Interactions with glutathione S-transferases of porphyrins used in photodynamic therapy and naturally occurring porphyrins

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Several naturally occurring porphyrins and porphyrins used in photodynamic therapy inhibit glutathione S-transferase isoenzymes either purified from rat liver or lung or in cytosol from normal and from cancerous (Morris 7288C hepatoma) liver. Although differences occur in the type and amount of transferases in normal and cancerous liver and in the liver of rats bearing an extrahepatic tumour, these enzymes are potential binding sites for porphyrins. Porphyrin structure is an important factor in determining the affinity of binding, as shown by the relative inhibitory effectiveness. Of the dicarboxylic porphyrins in the mixture used clinically, OO'-diacetylhaematoporphyrin and monohydroxyethylmonovinyldeuteroporphyrin are more effective inhibitors than haematoporphyrin and protoporphyrin IX. Of the naturally occurring porphyrins the order of effectiveness is protoporphyrin IX (dicarboxylic) > coproporphyrin (tetracarboxylic) > uroporphyrin (octacarboxylic) and type I > type III isomers of both uroporphyrin and coproporphyrin, and the synthetic tetra-*meso*-phenylporphinetetrasulphonate is a better inhibitor (apparent $K_i = 250$ nM) than coproporphyrin, which contains a comparable number of negative charges. In addition, iron–porphyrin chelates are more effective inhibitors of the transferases, with 25-fold decrease in K_i value, than the free porphyrins. These results indicate that one means whereby porphyrins accumulate in tissues is the occupation of intracellular binding sites, such as the transferases. Since porphyrins inhibit the activity of these important detoxifying enzymes, there will be metabolic consequences to the cell.

Porphyrin photodynamic therapy, previously termed 'photoradiation therapy', has been used with some success to treat several different neoplasias. This technique employs the intravenous injection of a complex mixture of porphyrins that can preferentially accumulate in neoplastic tissues compared with surrounding normal tissues (for recent reviews see Kessel, 1984; Dougherty *et al.*, 1984). Upon illumination the porphyrins fluoresce and highly reactive oxygen intermediates are produced intracellularly, resulting in cell death (Weishaupt *et al.*, 1976). However, the biochemical consequences to normal and cancerous cells of porphyrin photodynamic therapy require investigation, and the processes responsible for the retention of porphyrins by tumours remain to be established. In addition, the means whereby

endogenous porphyrins produce cutaneous photosensitivity in certain genetic porphyrias are not fully understood. We have previously addressed factors affecting cellular uptake (Smith & Neuschatz, 1983) and metabolism (Dailey & Smith, 1984) of representative exogenous porphyrins. We have also presented evidence for binding of haematoporphyrin and OO'-diacetylhaematoporphyrin to cytosolic proteins from normal rat liver and Morris hepatoma cells (Smith & Neuschatz, 1983); however, the cytosolic proteins responsible were not identified. In the present paper we report our investigation on the interactions of several porphyrins with GSH S-transferases, undertaken to determine the importance of protein binding for the retention of porphyrins by cells and to assess effects of this binding on cell metabolism.

We considered that GSH S-transferases (EC 2.5.1.18) might be involved in intracellular por-

Abbreviation used: GSH, reduced glutathione.

phyrin binding for the following reasons. First, they are abundant cytoplasmic proteins, particularly in liver, which is a major site of porphyrin metabolism (Kappas *et al.*, 1983). Secondly, ligandin (one GSH *S*-transferase isoenzyme) binds haematin (Meuwissen *et al.*, 1972), protoporphyrin IX and haematoporphyrin) (Tipping *et al.*, 1978; Ketterer *et al.*, 1978). Thirdly, several other transferases also bind non-substrate ligands, including haematin and bilirubin (Ketley *et al.*, 1975). Fourthly, GSH *S*-transferases are found in both normal and cancerous tissues (Arias, 1979; Arias *et al.*, 1979; Scully & Mantle, 1981). Further, protoporphyrin IX, haematoporphyrin and the other porphyrins present in the mixture of haematoporphyrin derivatives used in photodynamic therapy are similar in structure, suggesting that several exogenous porphyrins may interact with these proteins.

