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SHORT COMMUNICATIONS

A highly sensitive assay for glutathione transferase using 4,5-dihydro-epoxybenzo(*a*)pyrene as substrate

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Arene oxides, which arise from the enzymatic oxidation of polycyclic aromatic hydrocarbons (PAH), are electrophilically reactive agents which can alkylate biological nucleophiles (e.g. DNA) leading to toxic, mutagenic or carcinogenic effects [1, 2]. Besides their importance as direct noxious substances, they are, after hydration to dihydrodiols, the precursors of the ultimate carcinogens of PAH, the diol-epoxides [3].

Epoxides can be metabolized by epoxide hydrolase (EH; EC 3.3.2.3.) yielding the already mentioned dihydrodiols or by glutathione-transferase (GSH-T; EC 2.5.1.18) catalysing the nucleophilic attack of the sulphur anion of glutathione (GSH) on the epoxide, leading to the corresponding conjugate. Whereas the effect of EH can be considered as either toxification, providing the precursors of the diol-epoxides, or detoxification, providing a possible conjugative pathway of sulphotransferase action of the dihydrodiols, the GSH-T action is generally considered to act as detoxification of the parent compound.

In the present report we describe a fluorometric assay for the conjugation of 4,5-dihydro-epoxybenzo(*a*)pyrene (BPO) with glutathione. The method has several advantages over earlier published assays for this enzyme.

Materials and methods

Chemicals. BPO was obtained from the National Cancer Institute Chemical Repository at the IIT Research Institute (Chicago, IL). 17β-Estradiol-3-monosulphate was purchased from Sigma (St. Louis, MO). Reduced glutathione was from Boehringer Mannheim (Mannheim, F.R.G.). L-Glycine-2-^{3}H-glutathione (reduced; specific activity 1 Ci/mmole) was obtained from New England Nuclear (Dreieich, F.R.G.). TLC plates (silica gel 60, without fluorescence indicator) were from Merck (Darmstadt, F.R.G.).

Source of enzyme. Liver tissue was obtained from Wistar rats. After coarsely mincing the tissue with scissors the liver was homogenized in buffer (0.1 M potassium phosphate (pH 7.9), 10% (v/v) glycerol and 1 mM EDTA) using a Potter S homogenizer (Braun). The homogenate was centrifuged at 10,000 *g* for 1 hr at 4°. The resulting supernatant was used for preparation of the cytosol by centrifugation at 105,000 *g* for 1 hr at 4°. The protein content of the supernatant was determined by the method of Lowry *et al.* [4] with bovine serum albumin as the reference standard.

Assay procedure. All assays were performed in triplicate. For each assay 105,000 *g* supernatant (representing 5.5 μg protein) and 10 μl glutathione (20 mM) were added to 0.5 ml final volume 25 mM glycine (pH 10.0) in an Eppendorf reaction vial. The vials were pre-warmed for 5 min in a shaking water-bath at 37°. The reaction was initiated by addition of 4 μl BPO dissolved in DMSO/ethanol (1:1, v/v; final BPO concentration was 15 μM) and carried out in a water-bath at 37°. After the required incubation time (standard time: 5 min) the reaction was stopped by adding 0.5 ml trichloroacetic acid (TCA, 7.5%, w/v). After centrifugation for 2 min in an Eppendorf minifuge the reaction mixture was transferred to a glass tube and put on ice. To remove the unmetabolized BPO, 3 ml of hexane was added and the tubes were mixed on a Vortex mixer for 60 sec. After centrifugation for 5 min at 5000 *g*, the hexane fraction was removed and 0.5 ml aqua bidest added to the water phase. In control experiments, using ^{3}H-labelled glutathione, it was shown that only a negligible amount of conjugated glutathione was recovered in the hexane phase (less than 0.05%). The fluorescence of the conjugate was measured in a Perkin Elmer 650-40 spectrofluorometer at an excitation wavelength of 305 nm and an emission wavelength of 425 nm. Quinine HBr in 0.1 M H_2SO_4 was used for calibration of the fluorometer.

Calibration curve of the conjugate. Quantification of the adduct was performed by overnight TLC separation of the reaction mixture as described by Nemoto and Gelboin [5] using ^{3}H-labelled glutathione (0.3 μCi/incubation), diluted to the standard concentration of 400 μM. The reaction was stopped by putting the reaction vials on ice instead of adding TCA. The area of the conjugate ($R_f = 0.15$) was scraped off the plate and the conjugate isolated by extraction with water. A sample of the extract was analysed for radioactivity and the amount of conjugate formed was calculated from the specific activity of the compound. Fluorescence was correlated to the amount of conjugate by preparing dilutions of conjugate-extracts (i.e. without ^{3}H-glutathione) obtained simultaneously under standard conditions. Enzymatic activities were expressed as pmole conjugate/μg protein/min. This establishment of the relation between units of fluorescence and amount of conjugate has to be performed only once.

