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SHORT COMMUNICATIONS

The conversion of phenanthrene 9, 10-oxide and dibenz[a, h]anthracene 5, 6-oxide into dihydrodiols by a rat-liver microsomal enzyme

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PHENANTHRENE 9,10-OXIDE is converted by rats into 9-phenanthrol, *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene and *N*-acetyl-(9,10-dihydro-9-hydroxy-10-phenanthryl)-L-cysteine.[1] In rat-liver homogenate, the oxide yields the phenol, the dihydrodiol and S-(9,10-dihydro-9-hydroxy-10-phenanthryl)glutathione.[1] Dibenz[a,h]anthracene 5,6-oxide is similarly converted by rat-liver homogenates into 5-hydroxydibenz[a,h]anthracene, 5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene and S-(5,6-dihydro-6-hydroxy-5-dibenz[a,h]anthracenyl)glutathione.[2] Chemically, the oxides react with acid to yield phenols, with water to yield dihydrodiols and with *N*-acetylcysteine or glutathione to yield the corresponding mercapturic acids or glutathione conjugates.[1, 2] *In vitro* experiments showed that the conjugations of the epoxides with glutathione are catalysed by enzymes present in rat-liver supernatant.[3] The conversion of the epoxides into the phenols (with which they are isomeric) is presumably non-enzymic: benzene oxide is converted non-enzymically into phenol by proteins,[4] and both phenanthrene 9,10-oxide and dibenz[a,h]anthracene 5,6-oxide yield phenols spontaneously in aqueous media at neutral pH.[1, 2] The reactions of phenanthrene 9,10-oxide are summarised in Fig. 1.

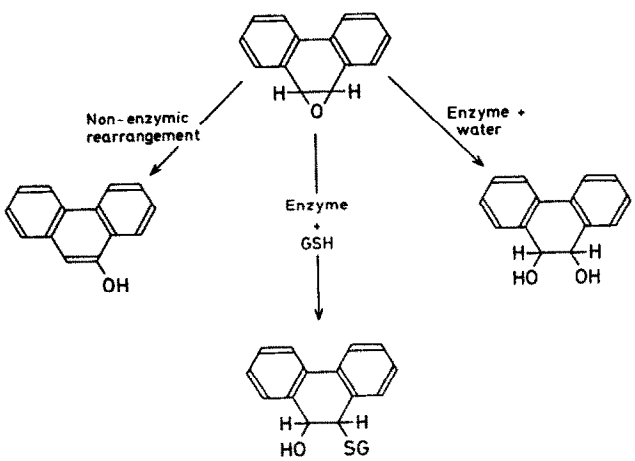

FIG. 1. Metabolic reactions of phenanthrene 9,10-oxide; GSH—glutathione.

Recent reports show that dihydrodiols are formed when certain chlorinated cyclodiene epoxides are incubated with pig-liver and rat-liver microsomes[5] and when benzene oxide[4] and naphthalene 1,2-oxide[6] are incubated with rabbit-liver microsomes. The usual microsomal co-factors (glucose 6-phosphate and TPNH) are not required. The present report is concerned with the conversion of phenanthrene 9,10-oxide (which is derived from a non-carcinogenic hydrocarbon) and dibenz[a,h]-anthracene (which is derived from a carcinogenic hydrocarbon) into dihydrodiols by rat-liver microsomes.

Samples of tritiated phenanthrene 9,10-oxide (sp. act. 29·8 mc/mM) and dibenz[a,h]anthracene 5,6-oxide (sp. act. 22·4 mc/mM) were prepared by methods previously described,[1, 2] except that

hydrocarbons generally labelled with tritium (Radiochemical Centre, Amersham, Bucks.) were used. The pooled livers from at least four male rats of the Chester Beatty strain (body wt. approx. 180 g) were homogenized in 4 vol. of ice-cold 1·15% (w/v) KCl as described.[7] A portion of the homogenate was reserved and the remainder separated by differential centrifugation into a nuclear, a mitochondrial, a microsomal and a supernatant fraction using methods previously described.[8] The first three fractions were resuspended in 0·1 M-phosphate buffer (pH 7·4, prepared from NaH_2PO_4 and Na_2HPO_4) so that 10 ml of the suspensions contained the fractions derived from the wet weight of liver shown in Table 1. The preparations (10 ml aliquots) were shaken in a water bath at 37° and tritiated phenanthrene 9,10-oxide (100 μg) in acetone (0·1 ml) added. After 15 min the mixtures were extracted with ice-cold ethyl acetate (10 ml) and portions (0·1 ml) of the extracts chromatographed for 15 cm in benzene–ethanol (19:1, v/v) on thin-layer chromatograms coated with silica gel G. (E. Merck A.-G., Darmstadt, Germany) of 0·25 mm thickness. Synthetic, unlabelled, *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene was added to each aliquot to act as a marker. The developed chromatograms were inspected in u.v. light and the regions of the chromatograms containing the dihydrodiol, which appeared as dark u.v. absorbing spots at R_f 0·17, were removed: the oxide itself has R_f 0·90 when chromatographed under these conditions. The silica gel was transferred to glass vials and the radioactivity present was determined by liquid scintillation counting. Control experiments in which phosphate buffer alone was used or in which the preparations were boiled for 5 min before incubation were also carried out. All experiments were carried out in duplicate and the results are shown in Table 1.

