Biochemical Pharmacology, Vol. 28, pp. 455–460.

0006-2952/79/0215-0455 $02.00/0

A NEW ASSAY FOR GLUTATHIONE *S*-TRANSFERASE USING [^{3}H]-BENZO(a)PYRENE 4,5-OXIDE AS SUBSTRATE.

INDUCIBILITY BY VARIOUS CHEMICALS IN DIFFERENT RAT TISSUES COMPARED TO THAT OF ARYL HYDROCARBON HYDROXYLASE AND EPOXIDE HYDRATASE

J. VAN CANTFORT, L. MANIL and J. E. GIELEN

Laboratoire de Chimie Médicale, Institut de Pathologie, B-4000 Sart-Tilman par Liège 1, Belgium

and

H. R. GLATT and F. OESCH

Section on Biochemical Pharmacology, Institute of Pharmacology, University of Mainz, D-6500 Mainz, West Germany

(*Received* 25 *April* 1978; *accepted* 3 *July* 1978)

Abstract—A new assay for glutathione *S*-transferase using [^{3}H]-benzo (a)pyrene 4,5-oxide as a substrate is presented. Compared to other methods, this assay presents the following advantages: (1) it is easy and rapid since only one extraction step is required and it does not necessitate the usual chromatographic separation of the substrate and products; (2) it uses an epoxide metabolically produced from a carcinogenic polycyclic aromatic hydrocarbon as a substrate; and (3) it is performed under conditions optimal with respect to both the pH and the concentration of the two substrates (glutathione and epoxide). Studies on the *in vivo* response of this enzyme to several chemicals show that glutathione *S*-transferase is remarkably constant and is only slightly influenced by various inducers. In this respect this enzyme behavior is very different from other enzymes involved in the polycyclic hydrocarbon metabolism. In effect, the aryl hydrocarbon hydroxylase appears in many organs to be very easily and strongly induced by several chemicals and epoxide hydratase is very significantly enhanced in the liver after phenobarbital and 16α-cyanopregnenolone treatment. These results constitute an additional argument in favor of a minor role of possible changes in the concentration of this enzyme in the regulation of polycyclic aromatic hydrocarbons elimination.

Glutathione *S*-transferase (E.C. 2.5.1.18) refers to a group of enzymes of broad specificity which, among others, catalyzes the addition of aliphatic epoxides and arene oxides to glutathione [1–3]. In mammalian tissues, the transformation of many exogenous compounds into electrophilically reactive epoxides is controlled by a microsomal multienzymatic complex, i.e. the monooxygenases [4–7]. In the case of polycyclic aromatic hydrocarbons, such reactive arene oxides and dihydrodiol epoxides are candidates for ultimate or proximate carcinogens [4, 5, 8]. Thus, the glutathione *S*-transferase as well as the microsomal epoxide hydratase [9, 10] play important roles in the metabolism of such harmful products.

A number of methods have been proposed to measure the glutathione *S*-transferase activity in the different organs using an epoxide as substrate. None of them are performed at optimal conditions with respect to substrate concentration, pH and incubation temperatures [1, 2, 11–15]. In addition, most of the assays require either tedious chromatographic steps [11–13] or complex calculation of the blank values [15]. In this paper, we describe a simplified assay which measures the enzyme activity under optimal conditions at 37°. The substrate and the product are separated by a simple differential extraction, a procedure which obviates the need for multiple blank determination. [^{3}H]-benzopyrene 4,5-oxide ([^{3}H]-BP4,5-oxide) was chosen as a substrate mainly because it is not subject to any detectable non-enzymatic hydration [16]. Moreover, it is a metabolite of a potent carcinogen ubiquitously present in our environment. Since many epoxides derived from polycyclic hydrocarbons are ultimate carcinogens or essential precursors of ultimate carcinogens [4, 5], estimation of glutathione *S*-transferase with an arene oxide as substrate may be an important approach to understand the susceptibility differences of various organs or species.

