rat liver enzyme), and the lack of inhibition by alanosine (up to 50 mM) as shown by Gale and Smith [9] was also observed. The ability of alanosine to serve as an alternative substrate for aspartate, as suggested by Gale and Smith [9], was also tested using the rat liver adenylosuccinate synthetase. The enzyme was incubated at 30° for 1 hr with 0.2 mM GTP, 10 mM alanosine, 0.01 mM IMP, 5 mM $MgCl_2$ in 0.05 M HEPES, pH 7.0, with 200,000 cpm of [8-^{14}C]IMP. The reaction mixture was chromatographed on PEI-cellulose sheets as described by Crabtree and Henderson [15] and also on a DEAE cellulose column. The column was equilibrated with H_2O. The reaction mixture (1:10 dilution) was then applied to the column and washed initially with H_2O. A gradient between H_2O and 1.0 M triethylamine–HCO_3, pH 8.0, was then run. This gradient allows elution of all nucleotide components of the reaction mixture. With both chromatographic systems no radioactive peaks were observed which would correspond to an alanosine analogue of adenylosuccinate as was suggested by Gale and Smith [9]. With aspartate present, a large amount of the [^{14}C]IMP was converted to adenylosuccinate. The possible formation of such an adenylosuccinate analogue was observed by Gale and Smith [9] by radiochromatographic techniques, but the product was not further characterized. The reason for the discrepancy in the results is not clearly understood, although the natural antibiotic might have contained contamination not found in the chemically synthesized compound, or the DL mixture used here may cause a difference.

The effect of alanosine and hadacidin on *E. coli* aspartase, asparaginase and aspartate transcarbamylase was the same. No significant inhibition was observed with any of the enzymes. Aspartase was assayed with 5 mM aspartate at pH 7.0. This aspartate level is at the K_m value [11] for aspartase so inhibition, if any, should be observed. Similar conditions were maintained for the other enzymes. Hadacidin (10 mM) and alanosine (4 mM) had no effect either in the ammonia assay or, in the case of hadacidin, in the spectrophotometric assay. Alanosine has a strong ultraviolet absorbance which makes the spectrophotometric assays unreliable. Asparaginase did not appear to be inhibited by 10 mM hadacidin or 4 mM alanosine at 0.1 mM asparagine with the assay done at pH 8.6.

Since Gale and Smith [9] had suggested that alanosine inhibited microbial pyrimidine synthesis possibly at the aspartate transcarbamylase step, the effect of the inhibitors on purified *E. coli* aspartate transcarbamylase was particularly interesting. At either 4 or 16 mM aspartate with 3.6 mM carbamyl phosphate, no inhibition was observed with either 12.5 mM hadacidin or 4 mM alanosine. These results suggest that, if microbial pyrimidine synthesis is inhibited by alanosine, it occurs at some other enzyme or by a metabolite of alanosine. Isolation of the active metabolite if present would allow determination of how alanosine effects nucleotide synthesis.

The specificity of hadacidin for adenylosuccinate synthetase is quite remarkable. It has no effect on the other three enzymes studied at concentrations 1000-fold higher than its K_I for the synthetase. This suggests a multiplicity of binding sites for aspartate and may allow future synthesis of very specific antimetabolites.

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REFERENCES

1. Y. K. S. Murthy, J. E. Thiemann, C. Coronelli and P. Sensi, *Nature, Lond.* **211**, 1198 (1966).
2. C. Coronelli, C. R. Pasqualucci, G. Tamoni and G. G. Gallo, *Farmaco, Ed. Sci.* **21**, 269 (1966).
3. D. Fumarola, *Pharmacology* **3**, 215 (1970).
4. G. C. Lancini, E. Lazzari and A. Diena, *Farmaco, Ed. Sci.* **24**, 169 (1969).
5. G. R. Gale, W. E. Ostrander and L. M. Atkins, *Biochem. Pharmac.* **17**, 1823 (1968).
6. J. C. Graff and P. G. W. Plagemann, *Cancer Res.* **36**, 1428 (1976).
7. H. T. Shigeura and C. N. Gordon, *J. biol. Chem.* **237**, 1932 (1962).
8. H. T. Shigeura and C. N. Gordon, *J. biol. Chem.* **237**, 1937 (1962).
9. G. R. Gale and A. B. Smith, *Biochem. Pharmac.* **17**, 2495 (1968).
10. F. B. Rudolph, *Ph.D. Thesis*, Iowa State University (1971).
11. F. B. Rudolph and H. J. Fromm, *Archs Biochem. Biophys.* **147**, 92 (1971).
12. S. W. Clark and F. B. Rudolph, *Biochim. biophys. Acta* **437**, 87 (1976).
13. J. C. Gerhart and H. Holoubek, *J. biol. Chem.* **242**, 2886 (1967).
14. F. B. Rudolph and H. J. Fromm, *J. biol. Chem.* **244**, 3832 (1969).
15. G. W. Crabtree and J. F. Henderson, *Cancer Res.* **31**, 985 (1971).
16. A. Meister, *Meth. Enzym.* **2**, 380 (1955).
17. G. R. Jacabson and G. R. Stark, *The Enzymes*, Vol. IX, pp. 225–308. (1973).