TABLE 1. THE ENZYMIC HYDRATION OF PHENANTHRENE 9, 10-OXIDE BY FRACTIONS FROM RAT LIVER

Cell fraction	Wet wt. of liver from which fraction was obtained (g)	Amount of 9,10-dihydro-9,10-dihydroxy phenanthrene formed/g wet wt. of liver (cpm × 10^{-5})
Whole homogenate	2·3	23·13
Boiled whole homogenate	2·3	0·11
Nuclei	35·0	1·69
Mitochondria	15·0	6·73
Boiled mitochondria	15·0	0·11
Microsomes	1·9	42·81
Boiled microsomes	1·9	0·11
Supernatant	4·0	0·44
Boiled supernatant	4·0	0·78
Phosphate buffer	—	(0·24)

100 μg of tritiated phenanthrene 9,10-oxide (110 × 10^5 cpm) in acetone (0·1 ml) was added to each fraction in 0·1 M-phosphate buffer (10 ml; pH 7·4). The mixtures were incubated for 15 min at 37° and the products isolated and estimated as described in the text. The results are the means of duplicate determinations.

The effect of substrate concentrations was investigated with rat-liver homogenates, each experiment using that derived from 1 g of liver in 0·1 M-phosphate buffer (10 ml). To each incubation was added 100, 200 or 300 μg of either tritiated phenanthrene 9,10-oxide or tritiated dibenz[a,h]anthracene 5,6-oxide (as solutions of 100 μg in 0·1 ml of acetone). The incubation, extractions, chromatography and estimations were carried out as before except that in the experiments with dibenz[a,h]anthracene 5,6-oxide, synthetic unlabelled 5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene was used as a marker on the thin-layer chromatograms. This dihydrodiol was detected as a violet fluorescent spot of R_f 0·16 when the chromatograms were examined in u.v. light. The results of these experiments are shown in Table 2.

In a third series of experiments, microsomes derived from 12·5 mg of rat-liver were suspended in 0·1 M-phosphate buffer (10 ml) and incubated at 37° for either 5, 10, 20 or 30 min with 100 mμmole of each of the epoxides added as solution in acetone (0·1 ml). The extractions and estimations of the dihydrodiols formed were carried out as before, except that 1 ml portions of the ethyl acetate extracts were evaporated and the residues chromatographed. Control experiments using boiled preparations

and experiments in which preparations were extracted with ethyl acetate immediately after the addition of the epoxide were also carried out. The latter determinations were necessary because small amounts of the dihydrodiol are formed from the epoxides during thin-layer chromatography.[1, 2] All experiments were carried out in duplicate. The results are shown in Fig. 2.

In another experiment, phenanthrene 9,10-oxide (10 mg) was incubated for 15 min at 37° with rat-liver homogenate prepared from 10 g of liver. The products were extracted with ethyl acetate and chromatographed on a silica gel preparative thin-layer chromatogram in benzene–ethanol (19:1, v/v). The band containing the dihydrodiol was removed and the product eluted with ether to yield

TABLE 2. THE EFFECT OF CONCENTRATION ON THE ENZYMIC HYDRATION OF PHENANTHRENE 9,10-OXIDE AND DIBENZ[a,h] ANTHRACENE 5,6-OXIDE BY RAT-LIVER HOMOGENATE

Substrate	Amount of substrate added (μg)	Amount of dihydrodiol formed/g wet wt. of liver (cpm $\times$ 10^{-5})
Phenanthrene 9,10-oxide	100	52·1
Phenanthrene 9,10-oxide	200	101·3
Phenanthrene 9,10-oxide	300	149·2
Dibenz[a,h]anthracene 5,6-oxide	100	1·52
Dibenz[a,h]anthracene 5,6-oxide	200	2·91
Dibenz[a,h]anthracene 5,6-oxide	300	4·45

Solutions containing tritiated phenanthrene 9,10-oxide [100 μg in acetone (0·1 ml); 110 $\times$ 10^5 cpm] or tritiated dibenz[a,h]anthracene 5,6-oxide [100 μg in acetone (0·1 ml); 85 $\times$ 10^5 cpm] were added to rat-liver homogenate (from 1 g wet wt. of rat liver) in 0·1 M-phosphate buffer (10 ml). The mixtures were incubated at 37° for 15 min and the products isolated and estimated as described in the text. The results are means of duplicate determinations.

