Jpn. J. Cancer Res. (Gann)
79, 556–572 ; May, 1988

REVIEW

GLUTATHIONE *S*-TRANSFERASES AND HEPATOCARCINOGENESIS

Kiyomi SATO
Second Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036

INTRODUCTION

Rat chemical hepatocarcinogenesis has been most extensively studied and sequential analysis of the processes involved has played an important role in generating the hypothesis that chemical carcinogenesis comprises at least two or three stages; initiation and promotion, together with further progression stages.[1-8] The initiation stage is generally understood to be an irreversible process(es), during which alterations of specific genes and in particular the activation of cellular proto-oncogenes occurs.[8-11] During the promotion stage, groups of preneoplastic cells have been observed in many organs prior to appearance of malignant cancers,[1,6] and in rat chemical hepatocarcinogenesis enzyme-altered foci and hyperplastic nodules (hepatocyte nodules) or neoplastic nodules have excited much interest as putative precursor populations.[1-8] They express specific enzymatic phenotypes; i.e. they are characterized by a number of alterations in enzyme expression which have been used as markers.[1-8,12-18] Drug-metabolizing enzymes, including the glutathione *S*-transferase (GST) family, which is one of the most important detoxifying systems in the liver having multifunctions performed by multimolecular forms,[18-21] are commonly altered. In particular, a novel neutral GST form purified from rat placenta by our group and named the placental form (GST-P)[22] has been identified as a new reliable marker enzyme for preneoplastic lesions in rat hepatocarcinogenesis.[22-25] In the present paper, the properties of GST-P (newly named GST 7-7[26,27]) as a marker for rat hepatic preneoplastic lesions, as well as hepatocellular carcinomas (hepatomas), will be reviewed mainly on the basis of our own and joint work with other groups. GST forms in other species which are related to rat GST-P will be also briefly reviewed.

Molecular Forms and Functions of Rat GSTs

GSTs are a family of multifunctional dimeric proteins and multimolecular forms are known to exist in the various organs of different species.[18-21] Rat GST forms have been most extensively studied and most of them are localized in the cytosol. However, one microsomal GST form has been purified from rat liver microsomal fractions,[28,29] and an unidentified GST form(s) involved in leukotriene C_4 synthesis was also reported to be present in particulate fractions from rat basophilic leukemia cells.[30-32] So far, at least 13 molecular forms have been identified in the cytosol from rat tissues, mainly in liver cytosol,[33] but new forms continue being discovered. The basic forms including subunits 1(Ya), 2(Yc), 3(Yb_1), and 4(Yb_2) (Table I) have been well studied, but more recently attention has concentrated on the neutral and acidic forms (Table I). Rat GST forms have been variously named in different laboratories,[21] and, although they were originally

Table I. Molecular Forms of Rat Glutathione *S*-Transferase

Names			pI as dimer		Subunit Mr	Subunit pI	Class
a)	*b*)	*b*)	*c*)	*d*)	*e*) *f*)	*g*)	*h*)
1-1	ligandin	YaYa	10	(9.8)	25,000(25,555 or 25,567)	7.7	*alpha*
1-2	B	YaYc	9.9	(9.7)			
2-2	AA	YcYc	9.8	(9.6)	28,000(25,322)	7.6	*alpha*
3-3	A	Yb_1Yb_1	8.9	(8.8)	26,500(25,915)	7.4	*mu*
3-4	C	Yb_1Yb_2	8.0	(8.4)			
4-4	D(X)	Yb_2Yb_2	6.9	(8.2)	26,500(25,705)	6.8	*mu*
5-5	E		7.3		26,500		
6-6		(YnYn)	5.8	(5.7)	26,000	6.6,6.0**	*mu*
7-7	P*	YpYp*(YfYf)	7.0	(8.3)	24,000(23,307)	6.8	*pi*
8-8		(YkYk)	6.1	(5.8)	24,500	6.3	*alpha*
3-6		(Yb_1Yn)	?	(8.3)			*mu*
4-6		(Yb_2Yn)	?	(6.2)			*mu*

a) Jakoby *et al.*[35] *b*) Mannervik[21] and Hayes & Mantle[33] for names in parentheses.
c) By isoelectric focusing. *d*) By chromatofocusing. *e*) Mannervik[21] and our results.
f) From cDNA (Tu *et al.*[98] and Sugioka *et al.*[88]).
g) By two-dimensional electrophoresis (our results). *h*) Mannervik *et al.*[36]
* Named by us.[22,24] ** Yn can be divided into Yn_1 and Yn_2 with different pIs.[48]

defined by their elution order on ion exchange chromatography,[19,20] they are now principally classified on the basis of isoelectric points (pIs) determined by either isoelectric focusing or chromatofocusing (see Fig. 2) and their subunit molecular weights (Mrs) on SDS-polyacrylamide gel electrophoresis, as first suggested by Bass *et al.*[34] (Table I). A new nomenclature was proposed by Jakoby *et al.*[35] in which GST subunits receive numbers referring to the order in which they were isolated and characterized (Table I), but this has not gained general approval. In another species-independent classification proposed by Mannervik *et al.*,[36] GST subunits in the rat, human and mouse were grouped into three classes with respect to amino-terminal amino acid sequence, and enzymatic and immunological properties.

GST species catalyze the conjugation of electrophilic compounds with reduced glutathione (GSH). Many compounds with electrophilic centers are produced from xenobiotics by biotransformation[20] and also may arise from endogenous substances.[21] The GSH conjugation reaction is the first step of the mercapturic acid pathway,[20,21] which is one of the most important detoxication reactions. The second step of this pathway is catalyzed by γ-glutamyl transpeptidase (γ-GTP), which has gained considerable attention as a useful marker for enzyme-altered foci in rat liver.[3,14,17,18] In addition, certain forms such as rat GST 1-2, 2-2 and 7-7 (GST-P) are known to possess Se-independent GSH peroxidase activity towards lipid peroxides.[23,26] Furthermore, since the finding that ligandin, identified as a protein capable of binding steroids, bilirubin, carcinogens and a number of exogenous organic anions,[37] is a basic form of GST[38] (GST 1-1 or together with 1-2 (Table I)), this and other forms have been shown to act as binding or carrier proteins for several dyes (bilirubin, bromosulfophthalein and indocyanine green), cholic acids, steroid hormones, hematin (heme) and carcinogens.[19,20,39] Thus, GSTs including ligandin have attracted the attention of many investigators studying chemical carcinogenesis (see reviews[19,20,39-42]).