MATERIALS AND METHODS

[^{3}H]-BP 4,5-oxide (Sp. act. 3895 dpm/nmol) was prepared according to the method of Dansette and Jerina [18]. This substrate (50 mg), dissolved in a mixture of benzene/hexane/triethylamine (60/40/2; 30 ml) was purified by chromatography on a silicagel column (Merck, Darmstadt, size C) eluted with the same mixture of solvents. The fractions corresponding to the peak of [^{3}H]-BP 4,5-oxide were pooled and evaporated under nitrogen. The dried product was stored at −20° under argon.

Conjugate of [^{3}H]-BP 4,5-oxide and glutathione was prepared by incubation under our optimal conditions. It was isolated by the procedure of Nemoto and Gelboin [12].

Other chemicals and solvents were purchased from Merck (Darmstadt, West Germany) except for glutathione (Boehringer, Manheim, West Germany).

Table 1. Incubation conditions adopted for the standard assay

Tissue	105,000 *g* Supernatant (μl/0.5 ml incub. mixt.)	Corresponding amount of tissue (mg)	Incubation time (min)
Liver	2	0.4	3
Lung	5	1	6
Kidney	10	2	8

The enzymatic preparations were made as described in the Methods section.

Aryl hydrocarbon hydroxylase [20] and epoxide hydratase [21] were assayed by isotopic methods in 9000 *g* supernatant fractions [19], as described in the indicated references. The organ homogenates were prepared in phosphate buffered (pH 7.4; 10 mM) isotonic KCl. The glutathione *S*-transferase activities were measured in the 105,000 *g* supernatant fraction of the same homogenates. From the various experiments described in the results section, the following optimal conditions for the standard assay were selected: in a final volume of 0.5 ml, the incubation mixture contains 25 mM glycine buffer (pH 10), 2 mM glutathione, 200 μg bovine serum albumin, 80 μM [^{3}H]-BP 4,5-oxide in 20 μl of ethanol and the enzymatic preparation. After incubation in a shaking incubator at 37°, the enzymatic reaction is stopped by the addition of 0.5 ml trichloracetic acid (7.5%). The mixture is agitated on a Vortex vibrator and centrifuged for 5 min at 2000 rpm. The clear supernatant fraction is transferred into a conical stoppered tube and extracted once with 5 ml of hexane. After centrifugation, the organic phase and the interphase are removed; 0.5 ml of the remaining water phase is diluted with 4 ml of Aqua luma (LUMAC, AG, Basel, Switzerland) and the radioactivity counted by liquid scintillation spectrometry. The optimal incubation time and enzyme concentration vary from tissue to tissue and are indicated in Table 1. The control samples (blanks) were always incubated in the presence of bovine serum albumin, the enzymatic preparation being added just before trichloracetic acid. These blanks corresponding to non-enzymatic conjugations plus non-specific binding of substrate to proteins, are always subtracted from the sample values.

RESULTS

1. *Analysis of the enzymatic reaction products.* The aqueous fraction which in the present assay is used for determination by scintillation spectrometry as described in Methods was analyzed by thin layer chromatography as described by Nemoto and Gelboin [12]. Besides a trace of [^{3}H]-benzo (a) pyrene 4,5-oxide, only one further labelled product was detected. This product was identified as a glutathione conjugate as (1) it was radioactive and fluorescent, (2) it was highly soluble in water, (3) it could not be extracted from water by hexane between pH 0 and 12, (4) it showed a ninhydrin positive reaction, and (5) it was formed only when both glutathione and benzo(a)pyrene 4,5-oxide were present.

Moreover, chromatographed on different supports (thin layer and paper chromatography) under different conditions, this metabolite always migrated as a single spot, possibly because the glutathione residue was conjugated to only one of the two possible positions (carbon 4 or carbon 5). Alternatively it cannot be excluded that none of the systems used resolved the positional isomers.

