Biochemical Pharmacology, Vol. 24, pp. 1569–1572. Pergamon Press, 1975 Printed in Great Britain.

RADIOCHEMICAL ASSAY OF GLUTATHIONE *S*-EPOXIDE TRANSFERASE AND ITS ENHANCEMENT BY PHENOBARBITAL IN RAT LIVER *IN VIVO*

JUKKA MARNIEMI and MAX G. PARKKI
Department of Physiology, University of Turku, Turku, Finland

(*Received* 6 *December* 1974; *accepted* 19 *March* 1975)

Abstract—A rapid and sensitive assay of glutathione *S*-epoxide transferase was developed using tritium-labeled styrene oxide as the epoxide substrate. The method is based on the separation of unreacted styrene oxide from the glutathione conjugate formed after the incubation by the extraction with light petroleum. The radioactivity of the conjugate remaining in the aqueous phase can be determined subsequently by liquid scintillation. Using the present radiochemical method we found, after the intraperitoneal administration of phenobarbital (80 mg/kg) *in vivo*, a significant enhancement (about 56%) in the conjugation of styrene oxide with glutathione catalyzed by rat liver soluble fraction. No enhancement in the enzyme activity could be detected, on the other hand, after administration with styrene (1 g/kg) or 20-methylcholanthrene (20 mg/kg).

Alkyl and aryl epoxides are known to be important intermediates in cytochrome P-450 dependent hydroxylation of many xenobiotics [1, 2]. The epoxide can be further metabolized by two enzymatic mechanisms of detoxification: (1) hydration to the corresponding dihydrodiol by epoxide hydrases (glycol hydrolyase, epoxide-forming, EC 4.2.1.63 and dihydrodiol hydrolyase, arene-oxide-forming, EC 4.2.1.64) as recently reviewed by Oesch [3] and (2) conjugation with glutathione by glutathione *S*-epoxide transferase [*S*-(2-hydroxyalkyl)glutathione alkyl-epoxide-lyase, EC 4.4.1.7] [4–9]. Epoxides of some polycyclic hydrocarbons have been reported to be more carcinogenic than the parent compound [10–12]. Because of this, interest in the enzymes converting the highly electrophilic epoxides to less harmful compounds has recently increased.

The measurements of glutathione *S*-epoxide transferase activity commonly used are based on the separation of the conjugate from the epoxide by chromatography [4, 5, 9] or on the spectrophotometric absorption of the reactants [7]. Unfortunately, these spectrophotometric assays are rather insensitive [9]. Recently, ^{35}S-labeled glutathione has been used as the substrate [9] but paper chromatographic separation of the products from substrates is also necessary in this method.

In the present study we have taken advantage of using ^{3}H-labeled styrene oxide as the substrate of glutathione *S*-epoxide transferase, which allowed the development of the current enzyme assay. The unreacted styrene oxide was extracted from the reaction mixture with light petroleum, and the glutathione conjugate remained in the aqueous phase, the radioactivity of which could be determined by liquid scintillation. Using this method we were able to demonstrate an increase in glutathione *S*-epoxide transferase activity of rat liver after phenobarbital administration *in vivo*.

EXPERIMENTAL

Male Wistar rats (weighing 200–250 g) about 3 months of age fed *ad lib.* on commercial pellets (Hankkija Oy, Turku, Finland) were used. Styrene (1 g/kg in corn oil, Koch-Light Laboratories Ltd., Colnbrook, England) and phenobarbital (80 mg/kg, dissolved in 1 N NaOH and adjusted to pH 8 with 1 N HCl; purchased from E. Merck AG, Darmstadt, West Germany) were administered intraperitoneally daily for 3 consecutive days and 20-methylcholanthrene (20 mg/kg in corn oil, Koch-Light) for 2 consecutive days to rats. Control animals in induction experiments received only corn oil (5 ml/kg). Livers of untreated rats were used in other experiments. The last dose was given 48 hr before killing the animals. The animals were killed by a blow on the head, bled and livers were removed and cooled in ice-cold aqueous 0·15 M KCl. The livers were homogenized in 0·15 M KCl with a glass–teflon Potter–Elvehjem homogenizer to give a 20% (w/v) homogenate. The homogenate was centrifuged for 10 min at 12,000 *g* for removal of the unbroken cells, cell debris and mitochondria. The microsomes were separated from the supernatant fraction by Ca^{2+}-aggregation [13, 14] as modified by Vainio and Aitio [15]. This soluble fraction was used as enzyme preparation.

The protein content of the enzyme preparation was estimated by the Folin–Ciocalteau method using bovine serum albumin (Armor Pharmaceutical Company Ltd., Eastbourne, England) as standard [16].