GHS *S*-transferases represent an important group of enzymes involved in the detoxification of various pharmacologically active compounds (Ketterer *et al.*, 1983) and in the metabolism of endogenous compounds (Booth *et al.*, 1961; Boyland & Chasseaud, 1969). Porphyrin binding to these enzymes might cause inhibition in a manner analogous to other hydrophobic ligands known to inhibit certain GSH *S*-transferases *in vitro* (Ketley *et al.*, 1975; Vander Jagt *et al.*, 1982), and thus be detrimental to cells. We therefore examined the interactions of representative exogenous porphyrins with GSH *S*-transferases, using purified enzymes and cytosol. We also compared the relative activities of transferase isoenzymes in normal liver with hepatoma tissue, using the Morris hepatoma 7288C. In addition, to assess whether the presence of this extrahepatic tumour alters the hepatic expression of these enzymes, we examined transferase activity in the liver of tumour-bearing rats.

Materials and methods

1-Chloro-2,4-dinitrobenzene, *p*-nitrophenyl acetate, cumene hydroperoxide, GSH and ethacrynic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bromosulphophthalein, *trans*-4-phenylbut-3-en-2-one and 1,2-dichloro-4-nitrobenzene were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and androst-5-ene-3,17-dione was from Research Plus, Denville, NJ, U.S.A. Protoporphyrin IX, haematoporphyrin, monohydroxyethylmonovinyldeuteroporphyrin, tetra-*meso*-phenylporphinetetrasulphonate and isomers I and III of coproporphyrin and uroporphyrin were purchased from Porphyrin Products, Logan, UT, U.S.A. *OO'*-Diacetylhaematoporphyrin was synthesized by the method of

Bonnett *et al.* (1981). The purity of the *OO'*-diacetylhaematoporphyrin was about 90%, as assessed by the h.p.l.c. method of Bonnett *et al.* (1978) with a C_{18} reverse-phase column (0.5 cm × 25 cm) with as solvent methanol/water (17:3 v/v) containing 2 mM-tetrabutylammonium dihydrogen phosphate as solvent. The flow rate was 1 ml/min, and the eluted porphyrins were monitored by their absorbance at 405 nm. Concentrations of porphyrins were determined by using published absorption coefficients (Fuhrhop & Smith, 1976; Tipping *et al.*, 1978; Bonnett *et al.*, 1981).

The Morris 7288C hepatoma, kindly provided by Dr. J. P. Mapes (Louisiana State University Medical Center) and originally supplied by Dr. Thompson (Thompson *et al.*, 1966), was passaged in adult male Buffalo rats (220–250 g body wt.) from Harland, Indianapolis, IN, U.S.A. Frozen tumour cells were rapidly thawed and injected (about 2×10^7 cells in 1.5 ml) intramuscularly into both hindlegs of ether-anaesthetized rats on the caudal side of the femur. Hepatomas were palpable at the site of injection in about 14 days, with a tumour mass of 7–10 g wet wt. Tumour cells were removed aseptically, necrotic haemorrhagic areas being avoided, and placed in ice-cold RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY, U.S.A.) containing penicillin (100 i.u./ml) and streptomycin (1000 μg/ml). These cells were then resuspended in fresh medium (1:4 ratio of cells to medium) and either repassaged *in vivo* as described above or made 10% (v/v) with respect to dimethyl sulphoxide and stored at −70°C. Cytosol was prepared as described below from hepatoma tissue after two passages of cells *in vivo*.

Liver from untreated or tumour-bearing rats was homogenized (2 ml/g) in 0.25 M-sucrose/25 mM-Hepes/NaOH buffer, pH 7.4. The homogenate was centrifuged at 1000 g for 20 min, and the supernatant was then centrifuged at 105000 g for 1 h (Davies *et al.*, 1979). Hepatoma cytosol was isolated after disruption of the cells by exposure to 1 s bursts with a Polytron (Brinkman Instruments, Westbury, NY, U.S.A.), with the same differential centrifugation procedures. Protein concentration in these cytosol preparations was in the range 26–37 mg of protein/ml, as determined by a modification of the Lowry procedure (Lowry *et al.*, 1951), with precipitation with trichloroacetic acid (Bensadoun & Weinstein, 1976) and with bovine serum albumin (fraction V; Sigma Chemical Co.) as standard.

Currently GSH *S*-transferase activity in rat liver is considered to be due to at least eight dimeric proteins formed by binary combinations of different subunits (Jakoby *et al.*, 1984). The nomenclature for GSH *S*-transferase isoenzymes used here is

the versatile numerical system devised by Jakoby *et al.* (1984). In this system, for example, a heterodimer composed of subunits 1 and 2 is referred to as glutathione transferase 1-2. For brevity, we refer below to the isoenzymes as form 1-1, form 1-2, form 2-2 and so forth. Transferases 2-2 and 3-3 referred to in Table 1 were isolated from rat liver by using published procedures (Awasthi *et al.*, 1980).

