < 10% (mean value 7.9%).

Figure 1 shows gas chromatograph tracings obtained from a typical experiment in which cells were incubated with 0.54 mM alphaxalone for 20 min at 37°. At the start of the incubation there is a single peak chromatographically identical with alphaxalone with an R_f value of 0.79 relative to the internal standard (Fig. 1a). After a 20 min incubation, the size of this peak was reduced and a single additional peak appeared with an R_f value of 1.11 (Fig. 1b). This peak represents the sole metabolic product of alphaxalone detected by this technique. The identity of this metabolite was not determined in the present investigation. However, other workers have shown that the primary metabolite of alphaxalone in the rat is 2α-hydroxy alphaxalone [12].

The time of course of alphaxalone disappearance from the incubation medium in a typical experiment is shown in Fig. 2. Alphaxalone was added at an initial concentration of 0.136 mM. Fifty per cent of the alphaxalone disappeared in 6.5 min and the appearance of the metabolic product followed a similar time course. In Fig. 2 the apparent concentration of the metabolite was calculated, assuming a similar chromatographic response factor to that of alphaxalone.

In order to use the isolated hepatocyte model to carry out quantitative studies on alphaxalone metabolism, it was necessary to establish that the initial rate of alphaxalone disappearance was proportional to cell protein concentration. In a series of experiments, alphaxalone was added at a near saturating concentration (136 μM) to suspensions of hepatocyte containing between 2 and 8 mg cell protein/ml. A linear relationship between initial rate of alphax-

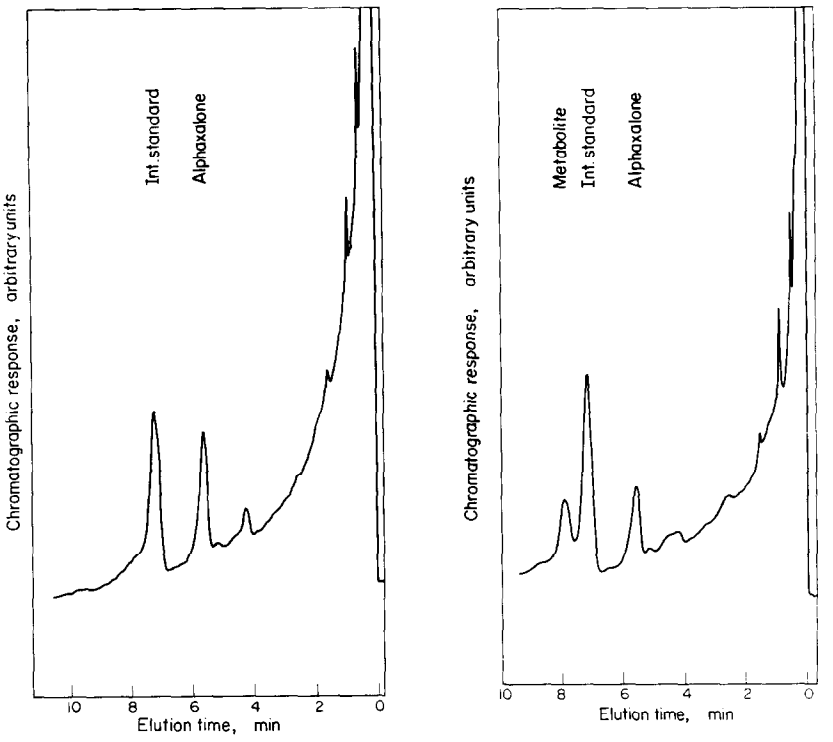

Fig. 1. Gas chromatograph traces of the protein-free acid extract of an isolated hepatocyte suspension. Alphaxalone (0.54 mM) was added at zero time; the cell protein concentration was 4.4 mg cell protein/ml. Fig. 1(a), sample taken at zero time; Fig. 1(b), sample taken after 20 min incubation.

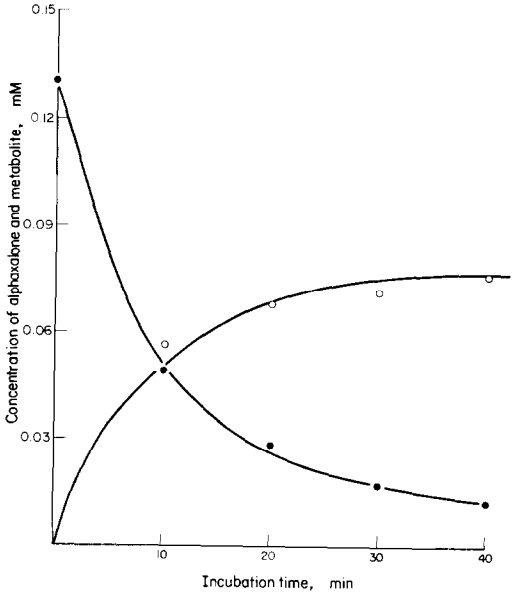

Fig. 2. The course of alphaxalone disappearance from a hepatocyte suspension. The initial concentration of alphaxalone was 0.136 mM and the cell protein concentration was 10.8 mg/ml. The results are from a single representative experiment. Closed circles represent alphaxalone concentration; open circles represent apparent concentration of the metabolite calculated as described in the text.

alone disappearance and cell protein concentration was observed (correlation coefficient = 0.971); the mean rate of alphaxalone metabolism was found to be 1.37 nmole/min/mg under these conditions.

