THE ENZYME-CATALYSED CONVERSION OF A NON-'BAY-REGION' DIOL-EPOXIDE OF BENZ[a]ANTHRACENE INTO A GLUTATHIONE CONJUGATE

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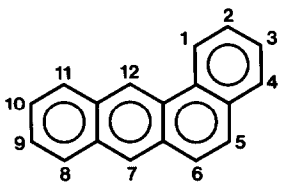
Received 9 July 1980

1. Introduction

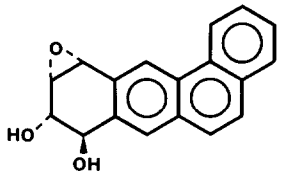
A major route of metabolism of carcinogenic polycyclic aromatic hydrocarbons involves the formation of simple epoxides. The epoxides, which arise by the action of microsomal monooxygenases on the hydrocarbon, can be converted either by the action of glutathione *S*-transferase into glutathione conjugates [1–3] or through the action of epoxide hydrolase into dihydrodiols [3]. Some dihydrodiols can then be metabolised further to form vicinal diol-epoxides [4] that can bind covalently to nucleic acids and that are believed to be responsible, in some cases, for the biological activities of the parent hydrocarbons. Although, for some hydrocarbons like benzo[a]pyrene [5], benz[a]anthracene [6] and 7-methylbenz[a]anthracene [7,8], the diol-expoxides formed by metabolism have been identified, relatively little attention has been paid so far to the further metabolism of diol-epoxides. This paper describes experiments designed to investigate the role of glutathione *S*-transferase in the further metabolism of *anti*-BA-8,9-diol 10,11-oxide, a metabolite of BA that contributes to the covalent binding of this hydrocarbon to nucleic acids in mouse skin [6,9], hamster embryo cells [6,9–11] and rat-liver microsomal systems [12]. All the evidence obtained is consistent with the idea that glutathione transferases, present in rat-liver supernatant, can catalyse the conversion of *anti*-BA-8,9-diol 10,11-oxide into a glutathione conjugate.

Abbreviations: BA, benz[a]anthracene; BA-8,9-diol, *trans*-8,9-dihydro-8,9-dihydroxybenz[a]anthracene; *anti*-8,9-diol 10,11-oxide, *r*-8, *t*-9-dihydroxy-*t*-10,11-oxy-8,9,10,11-tetrahydrobenz[a]anthracene; *anti*-BP-7,8-diol 9,10-oxide, *r*-7, *t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BA-8,9,10,11-tetrol, 8,9,10,11-tetrahydro-8,9,10,11-tetrahydroxybenz[a]anthracene

Benz(a)anthracene



anti-BA-8,9-diol 10,11-oxide



2. Materials and methods

2.1. *Materials*

L-[*glycine*-2-^{3}H]Glutathione was purchased from New England Nuclear Chemicals GmbH, FRG. *Anti*-BA-8,9-diol 10,11-oxide [13] and BA-8,9,10,11-tetrol [11] were synthesised as described. Glutathione was obtained from Sigma Chemical Co., St Louis, MO.

2.2. *Preparation of rat-liver supernatant*

A 100 000 × g_{av} rat-liver supernatant was prepared from livers (20 g) from rats that had been pretreated with 3-methylcholanthrene [7]. A 20% (w/v) homogenate prepared in phosphate buffer (0.1 M, pH 7.4) was centrifuged at 2000 × g_{av} for 10 min at 4°C. The supernatant was then centrifuged at 100 000 × g_{av} for 2 h at 4°C. A portion of the clear supernatant was removed from between the upper fat layer and the pellet and was dialysed against 100 vol. distilled water at 0°C for 24 h.

2.3. *The preparation of glutathione conjugates*

Standard reaction mixtures (30 ml) contained *anti*-BA-8,9-diol 10,11-oxide (1 mg), dialysed rat-liver supernatant (3 ml) and either non-labelled glutathione

(9 mg) or [^{3}H]glutathione (9 mg, 0.17 mCi/mmol) in phosphate buffer (50 mM, pH 7.4). Reaction mixtures were incubated at 37°C for 1 h and then extracted sequentially with ethyl acetate (2 X 2 vol.) and with diethyl ether (4 X 1 vol.). The aqueous phase was poured into ethanol (5 vol.) and the mixture heated to boiling point and then cooled. Precipitated protein was removed by filtration and the crude mixture of water-soluble materials was evaporated to dryness under reduced pressure.