2. *Selective extraction of the substrate from the incubation medium.* Based upon the partition coefficient of the substrate and the conjugate product between hexane and water, theoretically it should be easy

Table 2. Influence of bovine serum albumin on the remaining radioactivity in the aqueous phase

BSA concentration (mg/ml incubation medium)	Radioactivity (%) in the water phase		
	One extraction unchanged incubation medium	Two extractions unchanged incubation medium	One extraction after TCA precipitation
0	1.6	1.3	1.5
0.031	5.5	4.6	1.6
0.062	6.4	5.9	1.6
0.125	9.7	8.0	1.5
0.250	12.1	10.3	1.6
0.500	14.0	11.9	1.4
1.0	15.2	13.1	1.6
2.0	15.5	13.8	1.5

Twenty μg of [^{3}H]-benzo(a)pyrene 4,5-oxide were mixed with 300 μg of glutathione in one ml of Tris buffer (10 mM; pH 10). The medium also contained the indicated amount of bovine serum albumin. After 5 min of incubation (37°), the solution was extracted once or twice with 5 ml hexane., the radioactivity was counted in the remaining aqueous phase and expressed as the percentage of the whole radioactivity added into the incubation medium. The blank value obtained after one or two extractions are compared to those after precipitation by the concentration of TCA adopted for the standard assay followed by one extraction.

Table 3. Influence of the precipitation by trichloracetic acid on the remaining radioactivity in the aqueous phase

Trichloracetic acid final concentration (g/100 ml)	Radioactivity (%) in the water phase (pH 10)
0	9.1
0.6	6.5
1.25	4.3
2.5	2.1
3.75	1.6
5	1.9
7.5	2.2
10	2.6

After 5 min of incubation under the conditions described in Table 1 with 500 μg of bovine serum albumin per ml, trichloracetic acid was added to reach the indicated final concentration. After centrifugation, the supernatant was isolated and extracted twice with 5 ml of hexane. The radioactivity present in 0.5 ml of the aqueous phase was finally measured.

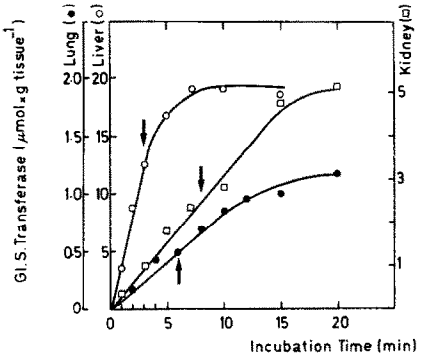

Fig. 1. Glutathione *S*-transferase activity as a function of time. Liver, lung and kidney 105,000 *g* supernatant fractions were incubated under the optimal conditions described in the Methods section. The arrows indicate the incubation times adopted in the standard assay for the different tissues.

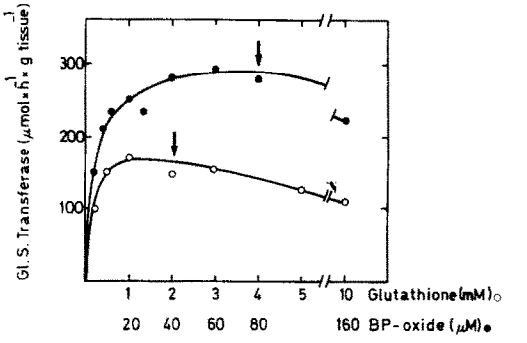

Fig. 2. Liver glutathione *S*-transferase activity as a function of glutathione and benzo(a)pyrene 4,5-oxide concentration. The arrows indicate the selected concentrations of glutathione or BP 4,5-oxide adopted in the standard assay.

to separate the two compounds by a simple extraction procedure. Nevertheless, it was not possible to extract quantitatively the substrate from the incubation mixture. This was most likely due to its unspecific binding to the proteins of the medium as suggested by the observation that the amount of contaminating substrate left in the water phase after hexane extraction increased with the protein concentration (Table 2). To overcome this difficulty, trichloracetic acid (TCA) was first added to the medium and the hexane extraction was performed on the protein free supernatant. Table 3 shows that a final concentration of 3.75 g TCA/100 ml is optimal to minimize the radioactive contamination in the water phase. Under these conditions, the yield of the [^{3}H]-BP 4,5-oxide extraction from the incubation medium was similar to that obtained from a protein free water suspension of the substrate (Table 2). Subsequent hexane extractions did not remove any additional radioactivity from the water phase. The concentration of the albumin added to the medium did not significantly influence the blank value. 400 μg of albumin per ml was thus maintained in our assay.