Determination of glutathione S*-epoxide transferase activity*: The radioactive substrate used, [7-^{3}H]styrene oxide (sp. act. 15·5 mCi/m-mole, NEN Chemicals GmbH, Dreieichenhain, West Germany), was purified from the water-soluble radioactivity formed during storage as follows: The labeled styrene oxide was diluted to the final specific activity with unlabeled styrene oxide and extracted from the incubation buffer with light petroleum. The petroleum ether

phase was evaporated under vacuum and styrene oxide remaining was dissolved into dried acetonitrile [17].

The reaction mixture of the enzyme assay consisted of 0·95 ml of 0·1 M Tris–HCl buffer, pH 8·0, containing 3·9 m-moles/l. reduced glutathione (Boehringer & Soehne GmbH, Mannheim, West Germany), of 1 μmole unlabeled styrene oxide (Koch-Light) and of 165,000 dis/min [7-^{3}H]styrene oxide added together in 25 μl of acetonitrile. This amount of acetonitrile did not cause any measurable change in the enzyme activity. The supernatant diluted with 0·15 M KCl was added in a volume of 25 μl containing about 100–120 μg of protein. Three kinds of blanks were necessary: one without added glutathione (formation of styrene glycol), the second without the enzyme preparation (non-enzymatic conjugation) and the third containing no added glutathione and enzyme (impurities of the radioactive substrate). The final background to be subtracted from the counts of the sample was: blank 1 + blank 2 − blank 3. After the incubation at 37° (5 min, if not mentioned otherwise) the reaction was stopped by cooling the tubes in water at 0° and by immediately extracting the unreacted styrene oxide three times with 10 ml of light petroleum (b.p. 40°–60°). The aqueous phase was frozen in an ethanol bath (−20°) between the extractions in order to enable the decantation of the organic phase. The radioactivity of the water phase (0·9 ml) was counted in Monophase 40 scintillation fluid (Packard Instrument Company Inc., Illinois, U.S.A.) using a Beckman 1650 liquid scintillation counter. The counting efficiency achieved was about 25% when determined with tritiated water (a kind gift from Dr. Niilo Kaartinen, Department of Physiology, University of Turku) as internal standard. The reaction was linear in regard to time and protein concentration under the conditions described above.

In order to determine the transfer of glutathione conjugate of styrene oxide into the organic phase, 420,000 dis/min of [7-^{3}H]styrene oxide and supernatant equivalent to 1·2 mg of protein was used and the incubation time was 30 min in order to increase the yield of the radioactive conjugate. The three light petroleum phases were combined after the extraction, evaporated to dryness and the remainder was dissolved in 150 μl of 50% (v/v) ethanol–water. One hundred μl of this solution was applied to Whatman No. 3 chromatography paper and the chromatograms were developed by descending chromatography in 1-butanol–acetic acid–water (12:3:5) [9]. The conjugate was localized with ninhydrin spray, cut out and the radioactivity of the spot was counted by liquid scintillation.

RESULTS AND DISCUSSION

Styrene oxide was extracted almost totally with light petroleum as also reported by Oesch *et al.* [17]. Less than 0·5% of unreacted styrene oxide remained in the aqueous phase after three washings. The background was usually, however, a little higher because the substrate converted slowly on storage to water soluble metabolite(s). These are possibly monohydroxyl derivatives, because at least arene oxides are known to be rearranged non-enzymatically to phenols [3]. Furthermore, the enzyme preparation caused a small increase in the radioactivity (about 1% of the total radioactivity of the reaction mixture) of the water phase in the absence of added glutathione. This obviously is partly due to the synthesis of styrene glycol catalyzed by epoxide hydrase of the microsomes remaining in the supernatant fraction and partly due to possible endogenous glutathione of the sample.

Less than 1% of the glutathione conjugate of styrene oxide was transferred into the organic phase in the extraction procedure. This 1% corresponded to about 100 cpm in the final counting in the conditions described Experimental. All the increase in the radioactivity in the aqueous phase after the addition of glutathione was found to be located in a ninhydrin positive spot (R_f-value about 0·6) as checked by paper chromatography in 1-butanol–acetic acid–water, and no other radioactive derivatives were found.

The activity of glutathione *S*-epoxide transferase was almost identical in the rapidly preparable Ca^{2+}-supernatant and in the conventional supernatant fraction obtained after centrifugation at 105,000 *g*. The glutathione conjugate of styrene oxide was also formed non-enzymatically, and this spontaneous conjugation enhanced by increasing pH (Fig. 1). The pH optimum of rat liver glutathione *S*-epoxide transferase was found to be pH 8 and at this pH the non-enzymatic conjugation was not yet disturbingly rapid. This is in accordance with earlier studies with sheep liver enzyme [9].