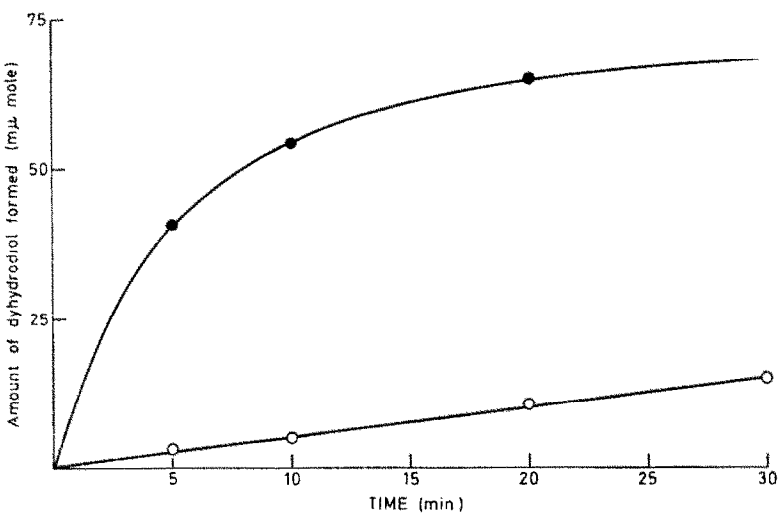

FIG. 2. Incubations and estimations of products were carried out as described in the text. 100 mμmole of tritiated phenanthrene 9,10-oxide or 100 mμmole of tritiated dibenz[a,h]anthracene were added to each incubation mixture. Formation of 9,10-dihydro-9,10-dihydroxyphenanthrene ●——●; formation of 5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene ○——○: the points represent means of two determinations. Boiled homogenates were incubated with the oxides (100 μmole) for 30 min: with phenanthrene 9,10-oxide, 3·4 mμmole and with dibenz[a,h]anthracene, 0·16 mμmole of the dihydrodiols were found.

(±)-*trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (2·8 mg) in silky needles, m.p. and mixed m.p. 187°. There was no evidence for the presence of an optically active isomer of the dihydrodiol.

The results in Table 1 show that the enzyme catalysing the hydration of phenanthrene 9,10-oxide is localised mainly in the microsomal fraction. The activity in the mitochondrial fraction may be due to contamination, although Jerina *et al.*[4] have found some activity towards benzene oxide in the mitochondrial fraction of rabbit liver. The control experiments show that under the conditions used little non-enzymic hydration of the oxiran ring occurs.

Figure 2 shows that phenanthrene 9,10-oxide is a much better substrate for the enzyme than dibenz[a,h]anthracene 5,6-oxide. Although the dibenzanthracene derivative is less soluble in aqueous media than the phenanthrene derivative, the results in Table 2 indicate that this is not an important factor in explaining the differences in activities. Tests in mice have shown that although dibenz[a,h]-anthracene 5,6-oxide is active and phenanthrene 9,10-oxide inactive[9] as a carcinogen, the dibenz-anthracene epoxide is less active under the conditions used than the parent hydrocarbon. It has been suggested[10] that epoxides are intermediates in the metabolism of aromatic hydrocarbons: the question as to whether there is any general relationship between the activities of epoxides of these types towards the microsomal enzyme and the carcinogenicity of the parent hydrocarbons is under investi-gation.

Jerina *et al.*[6] have demonstrated the formation of an optically active dihydrodiol by the enzymic hydration of naphthalene 1,2-oxide by rabbit liver microsomes, whereas in the present work phen-anthrene 9,10-oxide yielded only the racemic form of the dihydrodiol in rat-liver preparations. It is difficult to determine the relative amounts of the optical active isomers formed in experiments with whole animals because the dihydrodiols are either metabolised further or are conjugated with glu-curonic acid, but with phenanthrene[10] and naphthalene[11–13] both forms of the dihydrodiol must be formed in the body since racemates have been isolated from the urine.