The residue was redissolved in water (2 ml) and applied to the top of a Sephadex G-25 column (100 X 1.5 cm) and the column eluted with water (100 ml/h). Fractions (65 drops) were collected and the A_{260} of the eluted fractions was examined. Fractions containing UV-absorbing material were pooled and evaporated to dryness. The residue was dissolved in water (1 ml) and subjected to high-pressure liquid chromatography (HPLC) on Spherisorb 5 ODS. The column was eluted at a flow rate of 2 ml/min with water for 5 min and then with a linear gradient of methanol in water (0–60%) for 48 min. Fractions (2 ml) were collected and examined for the presence of UV-absorbing material of UV-absorbing material and, when appropriate, for the presence of radioactive material using liquid scintillation counting.

2.4. *Amino acid analysis*

Samples for analysis were hydrolysed in 6 M HCl (0.5 ml) at 100°C for 24 h and amino acids present in the hydrolysates were separated and detected using a Rank-Hilger Chromospek V180 amino acid analyser. Under these conditions of hydrolysis, glutamic acid and glycine are recovered intact whilst cysteine is recovered as cysteic acid.

3. Results and discussion

The metabolism of *anti*-BA-8,9-diol 10,11-oxide to form glutathione conjugates was investigated in experiments in which the diol-epoxide was incubated with glutathione and a rat-liver supernatant. The mixture was extracted with ethyl acetate and diethyl ether and the water-soluble material was first purified by chromatography on Sephadex G-25 and then subjected to HPLC on Spherisorb 5 ODS. Under the conditions used for HPLC, unreacted glutathione was recovered in eluted fractions 2–5 (fig.1), a major water-soluble metabolite was present in fractions 13–15 (product I,

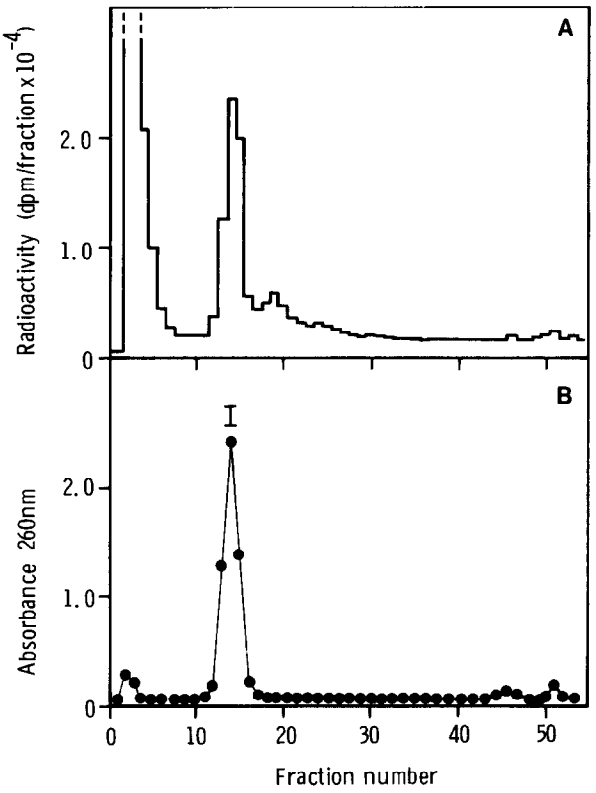


Fig.1. High-pressure liquid chromatography of water-soluble material. Water-soluble materials prepared from incubations of *anti*-BA-8,9-diol 10,11-oxide with L-[*glycine*-2-^{3}H]glutathione and 100 000 X g_{av} rat-liver supernatant were first purified by chromatography on Sephadex G-25 columns, then subjected to HPLC on Spherisorb 5 ODS columns. The Spherisorb 5 ODS columns were eluted with water–methanol gradients as in the text and (A) radioactivity (dpm X10^{-4}/fraction, histogram) and (B) UV A_{260} (•——•) determined.

fig.1B) and a small quantity of BA-8,9,10,11-tetrol, a hydrolysis product of *anti*-BA-8,9-diol 10,11-oxide, was present in fractions 51–52 (fig.1B).