Transferase activity measured as described below was linear over the range 1-100 μg of protein/ml of incubation mixture with 1-chloro-2,4-dinitrobenzene as substrate for all three types of tissue cytosol. Protein concentrations routinely employed were 5-25 μg of protein/ml. Initial rates of transferase activity were recorded with a Gilford 2600 spectrophotometer with an incubation volume of 1 ml at 30°C in 100 mM-potassium phosphate buffer by using published procedures. The conditions for the substrates investigated were: (*a*) 1 mM-1-chloro-2,4-dinitrobenzene, 1 mM-GSH, pH 6.5, monitored at 340 nm (Habig *et al.*, 1974); (*b*) 1 mM-1,2-dichloro-4-nitrobenzene, 5 mM-GSH, pH 7.5, monitored at 345 nm (Habig *et al.*, 1974); (*c*) 0.03 mM-bromosulphophthalein, 5 mM-GSH, pH 7.5, monitored at 330 nm; (*d*) 0.05 mM-*trans*-4-phenylbut-3-en-2-one, 0.25 mM-GSH, pH 6.5, monitored at 290 nm; (*e*) 0.2 mM-ethacrynic acid, 0.25 mM-GSH, pH 6.5, monitored at 270 nm (Habig *et al.*, 1974); (*f*) 0.2 mM-*p*-nitrophenyl acetate, 0.5 mM-GSH, monitored at 400 nm (Keen & Jacoby, 1978); (*g*) 12 mM-cumene hydroperoxide, 10 mM-GSH, pH 7.0, monitored at 366 nm (Wendel, 1981); (*h*) 0.07 mM-androst-5-ene-3,17-dione, 0.05 mM-GSH, 0.1 mM-dithiothreitol, monitored at 248 nm (Benson *et al.*, 1977). In order to measure initial rates with androst-5-ene-3,17-dione as substrate accurately, the transferase assay was carried out at 10°C in the presence of dithiothreitol to minimize oxidation of subunit 1. All reactions were initiated by the addition of the electrophilic substrate, and non-enzymic background rates were always subtracted to determine the initial enzyme-catalysed rate. Substrates of low solubility in water were dissolved in ethanol, and the final ethanol concentration was below 3% in all assays. To investigate the effect of porphyrins on enzymic activity, porphyrins (final concentration 0.1–10 μM) were incubated with the sample for 5 min at the standard incubation temperature before the addition of the electrophile. Stock porphyrin solutions (1 mM in dimethyl sulphoxide, stored at room temperature in the dark) were used within 1 week (*OO'*-diacetylhaematoporphyrin within 2 days) and diluted immediately before use. Dimethyl sulphoxide concentrations were kept below 2% because at higher concentrations enhanced non-enzymic acitivity was observed,

particularly with 1-chloro-2,4-dinitrobenzene as substrate.

Plots of the reciprocal initial velocities of transferase activity versus reciprocal substrate concentrations were linear. These data were then fitted, by using the FORTRAN programs of Cleland (1979), to the rate equation:

$$v = \frac{V[A]}{K_m(1+[I]/K_{is})+[A](1+[I]/K_{ii})}$$

where v is the initial velocity, V is the maximal velocity, $[A]$ is the substrate concentration, $[I]$ is the inhibitor concentration, K_m is the Michaelis constant, and K_{ii} and K_{is} are the inhibitor dissociation constants determined from the intercept and slope replots respectively.

GSH *S*-transferases were also isolated from rat tissue cytosol by using affinity chromatography on GSH-agarose (linked through the amino group to epoxy-activated agarose with 12-atom spacer) similarly to a published method (Mannervik & Jensson, 1982). After elution with GSH (10 mM), the proteins were dialysed and concentrated by freeze-drying before electrophoresis under reducing conditions on denaturing polyacrylamide gels containing sodium dodecyl sulphate (Laemmli, 1970). This procedure resolved three subunits of apparent M_r 28000, 26500 and 25000 after protein staining with Coomassie Blue R-250. Electrophoretic analysis on 3–17% polyacrylamide gels of portions of cytosol samples, before and after affinity chromatography, and of the GSH-eluted material revealed that subunit 2 was not quantitatively bound by this column. This screening procedure was therefore routinely employed to ensure that all the transferases in a sample were accounted for.