Purification and Protein Properties of Rat GST-P (GST 7-7)

During investigation of changes in GST isoenzymes in rat hepatocarcinogenesis, we first noted that GST-A (3-3) was increased

in hyperplastic nodule (HN)-bearing rat liver.[12, 43] Subsequently, we purified a new neutral form from rat placenta and named it the placental form (GST-P),[22] since this was almost only the form present in this tissue. Then, by using anti-GST-P antibody prepared in a rabbit, we noted that GST-P was markedly increased in HN-bearing rat liver,[23] and using this source we have prepared large quantities of rabbit antibody,[24] which has been used extensively by our and other groups.[44] Later, it was demonstrated using our anti-GST-P antibody by Mannervik and his co-workers[45, 46] that this form is immunologically identical with the new GST form (GST 7-7) purified from rat kidney and lung by them. GST-P (YpYp) seems to be related to GST-λ (YfYf) purified from human lung by Hayes and Mantle[33] but differs in subunit composition from the P form (YblYn, GST 3-6) named by them.[33] GST-P (7-7) is characterized by the smallest Mr and a neutral pI[22-24] (Table I). Immunologically, it is not crossreactive with other forms but does crossreact among many species: rat, mouse, hamster, dog, horse and human.[24, 47] By single radial immunodiffusion using the anti-GST-P antibody and later by immunohistochemical methods, it was found that this form is present at a very low level in normal rat liver (negative in parenchymal cells but weakly positive in bile ductular cells), and even in the placenta (trophoblast), but it is present in significant quantities in adult rat kidney (proximal and distal tubules) lung (bronchiolar epithelial cells), pancreas (ductular cells) and spleen,[24, 44] and also in brain (astroglia).[48] Immunohistochemically, GST-P is also detectable in small intestine (columnar epithelial cells), skin (epithelial cells) and ovarium (primary follicles). Thus, the GST-P (7-7) form seems to be almost universally albeit weakly expressed in various rat tissues. It is now grouped into the Class *pi* of the species-independent classification of GST[36] (Table I).

GST-P as a Rat Hepatic Preneoplastic Marker

Enzyme-altered foci and hyperplastic nodules are easily inducible in rat liver by chemical carcinogens using different protocols[1, 6, 8, 14] such as the Solt and Farber (Solt-Farber) model[49]; i.e. ip injection of diethylnitrosamine (DEN) (200 mg/kg of body weight) followed by administration of N-2-acetylaminofluorene (AAF) (0.02% in diet) for 2 weeks and partial hepatectomy (see Fig. 1).

In 1983, we observed by double immunodiffusion using the antibody to GST-P purified from the placenta that GST-P is markedly increased in HN-bearing rat liver.[50] This was confirmed using two-dimensional electrophoresis.[23, 44, 50, 51] In addition to basic

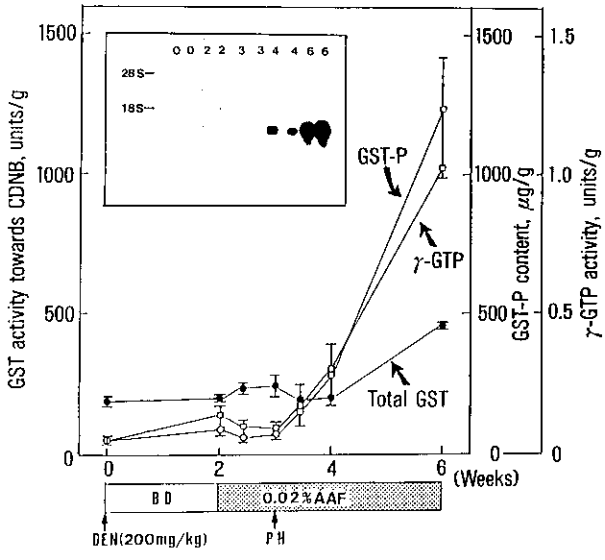

Fig. 1. Induction of GST-P and γ-GTP in early stages of rat chemical hepatocarcinogenesis using a Solt-Farber model modified by feeding AAF from week 2 for 4 weeks. CDNB, 1-chloro-2,4-dinitrobenzene; DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; BD, basal diet; PH, partial hepatectomy. GST-P content, and GST and γ-GTP activities were determined using 105,000g supernatants, as previously reported.[13, 24, 122] Northern blot analysis using GST-P cDNA probe (pGP5)[88] (generous gift of Prof. Muramatsu) is shown in the inserted figure, in which numbers at the top indicate number of weeks after DEN injection in the above model.

GST subunits such as Ya, Yc, Yb(Yb_1) and Yb′(Yb_2) (subunits, 1, 2, 3 and 4, respectively) present in normal rat liver cytosol, two polypeptides, 26000/6.8 (Mr/pI) (major) and 6.3 (minor), were detected, that were not detectable in normal liver.[23,44,51] These polypeptides were identified as charge isomers of the GST-P subunit by immuno-affinity column chromatography using the anti-GST-P antibody[23] and later by immunoblotting (Western blotting) using the antibody.[24,44] Sugioka *et al.*[52] (1982) had earlier detected increased polypeptides of 26/6.9 and 26/6.6 (kilodalton/pI) in hyperplastic nodules and hepatomas induced by AAF by using two-dimensional gel electrophoresis and were able to confirm these to be charge isomers of GST-P by using our GST-P antibody.[53] Roomi *et al.*[54] and Eriksson *et al.*[55] (1983) (both in the group of E. Farber) also reported the appearance of a polypeptide with Mr 21,000 (named p21) commonly detectable by SDS-polyacrylamide slab gel electrophoresis in isolated hyperplastic nodules induced by six different protocols including the Solt-Farber model,[49] and this polypeptide was found to be identical with the GST-P subunit by using our antibody.[56] Later p21 was described as p26; the subunit of a polypeptide of Mr 52,000, which was again confirmed to be GST-P.[57] A new GST form, GST 7-7, was reported to be increased in primary hepatomas induced by N,N-dimethyl-4-aminoazobenzene by Meyer *et al.*[26] and in hyperplastic nodules induced by AAF by Jensson *et al.*[27]

By single radial immunodiffusion using the anti-GST-P antibody it was established that the amounts of GST-P in HN-bearing livers and in primary hepatomas induced by different carcinogens and also a transplantable hepatoma (Morris 5123D) were between 10 and 100 times higher than in normal liver but negligible in transplantable AH 130.[24] It was also found to be very low in fetal and regenerating livers,[24] indicating that GST-P expression in hepatocarcinogenesis is not oncofetal and not simply a reflection of cell growth.