The effect of glutathione and styrene oxide concentrations on the conjugation is shown in Fig. 2. The non-enzymatic reaction increased almost linearly with substrate concentrations. The K_m-value for glutathione appeared to be about 0·84 m-moles/l. and for styrene oxide about 0·38 m-moles/l. when determined by the Lineweaver–Burk graphical method [18] (reciprocal velocity plots). Thus the substrate concentrations used, 3·9 m-moles/l. for glutathione and 1·0 m-moles/l. for styrene oxide, are high enough for the assay, and at these concentrations the non-enzymatic conjugation was not yet too rapid (Fig. 2).

Standard errors of the means of the current assay from fifteen experiments under conditions described

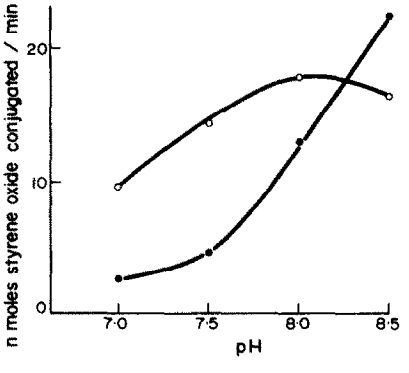

Fig. 1. The effect of pH on glutathione *S*-epoxide transferase of rat liver (open circles). The non-enzymatic reaction has been deducted from values of the enzyme activity. Black spots represent the non-enzymatic conjugation. The enzyme activity was measured using tritium labeled styrene oxide as substrate as described in Experimental.

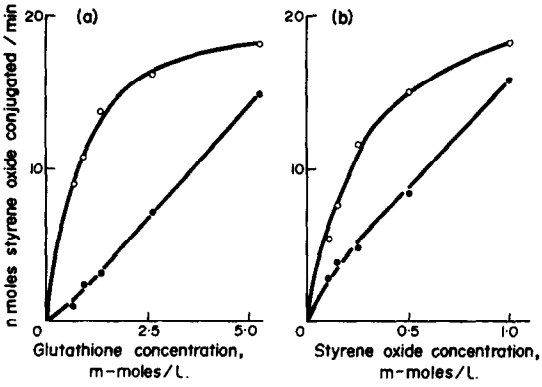

Fig. 2. The effect of glutathione (a) and styrene oxide (b) concentrations on glutathione S-epoxide transferase. Open circles represent the reaction catalyzed by rat liver supernatant fraction and black spots represent the non-enzymatic conjugation. For other explanations see Fig. 1.

in Experimental was 2·06%. This value has been calculated from the final enzyme activities and accordingly the corresponding blanks (3 × 15) are involved in this result. With the current method activity of the order about 20 nmoles of conjugate formed during 5 min could be detected corresponding to 1000 cpm above a total blank of about 2500 cpm. However, by lowering the incubation pH or glutathione concentration a little, thus reducing the non-enzymatic conjugation, the conjugation of at least 3 nmoles of styrene oxide with glutathione can be measured. This is of the same magnitude as with the radiochemical method involving chromatographic separation of the product as reported by Hayakawa *et al.* [9].

Thus the present method can be regarded as a rapid, sensitive and reproducible assay of glutathione S-epoxide transferase which in our opinion is suitable for routine determination. The only disadvantage at present is that it is limited to a single epoxide substrate. However, the same principle may be used when other epoxides of foreign compounds are commercially available in the future. The importance of epoxides in drug metabolism studies will certainly grow in the near future because it has recently appeared that in at least some cases the epoxide of the polycyclic hydrocarbon may be the ultimate carcinogen [10–12].

Phenobarbital administration *in vivo* appeared to enhance the measurable activity of glutathione S-epoxide transferase significantly (about 56%) in rat liver when assayed with the present radiochemical method (Table 1). All the increase in the radioactivity of the aqueous phase after phenobarbital treatment was found to be located in the ninhydrin positive spot after chromatography in 1-butanol–acetic acid–water. Styrene and 20-methylcholanthrene pretreatments of the animals did not cause, on the other hand, any increase in the enzyme activity. The activities were, conversely, slightly lower in rats pretreated with styrene or 20-methylcholanthrene (about 9% and 4%, respectively).

Table 1. The activity of rat liver glutathione S-epoxide transferase after the intraperitoneal administration of styrene (1 g/kg three times), 20-methylcholanthrene (20 mg/kg twice) and phenobarbital (80 mg/kg three times)

Compound administered	Glutathione S-epoxide transferase activity (nmoles styrene oxide conjugated/ min × mg protein)
Control	224 ± 13 (10)
Styrene	205 ± 22 (5)
20-Methylcholanthrene	215 ± 13 (5)
Phenobarbital	349 ± 32 (10) $2P < 0.005$

Means ± their standard errors are given. Numbers of animals are indicated in parentheses. For assay conditions, see Experimental.