Results

We first examined whether purified GSH *S*-transferases are inhibited by porphyrins present in the photodynamic-therapy mixture (Clezy *et al.*, 1980; Bonnett *et al.*, 1981) and also shown to be taken up by tumours in animals (Berenbaum *et al.*, 1982; Bonnett & Berenbaum, 1983). Transferase activity is inhibited by several exogenous porphyrins in a concentration-dependent and non-competitive manner. A typical Lineweaver–Burk plot together with slope and intercept replots are shown in Fig. 1, and the velocities and kinetic constants are summarized in Table 1. This non-competitive inhibition of transferase activity by *OO'*-diacetylhaematoporphyrin occurs with an inhibitor dissociation constant, K_i, of 1 μM with form 1-2 with 1-chloro-2,4-dinitrobenzene as substrate. We also examined whether these porphyrins could interact with other transferase subunits by inhibiting forms

A. Smith, I. Nuiry and Y. C. Awasthi

2-2, 3-3 and 3-4. The K_i values for forms 3-3 and 3-4 with OO'-diacetylhaematoporphyrin are both 1 μM. In general, all porphyrins investigated interact with the transferases with K_i values in the range
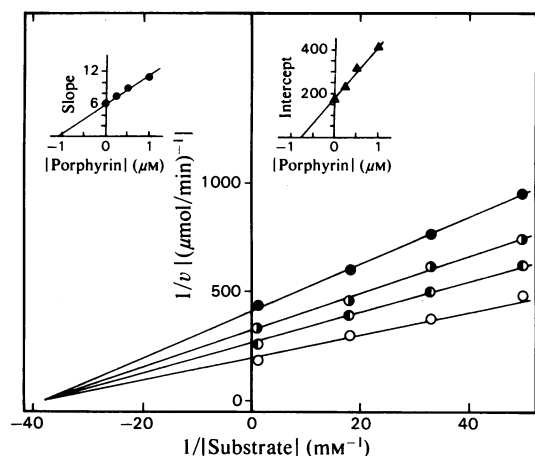

Fig. 1. *Non-competitive inhibition of purified GSH S-transferases by porphyrins*

Assays were carried out at 30°C in 0.1 M-potassium phosphate buffer, pH 6.5, with various 1-chloro-2,4-dinitrobenzene concentrations from 0.2 to 1.0 mM in the presence of a saturating concentration of GSH (10 mM). OO'-Diacetylhaematoporphyrin was added at 0 μM (○), 0.25 μM (◐), 0.5 μM (◑) and 1.0 μM (●) final concentration. Insets show the intercept and slope replots of the data for form 3-3. All data were fitted by computer to the equation (Cleland, 1979):

$$v = \frac{V[A]}{K_m(1+[I]/K_{is})+[A](1+[I]/K_{ii})}$$

which showed non-competitive inhibition with $K_{is} = K_{ii}$. The K_i values and other kinetic data for several porphyrins and purified transferase isoenzymes are summarized in Table 1.

1-2 μM, and K_{ii} equals K_{is} except for form 2-2 (see Table 1).

We next examined whether these porphyrins could still inhibit transferase activity in the presence of other proteins in cytosol. In addition, we investigated two other groups of porphyrins, either naturally occurring or found to localize in animal tumours, but not present in the mixture of haematoporphyrin derivatives used clinically. In normal rat liver cytosol monohydroxyethylmono-vinyldeuteroporphyrin and OO'-diacetylhaemato-porphyrin are more effective inhibitors than haematoporphyrin and protoporphyrin IX (Fig. 2). Uroporphyrin isomer I interacts only weakly with GSH S-transferases, as indicated by the low extents of inhibition (Fig. 2). This weak inhibition of transferases by uroporphyrin I, with eight carboxylate side chains, compared with the strong interaction with porphyrins like haemato-porphyrin and OO'-diacetylhaematoporphyrin, containing two carboxylate groups, suggested that the effects of tetracarboxylic porphyrins such as coproporphyrin be compared. As shown in Fig. 2, inhibition of transferase activity by copro-porphyrin I is greater than that by uroporphyrin I, but significantly less than that by the dicarboxylic acid porphyrins. Coproporphyrin I is slightly more effective than coproporphyrin III, producing 30% inhibition at 1 μM compared with 20% inhibition by coproporphyrin III. Tetra-*meso*-phenylporphin-etetrasulphonate was next examined because it accumulates in tumours in animals and, like coproporphyrin, it contains four negative charges but at different positions. Tetra-*meso*-phenylpor-phinetetrasulphonate produces a 50% inhibition of GSH S-transferase activity at 0.25 μM in rat liver cytosol with 1-chloro-2-dinitrobenzene as substrate.