In all these experiments not more than 75 per cent of the added phenanthrene 9,10-oxide was con-verted into the dihydrodiol by rat-liver microsomes. The remainder was probably converted into 9-phenanthrol by the non-enzymic rearrangement described above. This rearrangement does not occur with the oxide formed *in vivo* when animals are dosed with phenanthrene since neither 9-phenanthrol nor its conjugates are metabolites of the hydrocarbon:[10] presumably *in vivo* the enzymic reactions are faster than the chemical rearrangements. The amount of dihydrodiol formed from phenanthrene 9,10-oxide by homogenates is less than that formed by an equivalent weight of micro-somes: this is probably because some of the oxide is metabolised by the alternative pathway involving conjugation with glutathione.[3]

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REFERENCES

1. E. BOYLAND and P. SIMS, *Biochem. J.* **95**, 788 (1965).
2. E. BOYLAND and P. SIMS, *Biochem. J.* **97**, 7 (1965).
3. E. BOYLAND, P. SIMS and K. WILLIAMS, *Ann. Rep. Brit. Emp. Cancer Campg.* p. 33 (1964).
4. D. JERINA, J. DALY, B. WITKOP, P. ZALTZMAN-NIRENBERG and S. UDENFRIEND, *Archs Biochem. Biophys.* **128**, 176 (1968).
5. G. T. BROOKES, *World Review Pest Control* **5**, 62 (1966).

6. D. M. JERINA, J. W. DALY, B. WITKOP, P. SALTZMAN-NIRENBERG and S. UDENFRIEND, *J. Am. Chem. Soc.* **90**, 6525 (1968).
7. P. SIMS, *Biochem. J.* **98**, 215 (1966).
8. J. BOOTH, E. BOYLAND and P. SIMS, *Biochem. J.* **79**, 516 (1961).
9. E. BOYLAND and P. SIMS, *Int. J. Cancer* **2**, 500 (1967).
10. E. BOYLAND and P. SIMS, *Biochem. J.* **84**, 571 (1962).
11. J. BOOTH and E. BOYLAND, *Biochem. J.* **44**, 361 (1949).
12. E. D. S. CORNER and L. YOUNG, *Biochem. J.* **58**, 647 (1954).
13. P. SIMS, *Biochem. J.* **73**, 389 (1959).

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Increased ileal absorption of salicylic acid induced by carbonic anhydrase inhibition

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PRETREATMENT with the carbonic anhydrase inhibitor, acetazolamide, has been shown to enhance the uptake of certain drugs by the brain.[1,2] Recently it has been observed that acetazolamide treatment may affect the absorption of drugs through other body membranes.[3] Therefore, we have studied the absorption of ^{14}C-salicylic acid *in vivo* from intestinal sacs located in the ileum of rats after treatment with acetazolamide and CL-13,850, an inactive analogue of acetazolamide.[4]

Male, Holtzman albino rats (170–200 g), fasted for 24 hr, were pretreated with acetazolamide (50 mg/kg, s.c.), CL-13,850 (50 mg/kg, s.c.) or an equivalent volume of saline (pH adjusted to 8·5, the same as the solvent for acetazolamide). The animals were anesthetized with urethane (1·25 g/kg i.p.) and intestinal sacs 5–7 cm in length were formed by the method of Levine and Pelikan[5] in the ileum approximately 2 cm from the ileocecal junction. The ligatures were carefully placed so they did not interfere with the blood supply to the ileal sac.

The intestinal segment was carefully washed with warm saline, and then 1·0 ml of warmed (37°) saline containing 1 mM ^{14}C-salicylic acid (0·25 μc/ml) was introduced into the sac via a polyethylene cannula 30 min after the appropriate pretreatment. The cannula was then withdrawn and the ligature tightened. The sac was replaced into the abdominal cavity and the incision was closed.

After an appropriate interval, a blood sample was obtained by cardiac puncture. The ileal sac was removed and the pH of the contents was determined by using a Beckman hypodermic electrode assembly (No. 39022) which prevents exposure of the sample to air. The ileal contents were emptied into a beaker and the sac was washed several times with saline. The ileal contents and washings were quantitatively transferred to a 25 ml volumetric flask and brought to volume with distilled water. A 0·5 ml aliquot was mixed with 16 ml of TC scintillation fluid (6 g of 2,5-diphenyloxazole dissolved in a mixture of 600 ml ethyl cellosolve and 1000 ml toluene) and counted in a Beckman LS 100 scintillation spectrometer. Each sample was corrected for quenching. The extent of drug absorption was calculated from the difference between the amount of drug placed into the ileal sac and the amount recovered. Blood samples were prepared for liquid scintillation analysis by the method of Mahin and Lofberg[6] and counted for total radioactivity by the same method used for the ileal contents. Statistical analyses were performed using Student's *t*-test.

The results are presented in Fig. 1. There was a significant ($P < 0·05$) decrease in the pH of the ileal contents at all time intervals after treatment with acetazolamide when compared to either the saline control or CL-13,850 treatment. The pH values obtained from the CL-13,850-treated animals