The amount of GST-P protein together with that of the mRNA increased in parallel with increasing γ-GTP activity during the early stage of hepatocarcinogenesis with the Solt-Farber model (Fig. 1), with a remarkable sex-dependence in favor of males (Table II), similar to that found for foci development. However, as is evident in Table II, the amounts of basic GST subunits 1 plus 2 and 3 plus 4 are also enhanced in HN-bearing livers and to a much higher extent than for GST-P (7-7), even when the latter was maximally increased (at 4 weeks after administration of AAF in Fig. 1). Indeed, GST-P was found to be responsible for only a small proportion of

Table II. Changes in GST Subunit Content and GST Activity during Early Stages of Rat Hepatocarcinogenesis[a]

Treatment	Subunit 1 plus 2	Subunit 3 plus 4	Subunit 7	Total activity towards CDNB
	mg/g of liver			units/g of liver
Nontreated	3.8±0.2[b]	3.4±0.3	0.04±0.01	151±7
	(5.7±0.1	2.1±0.2	0.06±0.01	141±6)[c]
1 week[d]	3.9±0.2	4.0±0.5	0.22±0.01	157±9
	(5.9±0.4	2.3±0.1	0.18±0.02	179±23)[c]
3 weeks[d]	7.8±0.2	6.0±0.2	1.12±0.14	360±13
	(7.6±0.1	4.5±0.1	0.36±0.07	321±11)[c]
Lead nitrate[e]	6.3±1.3	4.0±0.3	0.29±0.11	303±41

a) N. Tateoka, *et al.* unpublished data. *b*) Mean±SD.
c) Female rats. *d*) After hepatectomy in Fig. 1.
e) Three days after ip injection of 100 μmol/kg.
Subunit amounts were determined by using antibodies to GST 1–2, 3–4 and GST-P (7–7), respectively, and the total activity towards CDNB (1-chloro-2,4-dinitrobenzene), as described previously.[24]

total activity (see Fig. 2). Thus, it should be borne in mind that basic GST forms may play important roles in detoxication and other functions in foci and nodules.

By using the peroxidase-antiperoxidase (PAP)[58] or the avidin-biotin-peroxidase complex (ABC)[59] immunohistochemical methods with anti-GST-P antibody, enzyme protein was demonstrated to be localized in almost all enzyme-altered foci detectable by γ-GTP activity staining.[22] In contrast, an appreciable number of GST-P-positive foci did not show increased γ-GTP activity,[25] suggesting it may be the more accurate marker. This was furher indicated by the fact that lesions induced by a large number of protocols using different hepatocarcinogens and promoters,[25, 60] and hepatomas induced by AAF,

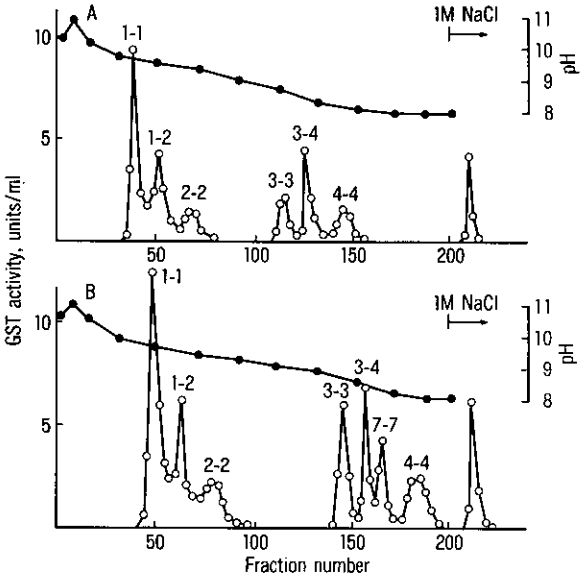

Fig. 2. Representative GST isoenzyme patterns in normal and hyperplastic nodule (HN)-bearing male rat livers separated by chromatofocusing (pH 10–7.0). In Fig. A, normal liver cytosol (105,000g supernatant) and in Fig. B, HN-bearing liver cytosol obtained from rats fed AAF for 4 weeks, as shown in Fig. 1, were used. Chromatofocusing was carried out as described previously.[106] The names of the GST forms are shown in Table I.

Table III. Induction of GST-P-positive Foci[71, 73]

Hepatocarcinogen and promoter	Single cells/mini-foci	Large foci/hepatoma
I) Carcinogen		
1) Genotoxic (mutagenic)[a]		
DEN, DMN, AfB_1	(+) (rapidly, within 7 days)	(+)
AAF, 3′-Me-DAB	(+) (slowly, 4–8 weeks)	(+)
2) Nongenotoxic		
Clofibrate (other peroxisome-proliferating agents)	(−)	(−)
Methapyrilene-HCl, orotic acid	(−)	?
Di(2-ethylhexyl)phthalate[b]	(−)	(−)
Ethynyl estradiol	(−)	?
Ethionine	?	(+)
II) Promoters	(−)	(−)
Phenobarbital, methylcholanthrene polychlorinated biphenyls, isosafrole		

a) By Ames test.
b) Recently, it was reported that di(2-ethylhexyl)phthalate, a peroxisome proliferator, lacks both initiating and promoting activities in rat liver.[74, 75]

3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), aflatoxin B_1 (AfB_1) and ethionine were all strongly stained using the anti-GST-P antibody.

Recently, however, several groups have reported that a new type of carcinogen (nongenotoxic) including the peroxisome-proliferating hypolipidemic agents, e.g. clofibrate,[61] is associated with GST-P- and/or γ-GTP-negative foci and hepatomas[62–68] and these results have been confirmed in our laboratory (Table III). However, ethionine, which is known to be nongenotoxic, does induce GST-P-positive foci and hepatomas.[69] The "initiated cells" induced by DEN (a mutagenic carcinogen) develop to form GST-P-positive foci by administration of AAF, as observed in the Solt-Farber model, but lead to GST-P-negative lesions after treatment with clofibrate, suggesting that expression of the enzyme may be controlled in the promotion in addition to initiation stages.

GST-P-positive Minifoci in an Early Stage of Hepatocarcinogenesis

Immunohistochemical staining has revealed GST-P-positive minifoci (composed of 2–5 cells) (Fig. 3B) or even single cells (Fig. 3A) in resected liver obtained at partial hepatectomy in the Solt-Farber model,[70] before the increase in GST-P content and parallel increase in γ-GTP activity become apparent, as is evident in Fig. 1. Furthermore, these cells can be detected at a very early stage, being observed 48 hr after single doses of the hepatocarcinogens DEN, dimethylnitrosamine (DMN), AfB_1, and methylazoxymethanol acetate.[71] The number of single cells correlates with increasing dose of initiator (e.g., DEN), but the cells are not induced by promoters of liver carcinogenesis (inducers of mixed-function oxidase) such as phenobarbital, methylcholanthrene, polychlorinated biphenyls and isosafrole.[71] Therefore, these GST-P-positive cells deserve consideration as "initiated cells" pointing to GST-P as a very early marker. GST-P-positive single cells, and more especially minifoci were also detectable for long periods (at least 6 months) after a single injection of DEN (200 mg/kg), whereas they were not visible using other marker enzymes such as γ-GTP. Furthermore, after such long periods, large foci (Fig. 3C) were inducible in the livers of the rats by feeding AAF (0.02%) for 2 or 3 weeks followed by partial hepatectomy (selection pressure), as performed in the Solt-Farber model. A significant direct correlation between number of single cells/