Since haem and haem analogues are probably metabolic products of these porphyrins (Dailey & Smith, 1984), and since haematin inhibits ligandin, i.e. form 1-1, (Meuwissen *et al.*, 1972), we

Table 1. *Kinetic parameters for the non-competitive inhibition of purified GSH S-transferase homodimers from rat liver*
The data were fitted to the equation (Cleland, 1979):

$$v = \frac{V[A]}{K_m(1+[I]/K_{is})+[A](1+[I]/K_{ii})}$$

Inhibition is clearly non-competitive with $K_{is} = K_{ii}$ (within experimental error), except that K_{is} does not equal K_{ii} for the non-competitive inhibition of form 2-2 by OO'-diacetylhaematoporphyrin. Measurement of transferase activity was determined with various concentrations of 1-chloro-2,4-dinitrobenzene and with saturating GSH concentrations. Purified transferases were identified by their subunit M_r and pI. Abbreviations: HpAc, OO'-diacetylhaematoporphyrin; HVD, monohydroxyethylmonovinyldeuteroporphyrin.

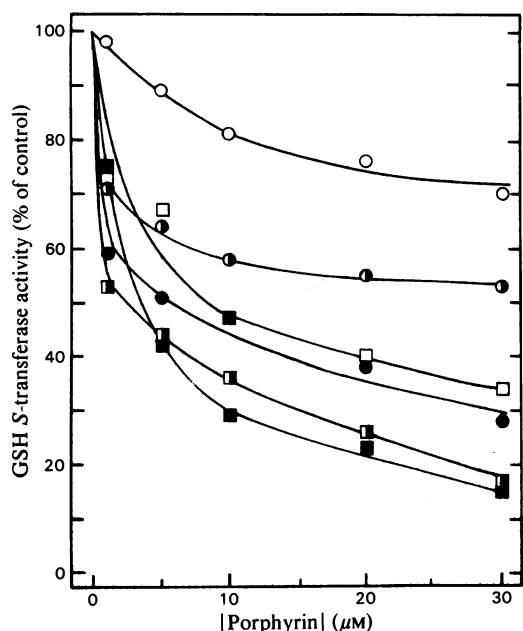| Form of enzyme	Inhibitor	V (μmol/min)	K_m (mM)	K_{is} (μM)	K_{ii} (μM)
2-2	HpAc	0.34 ± 0.005	0.04 ± 0.002	2.08 ± 0.42	0.97 ± 0.05
3-3	HVD	0.62 ± 0.02	0.43 ± 0.05	1.05 ± 0.36	1.44 ± 0.25
	Mesohaem	0.82 ± 0.05	0.06 ± 0.01	0.04 ± 0.02	0.07 ± 0.02

Fig. 2. *Inhibition of GSH S-transferases by porphyrins in cytosol from normal rat liver*

Porphyrins were incubated with cytosol samples (5–25 μg of protein) for 5 min at 30°C before initiation of the reaction by addition of 1-chloro-2,4-dinitrobenzene, a substrate metabolized by all forms of the transferases. Initial rates were measured as described in the Materials and methods section. The porphyrins investigated were monohydroxyethyl-monovinyldeuteroporphyrin (■), OO'-diacetyl-haematoporphyrin (▣), haematoporphyrin (□), protoporphyrin IX (●), coproporphyrin I (◑) and uroporphyrin I (○).

examined the effect of the protohaem analogue mesohaem on these enzymes. In addition, these iron–porphyrin chelates have quite different properties from those of the free porphyrins and are bound more tightly to serum proteins than are the free porphyrins (Morgan *et al.*, 1980). Mesohaem (containing ethyl groups on positions 2 and 4 of the porphyrin) is a potent non-competitive inhibitor of these transferases; for example, in cytosol from normal liver 50% inhibition is produced at 150 nM-mesohaem and for form 3–3 the K_{ii} is 70 nM (Table 1).

We next asked whether the amount and type of transferases are different in normal and cancerous liver (the Morris 7288C hepatoma) with substrates reported to be specific for the various transferase isoenzymes (Mannervik & Jenssen, 1982). Total transferase activity per mg of protein measured with 1-chloro-2,4-dinitrobenzene is only 18% of the normal liver values in the hepatoma (Table 2). In particular, forms 1–1 and 3–3 in the hepatoma show about one-quarter of the activity in normal liver as judged by the initial rates of conjugation of bromosulphophthalein, 1,2-dichloro-4-nitrobenzene and androst-5-ene-3,17-dione (summarized in Table 2). Similarly, the enzyme activities associated with the homodimeric forms 2–2 (with cumene hydroperoxide as substrate) and 4–4 (with *trans*-4-phenylbut-3-en-2-one and *p*-nitrophenyl acetate) are decreased to about 10% of those of normal liver. Both cytosols were examined by affinity chromatography on GSH–Agarose followed by polyacrylamide-gel electrophoresis of the eluted transferases. Consistent with these data, almost no subunit 2 was detected in electrophoretograms of

