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SHORT COMMUNICATIONS

Is phenobarbital a "gratuitous" inducer in male rats?

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THIS NOTE arises from an attempt to use the oxygen uptake in the presence of NADPH as a measure of hydroxylating enzymes in microsomes from the livers of rats treated with phenobarbital. The inducing effect upon liver enzymes was first recorded in 1959 by Conney and Burns[1] and Remmer.[2] In our hands, we could not observe the increased oxygen uptake described elsewhere on addition of phenobarbital, hexobarbital, laurate and testosterone[3] in 95 per cent of our experiments. In fact the microsomes took up much oxygen in the presence of NADPH alone, either when separated from the supernatant at 100,000 *g* or when collected with the supernatant after removal of the mitochondria and lysosomes at 12,000 *g*. This property usually increased after standing for 24 hr in the cold (−17°). The endogenous oxygen uptake of microsomes alone was sufficient to mask or to render unreliable any extra effect upon oxygen uptake of adding the above, or any fatty acid.

While we failed to see an increased oxygen uptake, we had no difficulty in confirming the increase in cytochrome P_{450} or in *N*-demethylation of aminopyrine. It was important to decide whether phenobarbital was inducing enzymes without being metabolized itself. Hence we studied phenobarbital metabolism *in vitro* using induced microsomes and both ordinary sodium phenobarbital and also 5-ethyl-5 phenyl barbituric-2-C^{14} acid.

Experimental

Chemicals. Radioactively labelled phenobarbital was from New England Nuclear. AnalaR reagents were used where possible.

Animals. For the experiments with the non-labelled phenobarbital, livers were obtained from Wistar rats ♂ (wt 150 g) were either injected intraperitoneally with phenobarbital (88 mg/kg) under light ether anaesthesia for 4 successive days or were dosed orally for 1 month (wt 100 g) with 0·5 mg/ml increased to 1·5 mg/ml of the compound within 8 days in the drinking water. In the case of the radioactive compound, animals had been dosed orally. All the animals were killed by decapitation, and their livers homogenized by hand in a Potter Teflon glass homogenizer, using 1·15 per cent KCl (1:5). The mixture was centrifuged at 12,000 *g* for 20 min, and the supernatant from this centrifuged at 100,000 *g* for 90 min in cases where separation of the final supernatant was required. The washed microsomal pellet was resuspended in 1·15 per cent KCl in the proportion of 0·5 g of original tissue in 1 ml KCl. The protein concentration of the microsomal suspension was measured by the procedure of Lowry *et al.*[4] Cytochrome P_{450} was estimated using its CO-binding properties, Omara and Sato.[5]

Exposure of microsomes to phenobarbital. The microsomal suspension (3 ml), contained 1·7 mg protein/ml, with additions of NADPH regenerating system (consisting of 50 mM Tris, 5 mM $MgCl_2$, 1 mM NADP, 5 mM isocitrate, 0·4 units isocitrate dehydrogenase) in 0·5 ml; ±10 μmoles total phenobarbital (Na salt) including radioactively labelled phenobarbital (90 μg). The whole was incubated for 1·0 hr at 37°, and then frozen, Gigon *et al.*[6]

Estimation of phenobarbital. Phosphate buffer pH 6·0 (1·0 ml) and 5 vol. diethylether were added to the frozen microsomal suspension, thawed, and the whole shaken for 1·0 min on a Whirlimixer. The ether layer was removed, poured through anhydrous Na_2SO_4 into a flask; this procedure was repeated twice more. The combined ether extracts were reduced in volume in a rotary evaporator and methylated as described below. Blank experiments in which 7·12 and 0·712 mg of phenobarbital (Na salt) were put through this procedure together with the salt mixture showed that 100 per cent was extracted, as judged by gas chromatography estimations.

The final extract was converted to methyl compounds to reduce the retention time. Methanol was added to the ether residues to a concentration of 10 per cent and the whole acidified with one drop of methanolic HCl. Diazomethane gas was bubbled through the solutions until they were yellow. After removal of excess diazomethane by boiling, aliquots were injected into the GLC apparatus. For the unlabelled phenobarbital a column 150 × 0·5 cm dia. of 10 per cent PEG 20 M on 100–120 mesh Diatomite CAW and an O_2 free N_2 flow rate of 60 ml /min. The column was maintained at 194°.

For the ^{14}C-labelled a good separation of all the components was obtained on a 180/0·5 cm dia. column of 10% polyethylene glycol adipate on Gas Chrom Z 80–100 mesh, and an argon carrier

gas flow rate of 40 ml/min. The column was maintained at 50° for 6 min, then temperature programmed at 6°/min to 200°. The effluent gas from the GLC apparatus was passed through a combustion tube and a proportional counter as designed by James and Piper[7] to detect ^{14}C-labelled components. The retention time of the methyl derivative of phenobarbital relative to methyl palmitate when chromatographed isothermally at 200° was 7·57.

Experimental results. The increase in the induced microsomes of cytochrome P_{450} over the normal varied from three to six times; in the labelled experiment it was four times.

Exposure of the phenobarbital to the liver microsomes, for one hour gave no decrease in the compound added, though there was a decrease in the amount extractable immediately after addition to the microsomes. Hence, though the method of extraction was completely efficient for the blank experiments, the total phenobarbital added to a microsomal preparation from a rat liver was not extracted completely. The average for a typical experiment for the average of duplicates not varying from the mean of more than $\pm 1{\cdot}2$ per cent, gave 72 per cent extracted before incubation and 77·4 per cent after 1 hr incubation at 37°. Hence 28 per cent became unextractable before and 22·5 per cent after incubation. We have not pursued this interesting point, which we suggest is due to binding of the drug to some component of the microsomes. The slight increase observed in extraction after incubation is consistent with an increase of some lipid substances.

Two experiments were done with the labelled compound; to one of these lecithin 10 ml of 0.02 per cent lecithin per 3·0 ml in flask was added (see Coon *et al.*).[8] In neither case was there any indication of a radioactive peak other than that due to unchanged phenobarbital.

Conclusions and discussion

In our experiments, there was no disappearance of phenobarbital (within experimental error) when exposed to microsomes from a rat liver induced with phenobarbital, though the induction had much increased the content of cytochrome P_{450} and of aminopyrine *N*-demethylase. No peak indicating a metabolic product could be detected by GLC chromatography, when [^{14}C]labelled phenobarbital was used, which we interpret to mean that there was no methoxy metabolic product.[9] In any case this was unlikely in view of the quantitative recovery.

Since phenobarbital induces large changes in the liver enzymes without undergoing detectable change, we consider that it is acting as a "gratuitous inducer"[10] to use a microbiological term. There is an apparent parallel in the action of spironolactone[11] though with further complications. In several recent papers, we note that authors say that phenobarbital is an inducer, which from our point of view is a strictly accurate statement.

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