parent compound (Naphthacene) in stimulating zoxazolamine metabolism. This does not fit in too well with our observation of the apparent ineffectiveness of the 6 formyl derivative of benzo[a]pyrene. However Buu-Hoi *et al.* used rats in their work and we have used mice. Our choice of animals was governed by the nature of some of the other biological properties we wished to investigate. It was clearly desirable to use the same species for all our work so that a meaningful comparison of the results could be made.

It is of interest that aromatic aldehydes are normally metabolised to acids.[11] The rates of metabolism in the mouse and the rat could be markedly different. The apparent lack of effect of 6 formyl benzo[a]-pyrene could arise from a combination of stimulation by the parent formyl compound coupled with inhibition produced by a metabolite. In this context the methyl, amide and to a lesser extent nitrile derivatives might all be expected to give carboxyl derivatives on metabolism.[12] Examination of Table 1, in which inhibition of drug metabolising systems would show up clearly; reveals that hypnosis times are prolonged, but not significantly, for formyl, nitrile and amide derivatives. In the case of the amide, if an inhibitory metabolite is formed then the inducing effect of the amide itself must be very great (see Table 2). It must be pointed out that the inhibitory effect is not necessarily a direct one but may operate via an indirect mechanism such as producing adrenal damage. This matter is being investigated further and attempts are being made to prepare the 6 carboxyl derivative of benzo[a]pyrene

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Department of Pharmacy,
Leicester Regional College of Technology,
The Newarke,
Leicester

F. DEWHURST
D. A. KITCHEN

REFERENCES

1. J. C. ARCOS, A. H. CONNEY and N. P. BUU-HOI, *J. biol. Chem.* **236**, 1291 (1961).
2. N. P. BUU-HOI and HIEN DO-PHUOC, *Naturwiss.* **53**, 435 (1966).
3. N. P. BUU-HOI, HIEN DO-PHUOC and G. SAINT-RUF, *Compt. rend.* **264**, 2414 (1967).
4. N. P. BUU-HOI and HIEN DO-PHUOC, *Z. Naturforsch.* **22** B, 532 (1967).
5. A. H. CONNEY, C. DAVISON, R. GASTEL and J. J. BURNS, *J. Pharmac. exp. Ther.* **130**, 1 (1960).
6. A. H. CONNEY, N. TROUSOF and J. J. BURNS, *J. Pharmac. exp. Ther.* **128**, 333 (1960).
7. J. R. COOPER and B. B. BRODIE, *J. Pharmac. exp. Ther.* **114**, 409 (1955).
8. D. B. CLAYSON, *Chemical Carcinogenesis*, pp. 135, 201. Churchill, London (1962).
9. W. C. HUEPER and W. D. CONWAY, *Chemical Carcinogenesis and Cancers*, pp. 197, 258. Thomas. Illinois (1964).
10. R. T. WILLIAMS, *Detoxication Mechanisms*, p. 318. Chapman and Hall, London, 2nd edn. (1959).
11. R. T. WILLIAMS, *Detoxication Mechanisms*, p. 332. Chapman and Hall, London, 2nd edn. (1959).
12. R. T. WILLIAMS, *Detoxication Mechanisms*, pp. 194, 156, 407. Chapman and Hall, London, 2nd edn. (1959).

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A comparison of hepatic drug-metabolizing enzyme activity in the germ-free and conventional rat*

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THE OXIDATIVE and reductive pathways of hepatic microsomal drug metabolism are generally deficient

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at birth.[1–4] Many factors directly or indirectly related to drug metabolism (NADPH oxidase, glycogen, microsomal protein, microsomal cytochromes) have been found to be deficient in the newborn.[1–6] It is not known whether the postnatal increase in drug-metabolizing enzyme activity and levels of these factors is caused by release of inhibition or by induction. Among the changes which occur in the environment of the fetus at birth is its introduction into nonsterile surroundings and the establishment of enteric microflora. It is possible that the development of drug-metabolizing systems occurs, at least in part, by virtue of enzyme induction by absorbed bacterial metabolities. If this is so, animals in which enteric flora do not develop during the postnatal period would be expected to develop this enzyme activity more slowly or not at all.