The dependence of the initial rate of alphaxalone metabolism on alphaxalone concentration is shown in Fig. 3. Alphaxalone metabolism appeared to follow normal Michaelis–Menten kinetics. From similar experiments on five separate cell preparations, the maximum rate of alphaxalone metabolism (V_{max}) was found to be 1.45 ± 0.26 nmole/min/mg cell protein and the drug concentration at half maximal velocity (apparent K_m) was 0.026 ± 0.015 mM (mean ± S.E.M.).

It should be noted that alphaxalone at high concentrations has been shown to be toxic to isolated hepatocytes [13]. However, at the concentrations used in the experiment shown in Fig. 3, little or no cytotoxic effects were detected.

The maximum rate of metabolism of alphaxalone by isolated hepatocytes (1.45 nmole/min/mg at 37°) is comparable with the rate at which the glucuronidation of harmol occurs in the same system [14]. The maximum rates at which a large number of different xenobiotic molecules are degraded in hepatocytes are in the range 0.01–1.2 nmole/min/mg under similar conditions [4,5]. Since the liver is known to be the site of metabolism of many of these compounds and also of many steroids, these results are consistent with the suggestion that the liver is the major organ responsible for the inactivation of alphaxalone.

The apparent Km value for alphaxalone metabolism in isolated hepatocytes is rather higher than the alphaxalone concentration measured in plasma of rat during recovery from a single sleep dose of althesin [12]. If the data obtained from the isolated hepatocytes reflect the properties of the intact liver, it appears that the rate of degradation of alphaxalone by the liver would be highly concentration dependent in the plasma concentration range observed during anaesthesia in the rat.

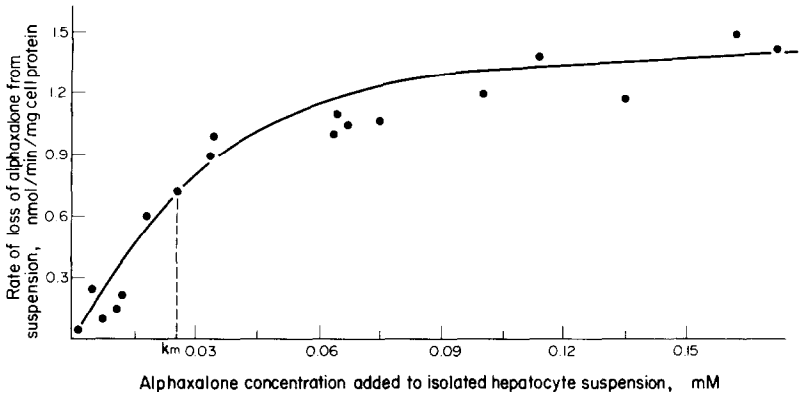

Fig. 3. Concentration dependence of alphaxalone disappearance as a function of alphaxalone concentration. The results shown are taken from five separate cell preparations, using cell concentration of between 2 and 8 mg/ml. Samples were taken at intervals over the first 5 min of incubation; the initial rates were evaluated from the first-order rate equation. Preliminary experiments similar to those in Fig. 2 had previously established that alphaxalone disappearance followed first-order kinetics.

Isolated hepatocytes appear to constitute a useful model system for the study of liver alphaxalone metabolism. A particular advantage of this preparation is that a large number of parameters can be varied using a single batch of cells, and also the reproducibility of results between different cell batches is high. However, insufficient data are at present available to establish adequately whether the rates of metabolism of alphaxalone in isolated hepatocyte are comparable with those in the isolated perfused liver or the whole animal.

In summary, a model system for the measurement of liver alphaxalone has been described and the rates of alphaxalone metabolism in this system have been determined. It is suggested that isolated hepatocytes may be of value in determining the pathway of alphaxalone metabolism in the liver, and also assessing the effect of factors such as administration of other drugs and variations in diet on this process. A particular use of this system may be in the study of alphaxalone metabolism during liver disease.

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University Departments of Anaesthetics and Biochemistry, Royal Infirmary, Bristol, Avon, U.K.

J. W. SEAR
J. D. MCGIVAN

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