Results

Comparison of the excitation and emission spectra of the BP-GSH conjugate isolated from TLC and from the aqueous phase after the enzymatic reaction using rat liver supernatant showed the similarity of the maximum excitation and emission wavelengths of both products. This demonstrates that the fluorescence in the aqueous phase almost entirely results from the BP–GSH conjugate. Addition of 17β-estradiol-3-monosulphate, which has been described as a selective inhibitor of several forms of GSH-T [6], results in a 25% decrease of enzyme activity. Hg, for which an inhibitory role, among other heavy metals, of GSH-T *in vitro* has been documented [7], results in about the same percentage of enzyme inhibition. The enzymatic nature of the reaction is further demonstrated by the low rate of conjugation using boiled supernatant, at 0° and in the absence of BPO (Table 1).

From Fig. 1 it can be concluded that linearity is maintained until 7 min of incubation (A) and until 7 μg protein (B). The pH-optimum is reached at pH 10.0 and the non-enzymatic addition of glutathione to BPO is strongly pH-dependent and particularly manifests at pH > 10 (Fig. 1C). BPO concentrations exceeding 10 μM (Fig. 1D) and GSH concentrations exceeding 300 μM (Fig. 1E) did not result in higher enzymatic rates. The K_m of the reaction is about 2.3 μM and the specific activity of the enzyme is 84 pmole/μg protein/min as can be derived from Fig. 1D. It is most likely that these results are an average of values of different isozymes of GSH-T present in the cytosolic fraction. DMSO/ethanol (1:1, v/v) concentrations used in the assay (0.8%) did not affect the enzyme(s) (Fig. 1F).

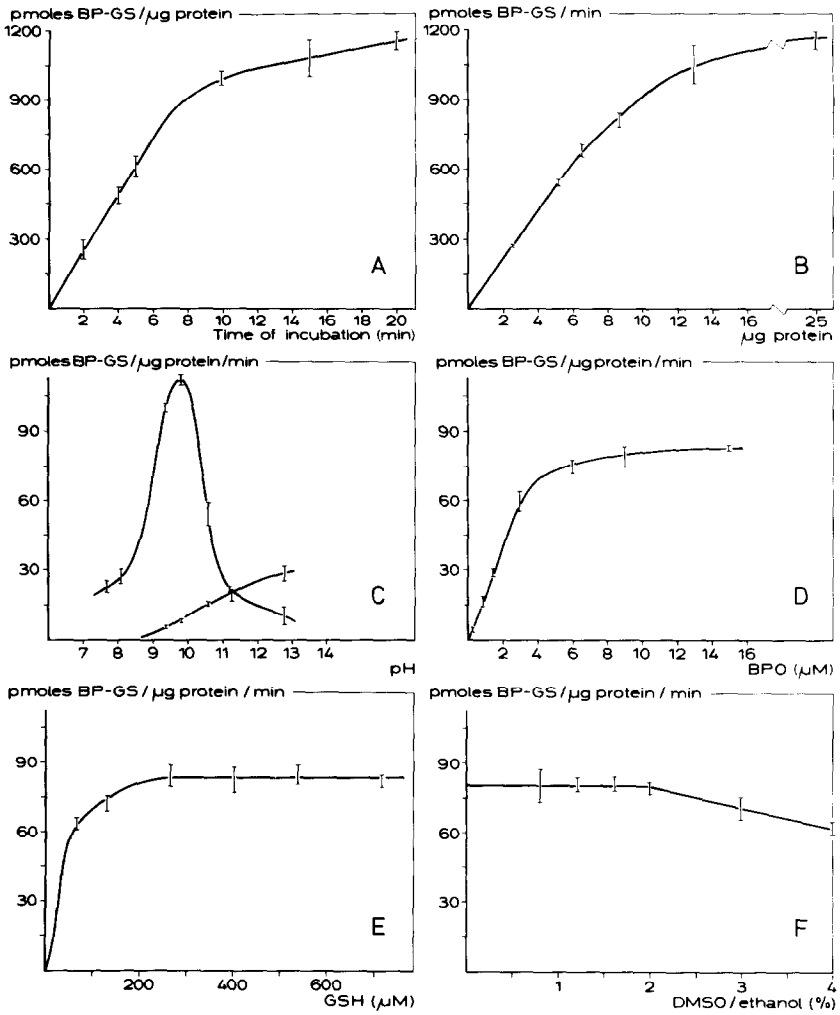

Fig. 1. Kinetics of the enzymatic conjugation of BPO to glutathione by rat liver supernatant. All data are expressed as mean (N = 3) ± S.D. and are obtained by substraction of the non-enzymatic blanks from the sample values. (A) Time–velocity relation (3 μl supernatant corresponding to 5.5 μg protein; 15 μM BPO; 400 μM GSH; pH 10.0) (B) protein–velocity relation (5 min incubation; 15 μM BPO; 400 μM GSH; pH 10.0); (C) pH–velocity relation (5 min incubation; 5.5 μg protein; 15 μM BPO; 400 μM GSH); (D) BPO–velocity relation (5 min incubation; 5.5 μg protein; 400 μM GSH; pH 10.0); (E) GSH–velocity relation (5 min incubation; 5.5 μg protein; 15 μM BPO; pH 10.0); (F) DMSO/ethanol–velocity relation (5 min incubation; 5.5 μg protein; 15 μM BPO; 400 μM GSH; pH 10.0).