Table 2. *Comparison of the relative activities of GSH S-transferases in liver from normal and tumour-bearing rats with that of the Morris 7288C hepatoma*

The initial rates of transferase activity (μmol/min per mg of cytosolic protein) were measured with each substrate as described in the Materials and methods section. The results are expressed as means ± S.D., with numbers of determinations in parentheses. Duplicate enzyme determinations are from two independent experiments. The liver of rats bearing the hepatoma is termed 'Experimental liver'.

Form of enzyme*	Substrate	Initial rates of GSH S-transferase (μmol/min per mg of protein)		
		Normal liver	Experimental liver	Hepatoma
All	1-Chloro-2,4-dinitrobenzene	1.13 ± 0.34 (6)	0.96 ± 0.29 (6)	0.29 ± 0.13 (6)
1–1	Androst-5-ene-3,17-dione	0.011 ± 0.001 (3)	0.005 ± 0.001 (3)	0.002 ± 0.0002 (3)
2–2	Cumene hydroperoxide	0.501 ± 0.014 (4)	0.332 ± 0.025 (4)	0.048 ± 0.008 (4)
2–2	Ethacrynic acid	0.047 ± 0.002 (2)	0.07 ± 0.01 (2)	0.042 ± 0.01 (2)
3–3	1,2-Dichloro-4-nitrobenzene	0.24 ± 0.07 (4)	0.18 ± 0.05 (4)	0.056 ± 0.02 (4)
3–3	Bromosulphophthalein	0.066 ± 0.002 (2)	0.048 ± 0.001 (2)	0.018 ± 0.001 (2)
4–4	*trans*-4-Phenylbut-3-en-2-one	0.022 ± 0.009 (5)	0.0204 ± 0.008 (5)	0.0017 ± 0.001 (5)
4–4	*p*-Nitrophenyl acetate	0.148 ± 0.041 (3)	0.094 ± 0.039 (3)	0.018 ± 0.009 (3)

* The form of transferase is listed, in the revised nomenclature system of Jakoby *et al.* (1984), that shows particularly high activity with the substrate indicated, on the basis of published reports (Mannervik & Jenssen, 1982).

the hepatoma cytosol. Unexpectedly, the hepatoma samples show high rates of metabolism of ethacrynic acid, equivalent to those in normal liver.

We extended these observations by measuring the amount and type of transferases in the liver of normal Buffalo rats and of Buffalo rats bearing the Morris hepatoma (Table 2). Although total transferase activity is only slightly lower than in normal liver, differences in the initial rate of transferase activity with several substrates are apparent, showing that extrahepatic tumours affect the liver transferase content. The reaction rates with cumene hydroperoxide, p-nitrophenyl acetate and androst-5-ene-3,17-dione are decreased by about 30%, whereas that with ethacrynic acid is significantly increased, compared with normal animals. Electrophoretic analysis of the subunits isolated by affinity chromatography revealed no discernible

difference in the amount and type of transferase subunits in these livers compared with normal livers. This suggests that the lower activity is due to altered dimer combination, rather than to decreased expression of transferases.

Finally, we determined the extent of porphyrin inhibition of GSH S-transferase isoenzymes in normal and cancerous liver with a variety of substrates. If porphyrin retention by cancerous cells is due to the presence of significant concentrations of porphyrin-binding molecules other than GSH S-transferases, porphyrin inhibition should be decreased in cytosol from the hepatoma compared with cytosol from normal or experimental liver. No such trends are apparent in the results summarized in Table 3. OO'-Diacetylhaematoporphyrin and the monohydroxymonovinyldenteroporphyrin are the most effective inhibitors, and haematoporphyrin and protopor-

Table 3. *Differential effects of porphyrin isomers on GSH S-transferases in liver from normal and tumour-bearing rats and the Morris 7288C hepatoma*

Porphyrins ($10\,\mu$M final concentration) were incubated in cytosol samples for 5 min at the appropriate incubation temperature before initiation of the enzyme reaction by addition of the electrophilic substrate. The extent of porphyrin inhibition varied no more than $\pm 5\%$ in duplicate determinations. Percentage of control refers to the ratio of the enzyme activity measured in the presence of each porphyrin to that in its absence. Initial rates were determined as described in the Materials and methods section after subtraction of non-enzymic rates. Abbreviations: Hp, haematoporphyrin; HpAc, OO'-diacetylhaematoporphyrin; HVD, monohydromethylmonovinyldeuteroporphyrin; PPIX, protoporphyrin IX.