Evidence that the major water-soluble metabolite (product I, fig.1B) is a glutathione conjugate is presented in fig.1 and in table 1. The results presented in table 1 (expt. 1) show that the formation of product I is completely dependent upon the presence of glutathione whilst those presented in fig.1 show that when [^{3}H]glutathione is used, radioactive material elutes from the Spherisorb 5 ODS column in exactly the same position as product I (fig.1). Additional evidence that product I is a glutathione conjugate was obtained by subjecting an acid hydrolysate of product I to amino acid analysis. The results showed that this

Table 1
Conversion of *anti*-BA-8,9-diol 10,11-oxide into a glutathione conjugate

Expt.	Incubation mixture	[a]Conjugate formed (μg)
1	[b]Complete	162
	No glutatione	0
2	[b]Complete	120
	No rat-liver supernatant	26
3	[b]Complete	276
	Heat-denatured rat-liver supernatant	28

[a] The amount of glutathione conjugate formed was calculated from the total amount of UV-absorbing material present in fractions 13–15 (fig.1B), after the water-soluble metabolites had been subjected to HPLC on Spherisorb 5 ODS, assuming that the extinction coefficient (260 nm) and relative molecular mass of the conjugate were 60 000 and 582, respectively

[b] The complete incubation mixture contained glutathione, rat-liver supernatant and *anti*-BA-8,9-diol 10,11-oxide (see text)

product contains the 3 amino acids that comprise glutathione: namely glutamic acid, glycine and cysteine.

Examination of the UV spectrum of the glutathione conjugate provides additional information about its structure. The UV spectrum (fig.2A) is almost identical to those of *anti*-BA-8,9-diol 10,11-oxide and BA-8,9,10,11-tetrol (fig.2B) but with small shifts in the maxima of the spectrum to longer wavelengths, which may be due to an interaction between the glutathione and hydrocarbon moieties in the conjugate. These observations indicate that, like the diol-epoxide and the tetrol, the conjugate possesses an intact phenanthrene aromatic system and are consistent with a mechanism for glutathione conjugate formation in which the sulphydryl group of glutathione reacts with a carbonium ion formed at either the C-10 or C-11 of the diol-epoxide.

Evidence for the involvement of glutathione *S*-transferase in the conversion of *anti*-BA-8,9-diol 10,11-oxide into its glutathione conjugate was obtained by determining the amounts of glutathione conjugates formed in the presence and in the absence of a rat-liver supernatant. When 1 mg diol-epoxide was incubated with glutathione and rat-liver supernatant, 120–276 μg conjugate was formed (table 1). However, when heat-denatured rat-liver supernatant was used or when the rat-liver supernatant was omitted from the incubation mixture the yield of conjugate was considerably less (<30 μg) (table 1). Some glutathione conjugate was formed, even in the absence of enzyme, however, an observation that is characteristic of many reactions that are catalysed by glutathione *S*-transferases since the second substrates are usually electrophilic.

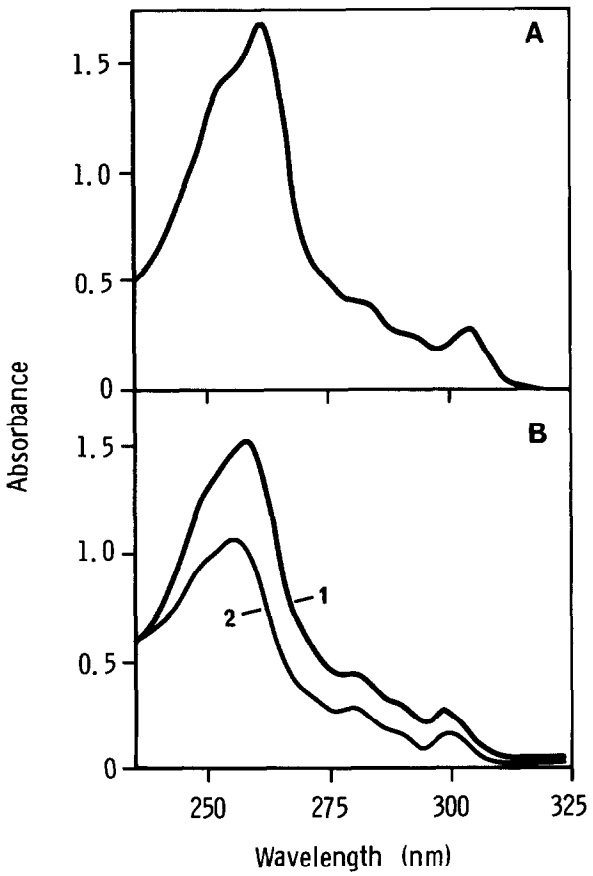


Fig.2. UV absorption spectra measured in water of (A) the glutathione conjugate formed by the further metabolism of *anti*-BA-8,9-diol 10,11-oxide (product I, fig.1B) and of (B) *anti*-BA-8,9-diol 10,11-oxide (1) and BA-8,9,10,11-tetrol (2).