Biochemical Pharmacology, Vol. 26, pp. 1354–1355. Pergamon Press, 1977. Printed in Great Britain.

Conjugation of hydroxyphenylhydantoin and hydroxyphenobarbital in rat liver microsomes. Induction by phenobarbital*

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Phenobarbital (PB) and diphenylhydantoin (DPH) are metabolized through hydroxylation [1, 2] followed by conjugation of 80% and 60–70% of the total drug, respectively, with UDP glucuronic acid (UDPGA). The hydroxylation has been studied in detail [3, 4] but the conjugation of the hydroxylated metabolites with UDPGA has never been examined in detail.

A suitable radiochemical procedure for determining the activity of UDP glucuronyltransferase (UDPGT (E.C. 2.4.1.17) unspecific acceptor) versus these metabolites was required and previously developed in our laboratory [5].

We studied the activity of rat liver UDPGT towards both metabolites and in addition the effect of phenobarbital pretreatment of rats was examined.

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MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats weighing 200–220 g from Domaine des Oncins (St Germain sur l'Arbresle, 69210 L'Arbresle, France) were fed *ad lib.* with commercial pellets from UAR (91360 Epinay-sur-Orge, France).

In vivo *treatment.* Rats were given phenobarbital (sodium salt, Gardenal (R), Specia, Paris, France) (80 mg per kg body weight) in a single daily i.p. injection (1 ml per 100 g body weight) at 1 p.m., for 5 consecutive days. Controls did not receive any injection. Animals were killed by decapitation after a 16-hr starvation.

Preparation of microsomes. The livers were quickly excised at 4°, cooled in sucrose medium containing 0.25 M sucrose, 1 mM Tris, HCl, pH 7.4 and 1 mM EDTA. Approximately 2-g portions of livers were weighed, minced, and homogenized (10% v/w) in sucrose medium with an ice-coated Braun glass-and-Teflon homogenizer, for 1 min at 2800 rpm.

Unbroken cells, nuclei, and mitochondria were removed by centrifuging the homogenates at 9000 *g* for 20 min at 5° (Beckman Spinco LB 50, rotor 50). The resulting supernatants were spun at 105,000 *g* for 60 min, and the pellets resuspended in sucrose medium at a concentration of 200 or 500 mg liver per ml. Proteins were determined by the method of Lowry *et al.* [6] with crystalline bovine albumin (Fluka, Bucks, CH) as a standard. Enzymes were activated with Triton X-100 (Sigma, St. Louis, MO, USA) added for a detergent–protein ratio of 0.25.

UDP glucuronyltransferase activity. The following reagents were used: UDP glucuronic acid (Boehringer, Manheim, Germany); *p*-nitrophenol; UDP glucuronic acid[^{14}C] (NEN, Frankfurt, Germany); hydroxyphenylhydantoin (HPPH) (hydroxyphenyl-5-phenyl-5-hydantoin), a gift from Dr A. J. Glazko (Parke Davis, Ann Arbor, Michigan, USA) and hydroxyphenobarbital (HPB) synthetised in the laboratory [7]. Conjugation of *p*-nitrophenol was determined both colorimetrically [8] and radiochemically [5] and was used as a reference. The conjugation of hydroxyphenobarbital and of hydroxyphenylhydantoin was measured radiochemically [5] using 0.14 mM (final concentration of the aglycone).