Table 4. Influence of the solvents for the BP 4,5-oxide on the enzymatic reaction and on the blank assays

Solvents	Enzymatic activity (% of the "Ethanol" assay)	Blank values (% of "Ethanol" blank)
Ethanol	100	100
Methanol	100	94
Ethylene glycol	103	105
Methyl glycol	83	190
n-propanol	24	160
n-butanol	17	110
Acetone	38	255
Acetonitrile	82	190
Dimethylsulfoxide	94	220
Tetrahydrofurane	0	250

The substrate, dissolved in 25 μ l of solvent, was introduced into the incubation mixture and incubated under the optimal conditions. The enzymatic activity as well as the counts measured in the blanks are expressed as a percentage of the values found in the assays where ethanol was used as a solvent.

3. *Optimal conditions.* The different enzymatic parameters were systematically studied in three tissues, liver, lung and kidney. Using the experimental conditons described in the methodological section, the reaction evolves linearly for four minutes with the liver enzymes and for 10 and 15 min, respectively, in the case of the lung and kidney glutathione *S*-transferases (Fig. 1). Tissue differences were also found regarding the dependence on the enzyme concentration in the incubation medium. The reaction was proportional to amounts of 105,000 *g* supernatants, respectively equivalent to 0.4 mg (60 μg of proteins per ml of incubation), 1.0 mg (30 μg of proteins per ml) and 2.0 mg (70 μg of proteins per ml of incubation) of wet liver, lung and kidney per assay.

Figure 2 shows the glutathione *S*-transferase activity of liver 105,000 *g* supernatant fraction as a function of the substrate concentrations. Thus, our standard substrate concentrations (2mM glutathione, 80 μM BP 4,5-oxide) saturate the enzyme. Very similar results were obtained with preparations from other organs. However, the optimal glutathione concentration was slightly higher in the lung than in the liver.

The optimal pH for the enzymatic conjugation was identical in the three tissues (Fig. 3). We observed also a variation of the blank with the pH of the incubation medium. The pH-dependence of the blank is compatible with the assumption that the concentration of glutathione dissociated at the thiol group (pK:9.11) is essential for the spontaneous conjugation.

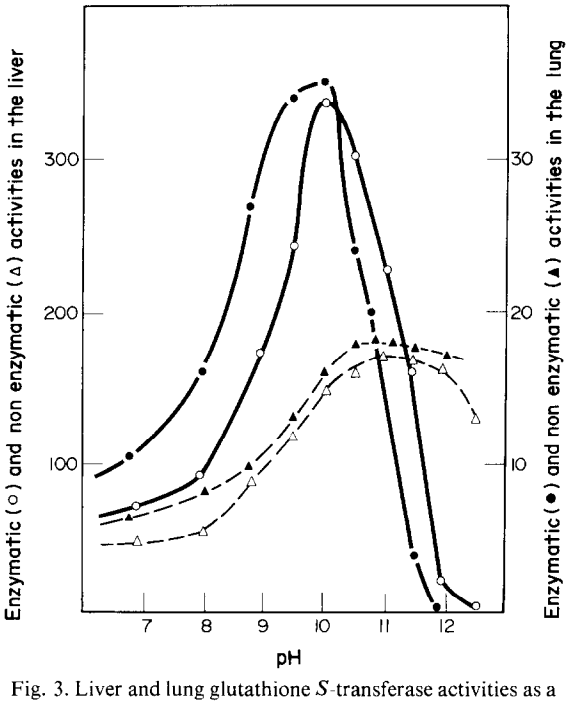

Fig. 3. Liver and lung glutathione *S*-transferase activities as a function of the incubation mixture pH. Glycine buffer (25 mM; pH 9–13) and Tris buffer (25 mM, pH 7–8) were used to cover the pH scale between pH 9 and 13 and for pH 7 and 8 respectively. The exact pH's indicated on the abscissa were measured in the incubation medium as the buffer capacity is limited at high pH. The blank values are indicated (dotted line) and are subtracted from the values of the enzymatic assays (solid line). The results are expressed in μmol × hr^{-1} × g $tissue^{-1}$ for the enzymatic activities. The blank is shown in its relative height to the enzymatic conversion under standard conditions.
Similar curves (data not shown) were obtained for the kidney tissue.