Table 1. The properties of GSH-T

Samples	Activity*
Complete	100
+ 17β-estradiol-3-monosulphate (50 μM)	75
+ $HgCl_2$ (37 μM)	76
Boiled liver supernatant	12
Incubation at 0°	11
− BPO	0

* The amount of BP–GSH formed in the control, complete experiment is expressed as 100%. The enzyme assay was performed as described in Materials and Methods.

The assay was also performed with freshly isolated hair follicles under standard conditions. It was found that glutathione-conjugation to PBO could be measured adequately in one single hair follicle. Inhibition of the reaction by 17β-estradiol-3-monosulfate and Hg, as well as absence of conjugation in boiled hair follicles, at 0° and in the absence of BPO, were comparable to that found with rat liver supernatant.

We wish to emphasize that the optimal signal for the glutathione-conjugate is only obtained after 3 min. The signal is then stable and 30% elevated. The following experiments were performed in order to establish the eventual consequence of this phenomen for the experimental procedure:

1. A spectrum was determined at several intervals between 0 and 15 min of irradiation. It was observed that the spectrum started to change only after 8 min of irradiation.

2. The observed enhanced signal that was obtained after 3 min was lost when the sample was stored on ice for 15 min. Renewed irradiation again showed the same enhancement.

It is evident that these factors do not negatively influence the accuracy of the determination. Although the exact molecular nature of the phenomenon is not understood, the phenomena mentioned above strongly suggest the

reversible formation of a hydroxy-benzo(*a*)pyrene radical upon irradiation (Prof. Laarhoven, personal communication).

Discussion

A number of assays for GSH-T has been described including titrimetric, colorimetric, spectrophotometric and radiochemical methods using a variety of substrates (for review see [8]). However, assays using substrates of the biologically important PAH have only employed radioactive substrates [5, 9–11], most of them using a time consuming TLC separation of the conjugate. In addition, enzyme activity is often measured at suboptimal pH due to the large contribution of non-enzymatic conjugation. The present method combines an extreme sensitivity with the easy separation of glutathione conjugated with a non-radioactive PAH substrate. Optimal conditions (substrate, enzyme and glutathione concentrations) were obtained at 1/5 of those described by Cantfort *et al.* [9] who have used the same extraction principle. pH-optimum, time-dependence and non-enzymatic conjugation were quantitatively comparable between the former assay and the present one. The high pH-optimum is rather unusual for cytosolic enzymes, but in accordance to results of others [5, 9, 11]. Although we have used BPO obtained as a gift from the National Cancer Institute, the compound can be synthesized relatively easily from benzo(*a*)pyrene [12].

Due to the extreme sensitivity the method is especially useful for application in extrahepatic tissues and cultured cells. Since human hair follicles have been introduced as indicator organs for assessment of individual differences in susceptibility to chemical carcinogenesis, possibly reflected by differences in activities of carcinogen metabolizing enzymes among which GSH-T [13–16], we have studied the applicability of the present assay for human hair follicles. It was found that the enzyme could be detected in only one freshly isolated hair follicle. Thus, the present method is particularly useful for screening of high risk populations to identify interindividual differences in carcinogen metabolism.

In summary, a method is described for the quantitative determination of the glutathione-conjugate of 4,5-dihydroepoxybenzo(*a*)pyrene. The sensitivity and practical convenience of the procedure is based on (a) the high specific fluorescence intensity of the product, (b) the very low background obtained by the efficient differential extraction of substrate and product, (c) the use of a non-radioactive substrate from the important class of polycyclic aromatic hydrocarbons and (d) the involvement of a single rapid transfer and extraction step. Due to the sensitivity of the method permitting measurement of 30 pmoles product the procedure is especially useful for assaying transferase activity in minute tissue samples such as human hair follicles or cultured cells.

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Tricyclic antidepressant drug effects on liposomal membranes

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Alterations of membrane lipid structure may be an important mechanism by which hormones and other small molecules may regulate cell function [1–4]. The tricyclic antidepressants inhibit lymphocyte mitogenesis [5–8] and reduce numbers of plaque forming cells [7] *in vitro*, and reduce elevated rheumatoid autoantibody levels [9] and produce leukopenia [10] *in vivo*. Tricyclic antidepressant drug-induced perturbations of membrane lipid structure may be related to tricyclic antidepressant-induced inhibition of lymphocyte mitogenesis [11].

Since biological membranes are enormously complex, phospholipid vesicles or liposomes, representing relatively