Substrate	Porphyrin	GSH S-transferase activity (% of control)		
		Normal liver	Experimental liver	Hepatoma
Androst-5-ene-3,17-dione	Hp	67	31	53
	HpAc	57	51	78
	HVD	58	61	43
	PPIX	62	90	26
1,2-Dichloro-4-nitrobenzene	Hp	67	67	84
	HpAc	24	20	21
	HVD	16	39	21
	PPIX	34	32	71
Bromosulphophthalein	Hp	91	83	49
	HpAc	56	50	49
	HVD	78	73	59
	PPIX	62	100	74
Cumene hydroperoxide	Hp	107	93	95
	HpAc	112	105	102
	HVD	104	114	102
	PPIX	94	94	121
Ethacrynic acid	Hp	88	95	96
	HpAc	53	71	57
	HVD	43	54	53
	PPIX	53	100	87
trans-4-Phenylbut-3-en-2-one	Hp	70	33	76
	HpAc	67	13	76
	HVD	57	27	100
	PPIX	84	25	100

phyrin IX the least effective ones. The respective extents of inhibition for all four porphyrins are similar in the three tissues examined, but a few exceptions are apparent. The lack of inhibition of androst-5-ene-3,17-dione and ethacrynic acid metabolism by protoporphyrin IX or of bromosulphophthalein metabolism by haematoporphyrin in the experimental liver are not due to low activities of enzyme but may reflect subtle differences in the transferase porphyrin-binding site or in mechanism of action. Interestingly, the metabolism of *trans*-4-phenylbut-3-en-2-one in the hepatoma cytosol and the metabolism of cumene hydroperoxide in all three cytosols are insensitive to inhibition by these porphyrins.

Discussion

The data presented above establish that several porphyrins inhibit GSH *S*-transferase isoenzymes and that there are differences in the type and amount of these isoenzymes in normal and cancerous liver. Four dicarboxylic porphyrins present in the mixture of haematoporphyrin derivatives used clinically inhibit all forms of the purified GSH *S*-transferases examined. In this context it should be noted that, although the active component of the photodynamic-therapy mixture is currently suggested to be a dimeric dihaematoporphyrin ether (Dougherty, 1984), monomeric porphyrins are considered to be the effective intracellular photosensitizers (Dougherty *et al.*, 1984; Kessel, 1984).

There are several possible mechanisms for the inhibition of transferase activity by haem and porphyrins. For example, if the interaction of the two substrates 1-chloro-2,4-dinitrobenzene and GSH with enzyme is ordered, with 1-chloro-2,4-dinitrobenzene bound first, then the inhibitor can bind either to the free enzyme or to an enzyme-1-chloro-2,4-dinitrobenzene complex. If the inhibitor binds to the active site of the free enzyme then competitive inhibition with 1-chloro-2,4-dinitrobenzene should be observed. If the mechanism is random or ordered with GSH bound first, an enzyme–GSH complex will be formed, and, again, if inhibitor bound to the same site as the substrate 1-chloro-2,4-dinitrobenzene, competitive inhibition would be expected. However, the intercept replots demonstrate non-competitive inhibition. Although inhibition at saturating substrate concentration could still be due to inhibitor binding to a central complex (i.e. GSH–enzyme-1-chloro-2,4-dinitrobenzene or enzyme–product adduct), the most likely explanation, since GSH was maintained at saturating concentrations, is that haem and porphyrins are binding to a site different from that for 1-chloro-2,4-dinitrobenzene. Al-

though a hydrophobic site for ligands, including haem and porphyrins, has been shown on subunit 1 (Ketterer *et al.*, 1982), the studies presented here with purified homodimers indicate that sites, presumably hydrophobic, for porphyrin binding exist on subunits 2 and 3 also. Interestingly, differences in the interaction of porphyrins with transferases monitored by changes in enzymic activity depend on the substrate used in the case of form 2–2. This form was inhibited by porphyrins with 1-chloro-2,4-dinitrobenzene as substrate, but the metabolism of cumene hydroperoxide, a relatively specific substrate for this form (Mannervik & Jenssen, 1982), was unaffected by the addition of porphyrins. Of the transferases studied form 2–2 was the only one in which K_{ii} does not equal K_{is}. How these observations reflect on the enzymic mechanism of form 2–2 remains to be determined.