Various agents were tested in order to dissolve and introduce the substrate into the incubation medium (Table 4). Three solvents gave identical results: ethanol, methanol, and ethylene glycol. They did not dissolve completely the substrate, but after vigorous shaking, gave a very fine suspension easy to transfer quantitatively. Ethanol was finally adopted for the standard assay as it provides us with the most stable suspension.

4. *Validation of the method.* By incubating known amounts of labelled conjugate under the standard experimental conditions, it was verified that more than 99 per cent of the product was always recovered at the end of the extraction procedure. The content of the hexane and the water phase was also analyzed by thin layer chromatography [12]. BP 4,5-oxide was the only compound detectable in the hexane phase while, besides the conjugate, small amounts (0.5 to 1%) of the incubated substrate, BP 4,5-oxide, were always found in the water phase and accounted for the rather high but reproducible blank values.

Finally, Fig. 4 demonstrates the good qualitative correlation between the results obtained by our simple assay and by the more sophisticated extraction procedure of Nemoto and Gelboin [12]. The sensitivity and reproducibility of this new method are good. The coefficient of variation (s/m), calculated on a series of 10 identical determinations, was 5 per cent, 7 per cent and 12 per cent for the liver, kidney and lung enzyme activities respectively. The limit of sensitivity was estimated to be 0.1 nmoles of conjugate.

5. *Effects of various chemical treatments on different benzo (a)pyrene metabolizing enzymes.* The assay developed in this study was used to determine the effect of

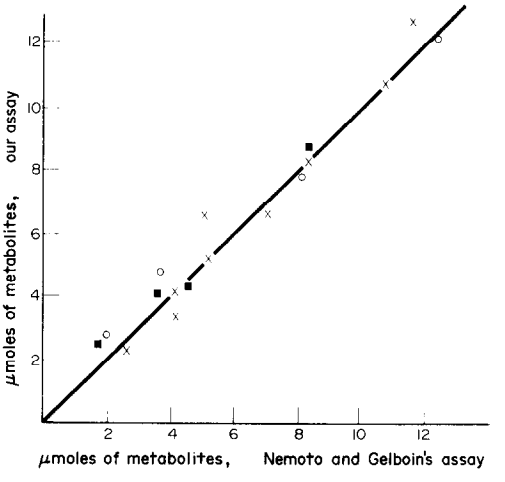

Fig. 4. Comparison of results obtained by our assay and the Nemoto and Gelboin assay [12]. Seventeen assays were performed in duplicate by both methods under different conditions, namely, pH (7.5, 9 and 10), time of incubation (3, 6, 10 and 15 min) and nature of the tissue (Liver (×), kidney (○) and lung (■)). The results are expressed in μmol of substrate metabolized in the assays. The correlation between both methods is highly significant ($r = 0.98$).

Table 5. Effect of different treatments on aryl hydrocarbon hydroxylase, epoxide hydratase and glutathione S-transferase activities