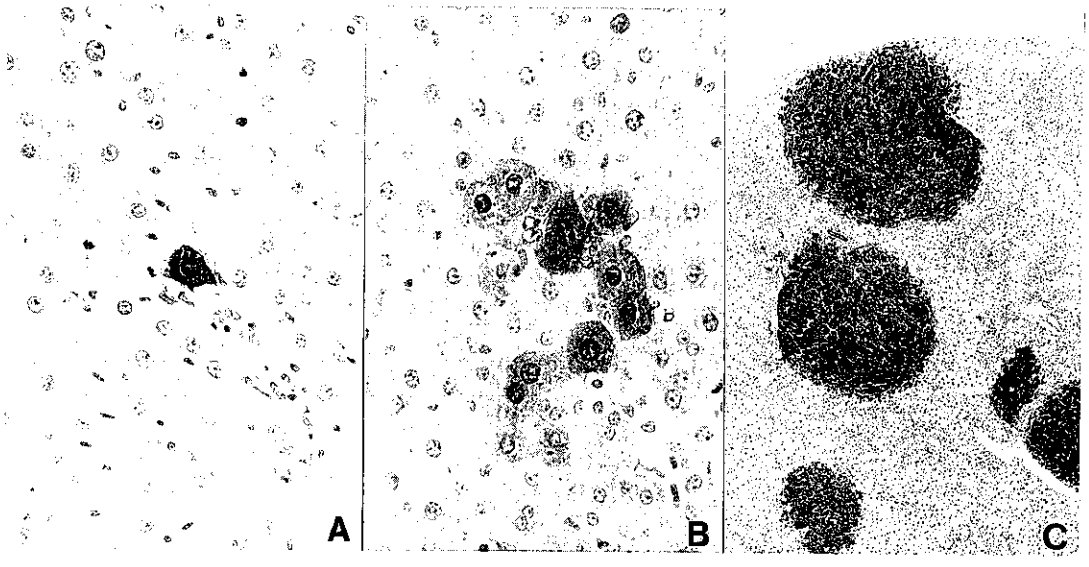

Fig. 3. GST-P-positive single cell and minifocus in resected liver and large focus in the remaining liver of the same rat subjected to "selection pressure" 6 months after a single injection of DEN (200 mg/kg). Single cell (A) and minifocus (B), ×400. Large foci (C), ×40. For "selection pressure," see text.

minifoci in the resected livers and number of large foci in the remaining livers of the same rats was observed (K. Satoh and I. Hatayama, unpublished data). Takahashi *et al.*[72] also observed small GST-P-positive foci 37 weeks after single treatment with even 10 mg/kg of DEN.

The fact that single doses (ip) of DEN, DMN and AfB_1 induced GST-P-positive single cells/minifoci very rapidly within 1 or 2 weeks, whereas AAF and 3′-Me-DAB in the diet bring about their development more slowly after 4 to 8 weeks (Table III), taken together with the finding that nongenotoxic hepatocarcinogens do not induce either GST-P-positive single cells/minifoci or GST-P-positive large foci[73] (Table III), strongly suggests that the GST-P-positive single cells represent precursor ("initiated") lesions of GST-P-positive large foci. Therefore, detection of GST-P-positive minifoci would appear to be of importance for characterization and screening of hepatocarcinogens, especially those incomplete examples such as benzo[*a*]pyrene and dimethylbenz[*a*]anthracene, which alone can not induce large enzyme-altered foci.

Inducibility of GST-P by Short-term Administration of Drugs

In direct contrast to a large number of other drug-metabolizing enzymes, GST-P was found not to be inducible by administration of a large number of carcinogens, tumor promoters or modulators (AAF, 3′-Me-DAB, ethionine, choline-methionine-deficient diet, clofibrate, phenobarbital, 3-methylcholanthrene, α-hexachlorocyclohexane, cyproterone acetate, carbon tetrachloride, and polychlorinated biphenyls) without appearance of enzyme-altered foci and hyperplastic nodules.[24,47] Although GST-P was found to be slightly induced by two antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene, in periportal areas, this was not to such an extent as to disturb detection of focal elevations.[25] Among the other drugs examined, lead nitrate, a hepatic mitogen, proved an exception in inducing significant amounts of GST-P in rat livers, but in this case the total biochemical pattern concerning drug-metabolizing enzymes was very similar to that exhibited in hepatic nodules.[76] Expression of GST-P was confirmed immunohistochemically in the whole liver but more strongly in the zone III and hepatic levels of GST-P 3 days after 100 μmol/kg (ip) of lead nitrate were almost the same as those in HN-bearing livers induced by the Solt-Farber model (see Table III), thereafter proving reversible and returning to normal levels after 2 weeks. Thus, it has been suggested that lead nitrate will be useful for future investigation of the mechanism of GST-P gene expression in chemical hepatocarcinogenesis.[76]

Enzymatic Properties of GST-P and Possible Functions in the Foci

The actual roles and functions of GST-P in (pre)neoplastic cells remain to be clarified. However, it has been reported that putative preneoplastic foci are resistant to cytotoxic agents including hepatocarcinogens, thus being termed "resistant cells,"[1,2,5,6] and basic GSTs in the liver are well known to have large capacities for binding of endogenous substances such as bilirubin, cholic acids and hematin as well as exogenous compounds.[19–21] As shown in Table IV, activities of GSTs 1-1, 1-2 and 2-2 were markedly inhibited by bilirubin and hematin, while those of GSTs 3-3, 3-4 and 4-4 were more sensitive to (glyco)-cholate. GST-P was, in contrast, almost insensitive to inhibition by these endogenous compounds and, therefore, increased activity of GST-P in foci might be accounted for by a requirement to replace, in part, the activity of basic GSTs for efficient detoxification of cytotoxic agents.

Robertson *et al.*[46] reported that the GST 7-7 purified from rat lung has the highest activity towards (+)-7β,8-diol-9,10-oxide of benzo[*a*]pyrene; it shows stereoselectivity for the (+) enantiomer. Hiratsuka *et al.*,[77] however, found that GST-P purified by our group from HN-bearing rat liver stereoselectively conjugates GSH at the benzylic carbon (C_7) of the (−) enantiomer (7S, 8R) of 9,10-dihydrobenzo[*a*]pyrene-7,8-oxide, while other basic forms preferred the (+) enantiomer. These results suggest that GST molecular forms may have different stereoselectivities towards the same substances, and GST-P may have unique properties.