RESULTS AND DISCUSSION

The conjugation rate of hydroxyphenylhydantoin was 0.47 nmol/min/mg microsomal protein, or approximately 0.78 μmol/g liver/hr. Kutt and Verebely [9] determined that 0.50 μmole of DPH/g liver/hr may be metabolized in microsomal preparations. In isolated liver cells Inaba *et al.* [10] determined rate of appearance of hydroxyphenylhydantoin glucuronide of about 0.29–0.32 μmol/g cells/hr. Our results may be high because the maximally activated form of UDPGT is not operating within the cells [11]. On the other hand, in unactivated microsomal preparations the rate of glucuronidation was slower than in isolated cells [12].

Table 1. Conjugation of hydroxyphenylhydantoin (HPPH) and hydroxyphenobarbital (HPB) in rat liver microsomal fractions after phenobarbital treatment

Rats	nmoles UDPGA Conjugation/mg protein/min HPB Glucuronide	HPPH Glucuronide
Controls	0.25 ± 0.06 (4)	0.44 ± 0.10 (6)
Phenobarbital treated (80 mg/kg body weight) 5 days	0.47 ± 0.04 (3)*	0.85 ± 0.20 (6)†

Mean values are given ± standard deviation. The number of animals is shown in brackets. Microsomal fractions were activated by Triton X-100 in the incubation medium contained 0.20 mg microsomal protein, 0.14 mM aglycone, 1 mM UDPGA, Tris–HCl buffer, pH 7.4, 50 mM UDPGA[^{14}C] 0.08 μCi, final volume 0.1 ml incubation 15 min, 37°.

Radioactivity was measured after separating synthetised glucuronides on XAD_2 column, eluted in methanol phase [8].

The activity was determined versus *p*-nitrophenol as standard = 1.21 ± 0.01 nmoles UDPGA conjugated/min/mg protein with the same incubation method.

* $P < 0.001$.

† $P < 0.005$.

The conjugation of hydroxyphenobarbital (0.21 m-mole min/mg protein) was about half that of hydroxyphenylhydantoin. This is probably not a limiting step in metabolism, for the half-lives of DPH and PB in rats are similar, respectively 2 hr [13] and 3 hr [14]. Although these compounds are chemically and pharmacologically related, some of the physical properties of their metabolites differ, such as liposolubility and pK_a, which could explain the observed difference. Phenobarbital increased conjugation of both hydroxyphenobarbital and hydroxyphenylhydantoin, as demonstrated in Table 1, by approximately 2 folds. With the help of our radiochemical method we could determine the conjugation rate of HPB and HPPH in microsomal preparations and determine the level of induction of the enzyme responsible for their own conjugation after *in vivo* administration of phenobarbital.

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REFERENCES

1. T. C. Butler, *J. Pharmac. exp. Ther.* **116,** 326 (1956).
2. E. W. Maynert, *J. Pharmac. exp. Ther.* **130,** 275 (1960).
3. T. E. Eling, R. D. Harbison, B. A. Becker, J. R. Fouts, *J. Pharmac. exp. Ther.* **171,** 127 (1970).
4. T. E. Eling, R. D. Harbison, B. A. Becker, J. R. Fouts, *Eur J. Pharmac.* **11,** 101 (1970).
5. J. M. Ziegler, B. P. Lisboa, A. M. Batt, G. Siest, *Biochem. Pharmac.* **24,** 1291 (1975).
6. D. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall *J. biol. Chem.* **193,** 265 (1951).
7. V. Loppinet, P. Greiveldinger, C. Guidon, G. Schiele and J. M. Ziegler, *J. Med. Chem.* (submitted).
8. A. M. Batt, *Thèse Pharmacie,* Université de Nancy I (1975).
9. H. Kutt, R. Verebely, *Biochem. Pharmac.* **19,** 675 (1970).
10. T. Inaba, T. Umeda, W. A. Mahon, J. Ho, K. N. Jeejee Bhoy *Life Sci.* **16,** 1227 (1975).
11. K. W. Bock and I. N. H. White, *Eur. J. Biochem.* **46,** 451 (1974).
12. P. Moldeus, R. Grundin, H. Vadi, S. Orrenius, *Z. Klin. Chem. Klin. Biochem.* **8,** 375 (1975).
13. N. Gerber, W. L. Weller, R. Lynn, R. E. Rangno, B. J. Sweetman and M. T. Bush, *J. Pharmac. exp. Ther.* **178,** 567 (1971).
14. D. R. Parke, in *Enzyme Induction, Basic Life Sciences,* Vol. 6, p. 217. Plenum Press, London—New York (1975).