The enhancement of glutathione S-epoxide transferase activity by inducers of drug metabolism has not been reported earlier and this enzyme has been thought to be non-inducible [3, 19]. The formation of glutathione conjugates from 7,12-dimethylbenzanthracene [20, 21] and bromobenzene [22] by rat liver preparations is increased in animals administered with 3-methylcholanthrene or phenobarbital, respectively. This enhancement may be, however, due to increased epoxide formation rather than induction of glutathione S-epoxide transferase [19]. Treatment of rats with barbiturates causes, on the other hand, a small increase in the activity of glutathione S-aryltransferase [22–25]. Pretreatment of animals with polycyclic hydrocarbons and also with phenobarbital, after the initial increase, considerably reduces glutathione S-aryl transferase activity [23] which is consistent with the non-inducibility of the epoxide conjugating enzyme by 20-methylcholanthrene found in the present study. Glutathione S-epoxide transferase has been demonstrated to differ from enzymes conjugating aryl and alkyl compounds [4]. On the other hand, even the most purified glutathione S-epoxide transferase preparations contain activities towards both alkyl and aryl epoxides [9].

Our results indicate that the elimination of the epoxide of a xenobiotic through conjugation with glutathione can be accelerated with an inducer of drug metabolism. If this also happened with epoxides of polycyclic hydrocarbons, there might be a possibility of protecting the organism against the ultimate chemical carcinogen (epoxide) by inducing glutathione S-epoxide transferase with some exogenous compounds. Unfortunately, barbiturates such as phenobarbital happen also to be strong inducers of the epoxide formation by the microsomal mono-oxygenase system [26].

Acknowledgement—This study has been financially supported by a grant from U.S. Public Health Service (AM HL 06018-13).

REFERENCES

1. K. C. Leibman and E. Ortiz, *J. Pharmac. exp. Ther.* **173**, 242 (1966).
2. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend, *Biochemistry* **9**, 147 (1970).
3. F. Oesch, *Xenobiotica* **3**, 305 (1973).
4. E. Boyland and K. Williams, *Biochem. J.* **94**, 190 (1965).
5. E. Boyland and P. Sims, *Biochem. J.* **95**, 788 (1965).
6. J. G. Wit and J. Snel, *Eur. J. Pharmac.* **3**, 370 (1968).

7. T. A. Fjellstedt, R. H. Allen, B. K. Duncan and W. B. Jakoby, *J. biol. Chem.* **248,** 3702 (1973).
8. P. L. Grover, A. Hewer and P. Sims, *FEBS Lett.* **34,** 63 (1973).
9. T. Hayakawa, R. A. LeMahieu and S. Udenfriend, *Archs. Biochem. Biophys.* **162,** 223 (1974).
10. P. L. Grover, A. Hewer and P. Sims, *Biochem. Pharmac.* **21,** 2713 (1972).
11. H. Marquardt, T. Kuroki, E. Huberman, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims, *Cancer Res.* **32,** 716 (1972).
12. E. Huberman, T. Kuroki, H. Marquardt, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims, *Cancer Res.* **32,** 1391 (1972).
13. S. A. Kamath, F. A. Kummerow and K. A. Narayan, *FEBS Lett.* **17,** 90 (1971).
14. D. Kupfer and E. Levin, *Biochem. biophys. Res. Commun.* **47,** 611 (1972).
15. H. Vainio and A. Aitio, *Acta pharmac. Toxicol.* **34,** 130 (1974).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
17. F. Oesch, D. M. Jerina and J. Daly, *Biochim. biophys. Acta* **227,** 685 (1971).
18. H. Lineweaver and D. J. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).
19. L. F. Chasseaud, *Drug Metab. Rev.* **2,** 185 (1973).
20. E. Boyland and P. Sims, *Biochem. J.* **95,** 780 (1965).
21. P. H. Jellinck, G. Smith and R. Fletcher, *Cancer Res.* **30,** 1715 (1970).
22. T. Suga and M. Akagi, *Chem. pharm. Bull.* **18,** 115 (1970).
23. P. L. Grover and P. Sims, *Biochem. J.* **90,** 603 (1964).
24. C. D. Klaasen and G. L. Plaa, *J. Pharmac. exp. Ther.* **161,** 361 (1968).
25. F. J. Darby and R. K. Grundy, *Biochem. J.* **128,** 175 (1972).
26. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).