Ketterer and co-workers[26] reported that the GST 7-7 purified from hepatoma has an Se-independent GSH-peroxidase activity to-

Table IV. Inhibitor Sensitivities (IC_{50}) of Seven Molecular Forms of Rat Hepatic Cytosolic GST[a]

Molecular form	Bilirubin (μM)	Hematin (μM)	Cholate (mM)	Glycocholate (mM)	Bile (%)	2-OH-AAF (mM)	Diepoxy-stearic acid(mM)	Sulfa-salazine[b] (μM)
1-1	2.4	0.02	0.8	1.5	0.7	0.5	0.45	0.42
1-2	5.0	0.6	1.6	4.1		0.54		
2-2	5.8	3.1	2.4	5		0.39	0.71	>25
3-3	28	1.1	0.13	0.5		0.34	0.09	2.0
3-4	36	1.4	0.15	0.55	0.9	0.24		
4-4	15	2.3	0.25	0.6		0.27	0.03	2.7
7-7[c]	>50	8.5	2.0	>5	6	>0.5	0.80	>25

IC_{50}, concentration of a test compound required to give 50% inhibition of GST activity assayed with 1mM GSH and 1mM 1-chloro-2,4-dinitrobenzene.
a) Satoh *et al.*, unpublished data.
b) Tsuchida *et al.*[48]
c) Purified from HN-bearing rat liver as previously reported.[24]

wards lipid peroxides, especially towards arachidonate and linoleate hydroperoxides. We also found that GST-P possesses significant activity towards 4-hydroxynonenal, which is one of the most potent lipid peroxides derived from arachidonate,[78] though GST 4-4 has the highest activity (our unpublished data). Thus, GST-P (7-7) may be related to the prevention of cytotoxic lipid peroxidation, which has been considered to play an important role(s) during tumor promotion, and may be of essential significance to the drug-resistant character of preneoplastic foci. The oxidized glutathione (GSSG) produced in the GSH peroxidase reaction can be supplied by GSSG reductase reaction, in which NADPH is necessary. The fact that GSSG reductase[79] and NADPH - generating G6P dehydrogenase,[72, 80-82] a key enzyme of the pentose phosphate pathway, are also increased in enzyme-altered foci is interesting in this respect. GST-P also has appreciable LTC_4 synthase activity,[48] but the significance of this finding remains to be clarified.

It is noteworthy that GST-P is detectable immunohistochemically in nuclei of preneoplastic lesions, especially in early stages of chemical carcinogenesis in rat liver[71] and hamster pancreas.[83] Similarly, human GST-π was detected in nuclei of human cervix dysplastic cells,[84] and mouse GST II, which is immunologically related to rat GST-P and is present abundantly in normal male mouse liver,[85] was also observed within hepatocyte nuclei. This phenomenon is not associated with antibodies to other GST forms,[84] and, therefore, appears to be specific. Thus, it can be speculated that GST-P (human GST-π and mouse GST II) in the nuclei either acts to detoxify genotoxic substances from the cytosol or functions as a carrier protein of some exogenous and endogenous substances such as carcinogens and hormones. With respect to this point, it is noteworthy that GST 7-7 has a high activity towards thymine hydroperoxide.[86]

Structure of GST-P and Regulation of GST-P Expression

The mechanism of GST-P gene expression during chemical hepatocarcinogenesis has been extensively investigated by Muramatsu and co-workers[87] in Japan. Sugioka *et al.*[88] cloned a cDNA (pGP5) of rat GST-P from a cDNA library prepared from poly(A)$^+$ RNA 2-acetylaminofluorene (AAF)-induced rat hepatocellular carcinoma by screening with synthetic DNA probes designed on the basis of a partial amino acid sequence (25 amino acids) of a GST-P subunit and determined the complete amino acid sequence. The subunit had 209 amino acids and the calculated Mr was 23,307 (Table I). They also showed by Northern blot and dot blot analysis using their cDNA probe (pGP5) that GST-P mRNA (about 750 nucleotides) is present abundantly

in hyperplastic nodules and Morris 5123D, 7316A and 7794A as well as chemically induced hepatocellular carcinomas, whereas it is almost absent in normal liver, fetal liver and regenerating liver and in the undifferentiated hepatoma AH 130. Lower amounts of mRNA were detected in lung, testis, kidney spleen and placenta generally in this order, indicating that the placenta is in fact not a representative tissue in which this gene is normally strongly expressed. The authors concluded from these data that the amount of GST-P in a tissue is primarily, if not completely, regulated at the transcriptional level.[87, 88] Knoll *et al.*[89] also cloned cDNA of GST-P from a cDNA library constructed from the mRNA species of a primary hepatoma induced by DEN and found that the nucleotide sequence of this cDNA was identical to that reported by Sugioka *et al.*[88] Pemble *et al.*[90, 91] and Taylor *et al.*[92] (both in the Ketterer group) also cloned a cDNA (pGSTr7) of GST 7-7 by using poly(A)$^+$ RNA isolated from N,N-dimethyl-4-aminoazobenzene-induced rat hepatoma and found that the amino acid sequence of GST 7-7 was identical with that reported by Sugioka *et al.*[88] Okuda *et al.*[93] in Muramatsu's group have isolated GST-P gene from a λ phage rat genomic library using a GST-P cDNA clone (pGP5) as a probe. The rat GST-P gene was found to be about 3 kilobase pairs long and to contain 7 exons and 6 introns, encoding the same GST-P protein specified by pGP5. The cap site was mapped 70 nucleotides upstream from the translation initiation site and the promoter "TATA" box was found 27 base pairs upstream from the putative cap site. The 200 base pairs upstream from the cap site were rich in G+C residues (61%), and the hexanucleotide sequence 5′-GGGCGG-3′ was found at position −47 to −42. Muramatsu and co-workers[87, 94] also analyzed the cis-acting regulatory DNA elements of the rat GST-P gene by the chloramphenicol acetyltransferase (CAT) activity assay method. Various regions of the 5′-flanking sequence were fused with a bacterial CAT gene and the transcriptional activity of each construct was determined by transient expression assay after introduction into a hepatoma cell line (dRLh 84). Two enhancing elements were found at 2.5 kilobase pairs and 61 base pairs upstream from the transcription start site.[94] The upstream enhancer, that is more potent, was divided into two domains; GPE I and GPE II. Both GPE I and the downstream enhancer sequence contained a 12-O-tetradecanoylphorbol-13-acetate (TPA) response element. GPE II contained two of the SV40 and one of the polyoma enhancer core-like sequence. A silencing element was found at 400 base pairs upstream from the cap site. TPA enhanced the expression of transfected GST-P gene in HeLa cells as well as in hepatoma cells. From these and other observations, they speculated that one of the causes for specific expression of GST-P gene during hepatocarcinogenesis is an activation of the protein kinase C-mediated signal cascade in an early stage of this process.[94] Furthermore, they are examining the factors transacting on the enhancer regions by gel-mobility shift analysis. The GST-P gene is located on rat chromosome 1 at band q43.[95] cDNAs and genomic DNAs of other rat GST subunits have been cloned by other groups. Pickett and his co-workers[96–98] have been investigating the expression of genes encoding GST subunits Ya (1), Yc (2) and Yb (3 and 4) in normal and preneoplastic liver and Tu and his co-workers[99, 100] have cloned cDNAs of Ya, Ha, Yb (basic) and Yb (anionic) and calculated the respective Mrs (Table I). Muramatsu and co-workers[87, 88] found that the GST-P subunit (Yp) and Ya had only a 32% amino acid homology, while Ya and Yc had 65% homology. Thus, they speculated that GST-P had evolved from the ancestral gene at a far earlier stage than that at which Ya and Yc separated.