The hepatic microsomes of the juvenile germ-free rat should not be able to metabolize drugs at a rate comparable to those of conventionally reared rats if microfloral products play any appreciable role in induction of these systems. In addition, if these products play an essential role in hepatic drug-metabolizing activity *per se*, the livers of germ-free animals should be deficient in this activity at all ages.

In this study, oxidation or reduction *in vitro* of four drugs was compared in liver obtained from adult and juvenile germ-free (GF) and conventional (CONV) Sprague–Dawley rats of both sexes.

Animals. Mature male (220–250 g) and female (190–230 g) and juvenile (35 days old) rats were purchased from A. R. Schmidt Co., Madison, Wis. GF rats were obtained from the same source and allowed to acclimate in sterile isolators for at least 1 week before use. CONV and GF animals were maintained on the same diet, with the exception that the diet autoclaved for the GF rats was fortified with additional amounts of vitamins A and B_1. The germ-free status of the GF rats was confirmed at the time of sacrifice by culturing cecal contents in thioglycollate broth and on blood agar plates.

Enzyme preparations. The rats were decapitated and the livers removed and homogenized with 2 vol. of 1·15% (isotonic) KCl solution in a Teflon-glass homogenizer at 4°. The homogenate was centrifuged at 9000 *g* for 15 min and the supernatant fluid was decanted and used as the enzyme source. An aliquot of the supernatant was centrifuged at 105,000 *g* for 60 min to obtain a microsomal pellet for protein determinations.

Assay system. Each incubation mixture contained 9000 *g* supernatant from 0·3 g liver, 20 μmoles, glucose 6-phosphate, 0·4 μmole NADP, 25 μmoles nicotinamide, substrate (2·5 μmoles hexobarbital, 1·5 μmoles zoxazolamine, 1·5 μmoles neoprontosil, 1·5 μmoles *p*-nitroanisole) and 0·2 M phosphate buffer, pH 7·4, to make a final volume of 2·5 ml. All incubations were carried out at 37° in a Dubnoff shaking incubator for 30 min under air (except for neoprontosil which was incubated under nitrogen).

The aliphatic hydroxylation of hexobarbital[7] and the aromatic hydroxylation of zoxazolamine[8] were estimated by measuring the disappearance of the substrate. The azoreduction of neoprontosil was determined by measuring the appearance of sulfanilamide.[9] The *o*-demethylation of *p*-nitroanisole was estimated by measuring the formation of *p*-nitrophenol.[10] Protein content of the microsomal pellet was determined by micro-Kjeldahl procedure. A comparison of activity was made between GF and CONV animals of each sex and age, with a *t*-test significance level set at $P \leq 0.05$.

Results. The amount of substrate metabolized or product formed is presented in Table 1. Enzyme activities were similar in GF and CONV animals of either sex or age, with no significant differences observed. The sex difference in hexobarbital metabolism reported by others[10,11] was seen in both GF and CONV mature rats, but not in the juvenile (prepubertal) rat. Microsomal protein values are presented in Table 2. Livers from 35-day-old GF rats contained significantly less protein than their conventional counterparts, while those from mature GF animals contained equivalent or greater amounts. This is in contrast to the relatively stable values observed in CONV rats with regard to age. When enzyme activity was calculated in terms of millimicromoles of drug metabolized per milligram of microsomal protein, the difference in one set of values (see Table 1) was significant.

Discussion. The cause of the greater age difference in microsomal protein in the GF rat than in the CONV rat is not clear. Protein content is a relative value and can be altered by the state of nutrition.[10] The diet fed to germ-free rats was autoclaved and may have influenced protein values.