Since GSH *S*-transferases are also inhibited in cytosol, these enzymes appear to be potential cytosolic binding sites for naturally occurring porphyrins and porphyrins used in cancer photodynamic therapy. Other porphyrins that are taken up by tumours in animals are also effective inhibitors of GSH *S*-transferase, e.g. uroporphyrin I, taken up by the KHJJ mouse mammary carcinoma (El-Far & Pimstone, 1983), and tetra-*meso*-phenylporphinetetrasulphonate, taken up by the Walker 256 carcinoma (Winkelman *et al.*, 1967), murine-sarcoma-virus-induced tumours (Carrano *et al.*, 1977) and by HEp-2 cells (Carrano *et al.*, 1978). It seems likely that these transferases also provide binding sites for uroporphyrin I and tetra-*meso*-phenylporphinetetrasulphonate.

Porphyrin structure is clearly a factor in determining the affinity of binding, as shown by the order of inhibitory effectiveness: protoporphyrin IX (dicarboxylic) > coproporphyrin (tetracarboxylic) > uroporphyrin (octacarboxylic) and type I > type III isomers of both uroporphyrin and coproporphyrin. Of particular interest is the greater effective inhibition by tetra-*meso*-phenylporphinetetrasulphonate compared with coproporphyrin. Tetra-*meso*-phenylporphinetetrasulphonate contains four negatively charged phenylsulphonic acid groups in the *meso* positions of the porphyrin macrocycle. This suggests that the porphyrin-binding site on the transferases contains positive charges in a particular orientation that facilitate binding. Furthermore, since several porphyrins are substrates for mitochondrial ferrochelatase (Dailey & Smith, 1984; Romslo & Husby, 1980), they are likely to be metabolized to haem analogues *in vivo*, which are even more effective inhibitors of the GSH *S*-transferases than are the metal-free porphyrins. If incorporated, for example, into apo-(tryptophan pyrrolase) and apo-

(cytochrome *P*-450), inactive haem-analogue proteins may be formed.

One postulated mechanism for the retention of exogenous porphyrins by tumours is, that, though both normal and cancerous tissues contain porphyrin-binding molecules, cancerous cells have a decreased ability to metabolize these porphyrins to haem analogues that are then catabolized (Dailey & Smith, 1984). In this regard the mode of cellular accumulation of uroporphyrin I (El-Far & Pimstone, 1983) may be different from that of exogenous dicarboxylic porphyrins. Unlike those porphyrins, uroporphyrin I binds only weakly to serum proteins (Morgan *et al.*, 1980; Smith & Neuschatz, 1983) and to cytosolic GSH *S*-transferases (the present work), and is not metabolized to haem (Kappas *et al.*, 1983).

Cellular protein binding of porphyrins, as described here for GSH *S*-transferases, may also play a role in determining the intracellular sites of photodamage after treatment with the photodynamic-therapy mixture of haematoporphyrin derivatives and in certain porphyrias. Although the cytosol from the Morris 7288C hepatoma contains lower amounts of the transferases, several isoenzymes that interact with porphyrins are present. Although the present studies concern the soluble transferases, these enzymes have also been located in the endoplasmic reticulum (Morganstern *et al.*, 1982), mitochondria (Kraus, 1980) and other organelles (Friedberg *et al.*, 1979). Porphyrins also have the potential to reside in the lipid phase of membranes, although the distribution between cytosolic protein and membrane *in vivo* is not known. This variety of binding sites could readily account for the extreme sensitivity observed in certain normal tissues, particularly liver (a major site of porphyrin metabolism), which currently hamper treatment of certain tumours, including hepatic metastases. Moreover, differences in porphyrin-binding and -metabolizing molecules in various types of tumours could explain in part the varied response to exogenously administered porphyrins observed in patients.

An interesting result in the present work is the apparent altered expression of these enzymes in the liver caused by an extrahepatic tumour. The presence of tumours in animals also alters the activity of haem-synthesizing enzymes (Woods, 1978) and causes induction of haem oxygenase (Schacter & Kurz, 1984). If similar changes occur in the liver of patients with tumours, alterations in the type and amount of these isoenzymes could affect not only the metabolism of drugs but also that of exogenous porphyrins.

Finally, since GSH *S*-transferases are important detoxifying enzymes and since porphyrin and haem binding inhibits the enzymic activity of the transferases, metabolic consequences to the cells of the administration of exogenous porphyrins are likely.

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