GST-P-related Forms as (Pre)neoplastic Markers in Other Species

GST-P was found to be immuno-crossreactive among many species and, therefore, the anti-rat GST-P antibody has proved useful for detection of preneoplastic lesions and neoplastic tissues in other animals. For example, hamster pancreatic ductal dysplasias and adenocarcinomas induced by N-bis(2-hydroxypropyl)nitrosamine (or methylnitrosourea), which are histologically similar to dysplasia and carcinoma in the human pancreas, were strongly stained.[83, 101] Cholangiocellular lesions[102] and lung preneoplastic lesions[103] induced by propylnitrosamine in Syrian ham-

sters were also found to be positive. In addition, acinar cell lesions of rat pancreas induced by hydroxyaminoquinoline 1-oxide[104] express an altered phenotype including enhanced GST-P expression.

An analogous approach has been taken to the investigation of human tissues. Several groups have applied the antibody raised by our laboratory against GST-π purified from human term placenta according to the method of Guthenberg and Mannervik,[105] modified by us by using S-hexylglutathione column chromatography and chromatofocusing,[106] and a number of precancerous states, or dysplasias, and differentiated carcinomas in several human organs have proved positive. For example, Kodate *et al.*[107] demonstrated that some human colon adenomas (about 50%) are positive and that colon carcinomas (nearly 90%) are stained even more strongly, while normal colon mucosa is negative or only slightly stained. In human uterine cervix, normal mucosa was similarly almost negative, whereas mild dysplasia including koilocytosis, suggesting infection with human papilloma virus, was mildly stained and severe dysplasia (about 70%), squamous cell carcinoma *in situ* and invasive carcinomas (about 80%) were strongly positive.[84] Similar results have been obtained on esophagus dysplasia and carcinoma (T. Nishihira *et al.*, unpublished data) and it has furthermore been immunohistochemically demonstrated that stomach carcinomas, with the exception of the signet ring cell type, express high levels of GST-π.[108] Interestingly, surface mucous cells and glandular cells of the stomach from a fetus aged 18 weeks demonstrated a high GST-π content, indicating that its expression in human stomach carcinoma may be oncofetal. Some parts of liver cirrhosis and of differentiated hepatomas were also stainable, albeit weakly. By using the single radial immunodiffusion method, the content of GST-π in esophagus, colon and cervix carcinomas was demonstrated to be about 10-fold higher than that of corresponding normal tissues (S. Tsuchida, *et al.*, unpublished data). It was also increased in cholangiocarcinomas and their metastases originating from the gallbladder, stomach and colon.[106] The finding that the levels of GST-π in sera of patients (30 to 50%) with stomach and esophagus carcinomas were significantly elevated (S. Tsuchida, *et al.*, unpublished data) may hopefully prove of diagnostic significance.

Cloning of cDNA of the human GST-π by Kano *et al.*[109] revealed a subunit Mr of 23,224 and also that GST-π and GST-P, both of which consist of 209 amino acids, have extensive homology (85.6%), differing in only 30 amino acids.

Although mouse GST M II is similarly related in immunological and other properties to both rat GST 7-7 (GST-P) and human GST-π,[36] the GST M II is basic, whereas rat GST 7-7 is neutral and human GST-π is acidic. The N-terminal amino acid sequences of all three, however, are quite similar, as pointed out by Mannervik *et al.*[36] and confirmed for the whole amino acid sequences of GST-P and GST-π.[109] Normal hepatic amounts of these forms differ among the three species[85] and the adult male mouse liver in particular contains exceptionally high levels of the GST II. Furthermore, it is inducible by injection of testosterone into females[85] or by administration of drugs such as BHA. In contrast, the level of GST-P (7-7) is very low in rat liver and this form is not easily inducible by drugs (including testosterone) prior to the appearance of preneoplastic nodular hepatocytes. Interestingly, the levels of GST M II in male livers proved reducible by castration to the low levels of female mice,[85] whereas the contents of GSTs M I and III were hardly affected by castration or by administration of testosterone. Variation of these GST forms and their roles in mouse hepatocarcinogenesis remain to be investigated.

CONCLUSIONS

So far, many marker enzymes for preneoplastic foci in rat liver have been reported.[8, 14, 16–18] Among them, glucose-6-phosphatase, calcium-dependent adenosine triphosphatase, γ-GTP and glucose-6-phosphate dehydrogenase have often been used for histochemical detection of enzyme-altered foci.[8, 17, 110, 111] Some of these alterations are known to be unstable,[110, 112] but GST-P expression is stable.[112] Thus, GST-P has

become established as one of the best markers for early detection of putative preneoplastic and neoplastic cells in rat chemical hepatocarcinogenesis, although it is not expressed in lesions associated with nongenotoxic (nonmutagenic) hepatocarcinogens. GST forms related to rat GST-P were also found to be possible markers for (pre)-neoplastic lesions in organs other than the liver in other species.

Thus, GST-P has been introduced as a marker for analysis of (hepato)carcinogenic processes and for screening of carcinogens and carcinogenic modifiers (promoters and inhibitors) inside and outside Japan. For example, Ito and his co-workers[60, 113, 114] have developed an *in vivo* short-term screening test for hepatocarcinogens, which includes DEN (200 mg/kg) ip injection (as an initiator) followed by a 6-week administration of test compounds starting 2 weeks later and hepatectomy at week 3. So far, they have investigated 112 chemicals for potential to modify GST-P-positive foci development initiated by DEN and, of the liver carcinogens, 10 out of 11 (90.9%) mutagenic, and 11 out of 13 (84.6%) non-mutagenic compounds gave positive results (mean, 87.5%). Carcinogens other than hepatocarcinogens gave less positive results (2 out of 17, 11.8%), but none of the compounds reported as non-carcinogenic was positive. This test is unique in screening not only (hepato)carcinogens but also modifiers (promoters and even inhibitors such as antioxidants) of hepatocarcinogenesis.