A glutathione conjugate similar to that described in this study is also formed when BA-8,9-diol, a major metabolite of BA, is incubated with rat-liver homogenates [14]. This conversion most probably occurs by the metabolism of the diol to form *anti*-BA-8,9-diol 10,11-oxide, which can then be converted into the glutathione conjugate. *Anti*-BA-8,9-diol 10,11-oxide is known to be a metabolite of BA-8,9-diol [4,15] and the results of this study show that this diol-epoxide is converted by the action of glutathione *S*-transferase into a glutathione conjugate. *Anti*-BA-8,9-diol 10,11-oxide also contributes to covalent bind-

ing to DNA in mouse skin and hamster embryo cells [5,8–10]. Consequently enzyme-catalysed conversions of this diol-epoxide into other metabolites including glutathione conjugates might, in part, control the extent of binding of BA to nucleic acids.

As an important corollary, these results appear to provide the first clear evidence that a diol-epoxide, formed from a polycyclic aromatic hydrocarbon by metabolism, can act as a substrate for glutathione *S*-transferase: the possible role of glutathione transferases in the metabolic activation of hydrocarbons may merit further investigation.

Acknowledgements

We thank Dr E. W. Johns and Dr J. Hastings for the amino acid analysis. The investigations were supported in part by grants to the Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital from the Medical Research Council and the Cancer Research Campaign and in part by grant no. CA21959 from the National Cancer Institute, USPHS.

References

[1] Chasseaud, L. (1973) Drug Metab. Rev. 2, 185–220.
[2] Grover, P. L. (1977) in: Drug Metabolism – from Microbes to Man (Parke, D. V. and Smith, R. L. eds) pp. 105–122, Taylor and Francis, London.
[3] Yang, S. H., Deutsch, J. and Gelboin, H. V. (1978) in: Polycyclic Hydrocarbons and Cancer (Gelboin, H. V. and Ts'o, P. O. P. eds) vol. 1, pp. 205–231, Academic Press, New York.
[4] Booth, J. and Sims, P. (1974) FEBS Lett. 47, 30–33.
[5] Sims, P., Grover, P. L., Swaisland, A., Pal, K. and Hewer, A. (1974) Nature 252, 326–328.
[6] Cooper, C. S., Ribeiro, O., Hewer, A., Walsh, C., Pal, K., Grover, P. L. and Sims, P. (1980) Carcinogenesis 1, 233–243.
[7] Tierney, B., Hewer, A., Walsh, C., Grover, P. L. and Sims, P. (1977) Chem.–Biol. Interact. 18, 179–193.
[8] Vigny, P., Duquesne, M., Coulomb, H., Lacombe, C., Tierney, B., Grover, P. L. and Sims, P. (1977) FEBS Lett. 75, 9–12.
[9] Vigny, P., Kindts, M., Duquesne, M., Cooper, C. S., Grover, P. L. and Sims, P. (1980) Carcinogenesis 1, 33–36.
[10] Cooper, C. S., MacNicoll, A. D., Ribeiro, O., Gervasi, P. G., Hewer, A., Walsh, C., Pal, K., Grover, P. L. and Sims, P. (1980) Cancer Lett. 9, 53–59.
[11] Cooper, C. S., Ribeiro, O., Farmer, P. B., Hewer, A., Walsh, C., Pal, K., Grover, P. L. and Sims, P. (1980) Chem.–Biol. Interact. in press.
[12] MacNicoll, A. D., Cooper, C. S., Ribeiro, O., Gervasi, P. G., Hewer, A., Walsh, C., Grover, P. L. and Sims, P. (1979) Biochem. Biophys. Res. Commun. 91, 490–497.
[13] Lehr, R. E., Schaefer-Ridder, M. and Jerina, D. M. (1977) Tetrahedron Lett. pp. 539–542.
[14] Keysell, G. R., Booth, J. and Sims, P. (1975) Xenobiotica 5, 439–448.
[15] Sims, P. and Grover, P. L. (1976) Med. Bull. Environ. 4, 315–329.