Organ	Treatment	A.H.H. (nmol × hr^{-1} × g $organ^{-1}$)	Epoxide hydratase (μmol × hr^{-1} × g $organ^{-1}$)	Glutathione transferase (μmol × hr^{-1} × g $organ^{-1}$)
Liver	No (Controls)	681 ± 117	7.75 ± 1.15	167 ± 19
	Cigarette smoke	998 ± 204 (NS)	10.5 ± 2.7 (NS)	186 ± 13 (NS)
	Phenobarbital	1827 ± 49 (**)	30.1 ± 2.7 (**)	202 ± 13 (*)
	16α-Cyanopregnenolone	2042 ± 480 (**)	19.0 ± 4.5 (**)	281 ± 48 (**)
	Methylcholanthrene	4915 ± 701 (**)	11.8 ± 3.1 (NS)	191 ± 18 (NS)
Kidney	No (Controls)	13.1 ± 2.9	0.28 ± 0.06	21.1 ± 0.8
	Cigarette smoke	39.0 ± 2.9 (**)	0.28 ± 0.08 (NS)	20.8 ± 1.3 (NS)
	Phenobarbital	10.4 ± 4.6 (NS)	0.23 ± 0.02 (NS)	22.9 ± 1.9 (NS)
	16α-Cyanopregnenolone	10.5 ± 4.2 (NS)	0.21 ± 0.04 (NS)	21.7 ± 2.0 (NS)
	Methylcholanthrene	121.5 ± 43.7 (**)	0.23 ± 0.04 (NS)	23.0 ± 1.3 (NS)
Lung	No (Controls)	4.1 ± 1.5	0.25 ± 0.06	16.8 ± 2.7
	Cigarette smoke	18.9 ± 6.8 (**)	0.24 ± 0.09 (NS)	15.4 ± 2.2 (NS)
	Phenobarbital	3.6 ± 1.0 (NS)	0.26 ± 0.04 (NS)	18.1 ± 2.7 (NS)
	16α-Cyanopregnenolone	6.3 ± 2.9 (NS)	0.23 ± 0.05 (NS)	18.6 ± 2.1 (NS)
	Methylcholanthrene	49.0 ± 6.1 (**)	0.28 ± 0.07 (NS)	19.5 ± 2.7 (NS)

The statistical significance is as follows: NS = $P > 0.01$; (*) = $P < 0.01$; (**) = $P < 0.001$.
Sprague–Dawley male rats weighing 200 g were treated as follows:
(a) Nothing (control rats)
(b) Three successive 15 min inhalations of cigarette smoke diluted by 15 volumes of air, at 2 hr intervals.
(c) Four successive i.p. injections of phenobarbital (80 mg/kg) in 1 ml of 0.9% NaCl at 24 hr intervals.
(d) Three successive i.p. injections of 16α-cyanopregnenolone (40 mg/kg) in 2 ml of peanut oil at 24 hr intervals.
(e) One i.p. injection of methylcholanthrene (80 mg/kg) in 2 ml of peanut oil.
The rats were killed 24 hr after the last treatment except for cigarette smoke (5 hr).
The enzyme assays were performed in duplicate either on a 9000 *g* supernatant (aryl hydrocarbon hydroxylase and epoxide hydratase), or on a 105,000 *g* supernatant fraction (epoxide glutathione S-transferase).
The results represent the mean ± SD obtained from 5 rats.

cigarette smoke inhalation on glutathione S-transferase activity in liver, kidney and lung and to compare this effect with that of three well known drug metabolizing enzyme inducers (phenobarbital, 3-methylcholanthrene and 16α-cyanopregnenolone) and with the effect on two other enzymes involved in the polycyclic hydrocarbon metabolism: aryl hydrocarbon hydroxylase and epoxide hydratase (Table 5). Aryl hydrocarbon hydroxylase activity was enhanced by the three classical inducers in the liver, while only 3-methylcholanthrene and cigarette smoke significantly induced it in the lung and kidney. In the liver, epoxide hydratase activity was induced by 16α-cyanopregnenolone and especially by phenobarbital. In the other tissues, epoxide hydratase was never induced. This is very different from the induction of aryl hydrocarbon hydroxylase activity which was induced best by 3-methylcholanthrene.

Glutathione S-transferase activity was also inducible. However, the inductions were quantitatively much weaker than those of the other two enzyme activities. Induction was limited to the liver. The increases by 3-methylcholanthrene and cigarette smoke were very small and statistically not significant. Phenobarbital showed a weak, but clear induction. 16α-cyanopregnenolone was the most effective agent and nearly doubled the glutathione S-transferase activity.

Comparing the basal or control levels of the different enzymes, it is quite interesting that under the optimal conditions of the assays, glutathione S-transferase had a much higher specific activity than the other enzymes. For example, in control liver, it was 21 times and 245 times more active than epoxide hydratase and the aryl hydrocarbon hydroxylase respectively. The differences in specific activities are even more pronounced in kidney and lung.