It is interesting that GST-P is expressed not only in putative preneoplastic hepatic foci induced by chemicals but also in spontaneous lesions. Thus, the isoenzyme was observed in spontaneous "altered cell foci" in the livers of aged Fischer 344 rats, especially in males.[115] Furthermore, GST-P-positive foci spontaneously appeared in the livers of LEC rats, predominantly in males, without administration of any exogenous carcinogen.[116]

The GST-P gene is also a good model for gene expression in chemical carcinogenesis, as shown by the investigations of Muramatsu and co-workers. It is important to clarify whether or not GST-P expression is related to the activation of specific oncogenes and, in this respect, it is interesting to note that GST-P is highly expressed with the malignant transformation *in vivo* of primary hepatocytes either by transfection with *ras* oncogenes or by treatment with metabolically activated AfB_1.[117]

Recently, GST-P (-π) and other GST forms have further attracted attention in studies of (multi-)drug resistance mechanisms in experimental and also human tumors[118, 119] along with other multidrug-resistance genes.[120, 121] Further investigation of this enzyme family would appear warranted.

Acknowledgments

Our studies were supported in part by a Grant-in-Aid for the Special Project Research-Cancer Bioscience from the Ministry of Education, Science and Culture of Japan, a grant from the Karoji Memorial Fund for Medical Research in Hirosaki University, and a research grant from the Princess Takamatsu Cancer Research Fund.

(Received January 30, 1988/Accepted April 5, 1988)

References

1) Farber, E. and Cameron, R. The sequential analysis of cancer development. *Adv. Cancer Res.*, **31**, 125–226 (1980).
2) Farber, E. The sequential analysis of liver cancer induction. *Biochim. Biophys. Acta*, **605**, 149–166 (1980).
3) Pitot, H. C. and Sirica, A. E. The stages of initiation and promotion in hepatocarcinogenesis. *Biochim. Biopshys. Acta*, **605**, 191–215 (1980).
4) Bannasch, P. Sequential cellular changes during chemical carcinogenesis. *J. Cancer*

Res. Clin. Oncol., **108,** 11–22 (1984).
5) Farber, E. Cellular biochemistry of the stepwise development of cancer with chemicals: G. H. A. Clowes memorial lecture. *Cancer Res.,* **44,** 5463–5474 (1984).
6) Farber, E. Pre-cancerous steps in carcinogenesis: their physiological adaptive nature. *Biochim. Biophys. Acta,* **738,** 171–180 (1984).
7) Scherer, E. Neoplastic progression in experimental hepatocarcinogenesis. *Biochim. Biophys. Acta,* **738,** 219–236 (1984).
8) Sell, S., Hunt, J. M., Knoll, B. J. and Dunsford, H. A. Cellular events during hepatocarcinogenesis in rats and the question of premalignancy. *Adv. Cancer Res.,* **48,** 37–111 (1987).
9) Makino, R., Hayashi, K., Sato, S. and Sugimura, T. Expressions of the c-Ha-*ras* and c-*myc* genes in rat liver tumors. *Biochem. Biophys. Res. Commun.,* **119,** 1096–1102 (1984).
10) Tashiro, F., Morimura, S., Hayashi, K., Makino, R., Kawamura, H., Horikoshi, N., Nemoto, K., Ohtsubo, K., Sugimura, T. and Ueno, Y. Expression of the c-Ha-*ras* and c-*myc* genes in aflatoxin B_1-induced hepatocellular carcinomas. *Biochem. Biophys. Res Commun.,* **138,** 858–864 (1986).
11) McMahon, G., Hanson, L., Lee, J-J. and Wogan, G. N. Identification of an activated c-Ki-*ras* oncogene in rat liver tumors induced by aflatoxin B_1. *Proc. Natl. Acad. Sci. USA,* **83,** 9418–9422 (1986).
12) Sato, K., Kitahara, A., Yin, Z., Ebina, T., Satoh, K., Tsuda, H., Ito, N. and Dempo, K. Molecular forms of glutathione S-transferase and UDP-glucuronyltransferase as hepatic preneoplastic marker enzymes. *Ann. N. Y. Acad. Sci.,* **417,** 213–223 (1983).
13) Sato, K., Tsuchida, S., Waragai, F., Yin, Z. and Ebina, T. Properties of molecular forms of γ-glutamyl transpeptidase and uridine diphosphate-glucuronyltransferase as hepatic preneoplastic marker enzymes. *Gann Monogr. Cancer Res.,* **29,** 23–31 (1983).
14) Farber, E. The biochemistry of preneoplastic liver: a common metabolic pattern in hepatocyte nodules. *Can. J. Biochem. Cell Biol.,* **62,** 486–494 (1984).
15) Roomi, M. W., Ho, R. K., Sarma, D. S. R. and Farber, E. A common biochemical pattern in preneoplastic hepatocyte nodules generated in four different models in the rat. *Cancer Res.,* **45,** 564–571 (1985).
16) Bannasch, P. Preneoplastic lesions as end points in carcinogenicity testing. I. Hepatic preneoplasia. *Carcinogenesis,* **7,** 689–695 (1986).
17) Moore, M. A. and Kitagawa, T. Hepatocarcinogenesis in the rat: the effect of promoters and carcinogens *in vivo* and *in vitro. Int. Rev. Cytol.,* **101,** 125–173 (1986).
18) Sato, K. Glutathione S-transferases as markers for preneoplasia. *Adv. Cancer Res.,* in press.
19) Jakoby, W. B. The glutathione S-transferases: a group of multifunctional detoxification proteins. *Adv. Enzymol.,* **46,** 383–414 (1978).
20) Chasseaud, L. F. The role of glutathione and glutathione-S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. *Adv. Cancer Res.,* **29,** 175–274 (1979).
21) Mannervik, B. The isoenzymes of glutathione transferase. *Adv. Enzymol. Relat. Areas Mol. Biol.,* **57,** 357–417 (1985).
22) Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M. and Ito, N. The placental form of glutathione *S*-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Gann,* **75,** 199–202 (1984).
23) Kitahara, A, Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. and Ito, N. Changes in molecular forms of rat hepatic glutathione S-transferase during chemical hepatocarcinogenesis. *Cancer Res.,* **44,** 2698–2703 (1984).
24) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA,* **82,** 3964–3968 (1985).
25) Tatematsu, M., Mera, Y., Ito, N., Satoh, K. and Sato, K. Relative merits of immunohistochemical demonstrations of placental, A, B and C forms of glutathione S-transferase and histochemical demonstration of γ-glutamyltransferase as markers of altered foci durig liver carcinogenesis in rats. *Carcinogenesis,* **6,** 1621–1626 (1985).
26) Meyer, D. J., Beale, D., Tan, K. H., Coles, B. and Ketterer, B. Glutathione transferases in primary rat hepatomas: the isolation of a form with GSH peroxidase activity. *FEBS Lett,.* **184,** 139–143 (1985).
27) Jensson, H., Eriksson, L. C. and Mannervik, B. Selective expression of glutathione transferase isoenzymes in chemically induced preneoplastic rat hepatocyte nodules. *FEBS Lett.,* **187,** 115–120 (1985).