The rate of enzymatic transformation of the substrates studied appears similar in mature and 35-day-old GF and CONV rats. We infer from this that the postnatal establishment of intestinal flora did not appreciably induce or inhibit microsomal oxidation or reduction of these drugs. This, of course, may not be true for other drug-metabolizing systems. In this regard, studies on the synthetic pathways may prove interesting, particularly sulfanilamide acetylation, which has been shown to occur in the hepatic reticuloendothelial cell of rabbits.[12] Most aspects of the reticuloendothelial system of germ-free animals are functionally and/or quantitatively underdeveloped.[13]

TABLE 1. COMPARISON OF THE ENZYME ACTIVITY* IN 9000 *g* SUPERNATANT PREPARATIONS FROM LIVERS OF CONVENTIONAL AND GERM-FREE RATS

Substrate	Female		Male	
	Conventional	Germ-free	Conventional	Germ-free
Mature				
Hexobarbital	223 ± 45	194 ± 50	584 ± 148	647 ± 192
Zoxazolamine	171 ± 24	118 ± 38	139 ± 62	214 ± 61
Neoprontosil	700 ± 170	544 ± 140	793 ± 132	743 ± 186
Nitroanisole	113 ± 20	96 ± 29	120 ± 19	100 ± 24†
Juvenile‡				
Hexobarbital	265 ± 86	322 ± 114	392 ± 119	438 ± 127
Zoxazolamine	191 ± 71	174 ± 34	208 ± 85	226 ± 38
Neoprontosil	809 ± 181	650 ± 154	803 ± 204	767 ± 67
Nitroanisole	130 ± 22	121 ± 46	133 ± 25	134 ± 18

* Enzyme activity (mμmoles/0·3 *g* liver and 30 min) ± S.D. for a minimum of 5 preparations.

† Significant (mμmoles/mg protein).

‡ The livers from two rats were pooled for each preparation.

TABLE 2. MICROSOMAL PROTEIN*

Rats	Female		Male	
	Conventional	Germ-free	Conventional	Germ-free
Mature	28·0 ± 1·4	27·0 ± 3·0	27·9 ± 4·1	32·1 ± 2·5†
Juvenile	26·9 ± 1·9	23·8 ± 2·1†	27·3 ± 2·1	23·9 ± 2·5†

* Expressed as mean (mg N × 6·25/*g* liver) ± S.D. for a minimum of 5 preparations.

† Significantly different ($P < 0·05$) values between GF and CONV rats of each age and sex.

Section of Comparative Pharmacology,
School of Veterinary Medicine,
University of Missouri,
Columbia, M., U.S.A.

C. R. SHORT
L. E. DAVIS

REFERENCES

1. W. R. JONDORF, R. P. MAICKEL and B. B. BRODIE, *Biochem. Pharmac.* **1**, 352 (1958).
2. J. R. FOUTS and R. H. ADAMSON, *Science, N.Y.* **129**, 897 (1959).
3. J. R. FOUTS, *Perinatal Pharmacology*, Report of 41st Ross Conference on Pediatric Research, p. 54. Ross Laboratories (1962).
4. G. DALLNER, P. SIEKEVITZ and G. E. PALADE, *Biochem. Biophys. Res. Commun.* **20**, 135 (1965).
5. R. KATO, P. VASSANELLI, G. FRONTINO and E. CHIESARA, *Biochem. Pharmac.* **13**, 1037 (1964).
6. J. R. FOUTS and L. G. HART, *Ann. N.Y. Acad. Sci.* **123**, 245 (1965).
7. J. R. COPPER and B. B. BRODIE, *J. Pharmac. exp. Ther.* **114**, 409 (1955).
8. J. J. BURNS, T. F. YU, L. BERGER and A. B. GUTMAN, *Am. J. Med.* **25**, 401 (1958).
9. J. R. FOUTS, J. J. KAMM and B. B. BRODIE, *J. Pharmac. exp. Ther.* **120**, 291 (1957).
10. R. KATO and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **150**, 279 (1965).
11. G. P. QUINN, J. AXELROD and B. B. BRODIE, *Biochem. Pharmac.* **1**, 152 (1958).
12. W. C. GOVIER, *J. Pharmac. exp. Ther.* **150**, 305 (1965).
13. H. A. GORDON, *Ann. N.Y. Acad. Sci.* **78**, 208 (1959).