DISCUSSION

The potential importance of glutathione S-transferase for the elimination of cancerogenic epoxides from the organism led to numerous methods for measuring this enzyme. Most of these methods present several disadvantages or limitations among them:

(a) Impossibility of measuring the enzyme under the optimal conditions (saturation of the enzyme by the epoxide and glutathione, optimal pH) because non-enzymatic conjugation with glutathione is often more important than the enzymatic reaction. The existence of this non-enzymatic reaction led other authors to work at pH 6.5–8.0 [1, 2, 11–15], which is far below the optimal pH (see Fig. 2), at non-saturating glutathione concentrations [11–13] or at room temperature [1, 11, 13].
(b) Length of the procedure requiring at least one chromatographic step [11–13] or several difficult blank assays [15].
(c) Use of a substrate that is not derived from a carcinogenic polycyclic hydrocarbon.

To overcome most of these disadvantages and limitations, Nemoto and Gelboin have proposed a new assay using BP 4,5-oxide as substrate [12]. With this particular epoxide, the non-enzymatic reaction is low even at saturating concentrations of both substrates and at the optimal pH (pH 10). Unfortunately, the method developed by these authors is time consuming since it re-

quires thin layer chromatography with the tedious deposit of an aqueous phase on silicagel. Furthermore, the assay does not use optimal conditions for the enzyme.

A much simpler extraction assay with BP 4,5-oxide (or other epoxides) as substrate(s) was proposed by Bend *et al.* [13]. Again, the assay is not performed at optimal pH. Although this assay works very well when tissue preparations with a high specific glutathione transferase activity are used, problems due to binding of the epoxides to the proteins occur when higher amounts of protein have been added to the incubation medium. In this case, several blanks (dialyzed protein without glutathione, glutathione without protein, no glutathione and no protein) have been performed. Therefore, we have now introduced a protein precipitation step which eliminates this requirement of multiple blanks. Furthermore, we use optimal conditions with respect to pH and substrate concentrations.

Compared to the original assay of Nemoto and Gelboin [12], our assay is by far simpler and gives qualitatively similar results, but on a quantitative basis, we obtained results 6 to 7 times higher; this is easily explained by the fact that contrary to these authors, we assay the enzyme under optimal conditions, i.e. saturation concentration of both substrates and optimal pH (10). Such a high optimal pH is rather unusual, but is in agreement with results obtained by others [1, 12, 13]. However, with the majority of substrates, it is impossible to work at optimal pH because the non-enzymatic conjugation increases more rapidly with the pH than the enzymatic activity. This is fortunately not true when BP 4,5-oxide is the substrate.

Due to its sensitivity, simplicity, precision and reproducibility, our glutathione *S*-transferase assay is well adapted to study the role of this enzyme in the detoxification of carcinogenic metabolites.

Our biological results indicate that, compared to epoxide hydratase and aryl hydrocarbon hydroxylase, glutathione *S*-transferase is quite insensitive to the administration of chemicals to the animals. Also, its activity is remarkably constant from animal to animal, the standard deviation of the results usually being less than 10 per cent of the mean value in the different groups of rats. From the observation that the specific activity of the glutathione *S*-transferase is much higher than that of the other two measured enzymes, one would expect that the absolute amount of glutathione *S*-transferase present in the cell is not a rate limiting step in the polycyclic hydrocarbon metabolism. Nevertheless, it should also be considered that the activity of the glutathione transferase is also dependent on the presence of glutathione, the second substrate of the enzymatic reaction. It is well known that the glutathione concentration can be drastically modified in the cells by a number of chemical agents and could almost reach zero in extreme conditions [23]. The conjugation to glutathione might thus be completely abolished in those cells. The consequences of such a situation have been extensively studied in the case of the halobenzenes or acetaminophen produced hepatocytolysis [24], but are still poorly documented in that of the polycyclic hydrocarbon mediated toxicity.

Due to the high lipophylic character of epoxides derived from polycyclic hydrocarbons, it is likely that they will preferentially stay in the membranes of the endoplasmic reticulum and preferentially be further metabolized by the epoxide hydratase compared to conjugation to glutathione by the cytosolic glutathione *S*-transferase. As mentioned in a previous paper [25], the role of this enzyme in the metabolism of the polycyclic hydrocarbon might therefore be very limited.

Acknowledgement—This research has been supported by grants (No. 1072 and No. 1097) from the American Tobacco Council.

REFERENCES

1. T. A. Fjellstedt, R. H. Allen, B. K. Suncan and W. B. Jacoby, *J. biol. Chem.* **248**, 3702 (1973).
2. T. Hayakawa, S. Udenfriend, H. Yagi and D. M. Jerina, *Archs Biochem. Biophys.* **170**, 438 (1975).
3. N. Nemoto, H. V. Gelboin, W. H. Habig, J. N. Ketley and W. B. Jakoby, *Nature, Lond.* **255**, 512 (1975).
4. D. M. Jerina and J. W. Daly, *Science* **185**, 573 (1974).
5. P. Sims and P. L. Grover, *Adv. Cancer Res.* **20**, 165 (1974).
6. O. Hayaishi, *A. Rev. Biochem.* **38**, 21 (1969).
7. D. W. Nebert, J. R. Robinson, A. Niwa, K. Kumaki and A. P. Poland, *J. cell. comp. Physiol.* **83**, 393 (1975).
8. P. G. Wislocki, A. W. Wood, R. L. Chang, W. Levin, H. Yagi, O. Hernandez, P. M. Dansette, D. M. Jerina and A. H. Conney, *Cancer Res.* **36**, 3350 (1976).
9. F. Oesch, *Xenobiotica* **3**, 305 (1973).
10. F. Oesch, P. Bentley and H. R. Glatt, in *Biological Reactive Intermediates* (Eds D. J. Jolley, J. J. Kocsis, R. Snyder and H. Vainio) pp. 181–206. Plenum Press, New York (1977).
11. H. Mukhtar and E. Bresnick, *Biochem. Pharmac.* **25**, 1081 (1976).
12. N. Nemoto and H. V. Gelboin, *Archs Biochem. Biophys.* **170**, 739 (1975).
13. T. Hayakawa, R. A. Lemahieu and S. Udenfriend, *Archs Biochem. Biophys.* **162**, 223 (1974).
14. J. R. Bend, Z. Ben-Zvi, J. Van Anda, P. M. Dansette and D. M. Jerina, in *Polynuclear Aromatic Hydrocarbons* (Eds R. Freudenthal and P. W. Jones) pp. 63–75. Raven Press, New York (1976).
15. J. Marniemi and M. G. Parkki, *Biochem. Pharmac.* **24**, 1569 (1975).
16. P. Bentley, H. Schmassmann, P. Sims and F. Oesch, *Eur. J. Biochem.* **69**, 97 (1976).
17. J. K. Selkirk, R. G. Croy and H. V. Gelboin, *Archs Biochem. Biophys.* **168**, 322 (1975).
18. P. M. Dansette and D. M. Jerina, *J. Am. chem. Soc.* **96**, 1224 (1974).
19. J. Van Cantfort, J. Renson and J. Gielen, *Eur. J. Biochem.* **55**, 23 (1975).
20. J. Van Cantfort, J. De Graeve and J. E. Gielen, *Biochem. biophys. Res. Commun.* **79**, 505 (1977).
21. H. U. Schmassmann, H. R. Glatt and F. Oesch, *Analyt. Biochem.* **74**, 94 (1976).
22. J. Van Cantfort and J. Gielen, *Int. J. Cancer* **19**, 538 (1977).
23. G. H. Lambert and S. S. Thorgeirsson, *Biochem. Pharmac.* **25**, 1777 (1976).
24. J. R. Gilette, J. R. Mitchell and B. B. Brodie, *A. Rev. Pharmac.* **14**, 271 (1974).
25. H. R. Glatt and F. Oesch, *Arch. Tox.* **39**, 87 (1977).