28) Morgenstern, R., Guthenberg, C. and DePierre, J. W. Microsomal glutathione S-transferase. Purification, initial characterization and demonstration that it is not identical to the cytosolic glutathione S-transferases A, B and C. *Eur. J. Biochem.*, **128,** 243–248 (1982).
29) Morgenstern, R. and DePierre, J. W. Membrane-bound glutathione transferase. *Biochem. Soc. Transact.*, **15,** 719–721 (1987).
30) Jakschik, B. A., Harper, T. and Murphy, R. C. Leukotriene C_4 and D_4 formation by particulate enzymes. *J. Biol. Chem.*, **257,** 5346–5349 (1982).
31) Bach, M. K., Brashler, J. R. and Morton, D. R., Jr. Solubilization and characterization of the leukotriene C_4 synthetase of rat basophil leukemia cells: a novel, particulate glutathione S-transferase. *Arch. Biochem. Biophys.*, **230,** 455–465 (1984).
32) Yoshimoto, T., Soberman, R. J., Lewis, R. A. and Austen, K. F. Isolation and characterization of leukotriene C_4 synthetase of rat basophilic leukemia cells. *Proc. Natl. Acad. Sci. USA*, **82,** 8399–8403 (1985).
33) Hayes, J. D. and Mantle, T. J. Use of immuno-blot techniques to discriminate between the glutathione S-transferase Yf, Yk, Ya, Yn/Yb and Yc subunits and to study their distribution in extrahepatic tissues. *Biochem. J.*, **233,** 779–788 (1986).
34) Bass, N. M., Kirsch, R. E., Tuff, S. A., Marks, I. and Saunders, S. J. Ligandin heterogeneity: evidence that the two non-identical subunits are the monomers of two distinct proteins. *Biochim. Biophys. Acta*, **492,** 163–175 (1977).
35) Jakoby, W. B., Ketterer, B. and Mannervik, B. Glutathione transferases: nomenclature. *Biochem. Pharmacol.*, **33,** 2539–2540 (1984).
36) Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. and Jornvall, H. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. *Proc. Natl. Acad. Sci. USA*, **82,** 7202–7206 (1985).
37) Litwack, G., Ketterer, B. and Arias, I. M. Ligandin: a hepatic protein which binds steroids, bilirubin, carcinogens and a number of exogenous organic anions. *Nature*, **234,** 466–467 (1971).
38) Habig, W. H., Pabst, M. J., Fleischner, G., Gatmaitan, Z., Arias, I. M. and Jakoby, W. B. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. *Proc. Natl. Acad. Sci. USA*, **71,** 3879–3882 (1974).
39) Smith, G. J., Ohi, V. S. and Litwack, G. Ligandin, the glutathione S-transferases, and chemically induced hepatocarcinogenesis: a review. *Cancer Res.*, **37,** 8–14 (1977).
40) Ketterer, B. Detoxication reactions of glutathione and glutathione transferases. *Xenobiotica*, **16,** 957–973 (1986).
41) Ketterer, B., Meyer, D. J., Coles, B., Taylor, J. B. and Pemble, S. *In* "Antimutagenesis and Anticarcinogenesis Mechanisms," ed. D. M. Shankel, P. E. Hartman, T. Kada and A. Hollaender, pp. 103–126 (1986). Plenum Publishing, London.
42) Mantle, T. J., Pickett, C. B. and Hayes, J. D. (ed.) "Glutathione S-Transferases and Carcinogenesis," pp. 1–267 (1987). Taylor & Francis, London, New York and Philadelphia.
43) Kitahara, A., Satoh, K. and Sato, K. Properties of the increased glutathione S-transferase A form in rat preneoplastic hepatic lesions induced by chemical carcinogens. *Biochem. Biophys. Res. Commun.*, **112,** 20–28 (1983).
44) Sato, K., Satoh, K., Hatayama, I., Tsuchida, S., Soma, Y., Shiratori, Y., Tateoka, N., Inaba, Y. and Kitahara, A. Placental glutathione S-transferase as a marker for (pre) neoplastic tissues. *In* "Glutathione S-Transferases and Carcinogenesis," ed. T. J. Mantle, C. B. Pickett and J. D. Hayes, pp. 127–137 (1987). Taylor & Francis, London, New York and Philadelphia.
45) Guthenberg, C., Jensson, H., Nystrom, L., Osterlund, E., Tahir, M. K. and Mannervik, B. Isoenzymes of glutathione transferase in rat kidney cytosol. *Biochem. J.*, **230,** 609–615 (1985).
46) Robertson, I. G. C., Jensson, H., Mannervik, B. and Jernstrom, B. Glutathione transferase in rat lung: the presence of transferase 7-7, highly efficient in the conjugation of glutathione with the carcinogenic (+)-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. *Carcinogenesis*, **7,** 295–299 (1986).
47) Roomi, M. W., Satoh, K., Sato, K. and Farber, E. Natural distribution of placental glutathione S-transferase and its modulation by chemicals *Fed. Proc.*, **44,** 521 (1985).
48) Tsuchida, S., Izumi, T., Shimizu, T., Ishikawa, T., Hatayama, I., Satoh, K. and

Sato, K. Purification of a new acidic glutathione S-transferase, GST-Yn_1Yn_1, with a high leukotriene-C_4 synthase activity from rat brain. *Eur. J. Biochem.*, **170,** 159–164 (1988).

49) Solt, D. and Farber, E. A new principle for the analysis of chemical carcinogenesis. *Nature,* **263,** 701–703 (1976).

50) Ishikawa, T., Satoh, K., Kitahara, A. and Sato, K. A markedly increased placental molecular form of glutathione S-transferase in preneoplastic hepatic lesions of the rat. *Proc. Jpn. Cancer Assoc., 42nd Annu. Meet.,* 314 (1983) (in Japanese).

51) Sato, K., Satoh, K., Kitahara, A., Ishikawa, T., Soma, Y., Tatematsu, M. and Ito, N. Identification of the placental form of glutathione S-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Proc. Am. Assoc. Cancer Res.,* **25,** 7 (1984).

52) Sugioka, Y., Fujii-Kuriyama, Y., Kitagawa, T. and Muramatsu, M. Changes in gene expression during the course of hepatocarcinogenesis. Analysis of proteins with 2-dimensional gel. *Proc. Jpn. Cancer Assoc., 41st Annu. Meet.,* 276 (1982).

53) Sugioka, Y., Fujii-Kuriyama, Y., Kitagawa, T. and Muramatsu, M. Changes in polypeptide pattern of rat liver cells during chemical hepatocarcinogenesis. *Cancer Res.,* **45,** 365–378 (1985).

54) Roomi, M. W., Sharma, R. N., Ho, R. K., Eriksson, L., Farber, E. and Murray, R. K. The polypeptides of the cytosol of hepatocyte nodules may exhibit a unique electrophoretic pattern. *Proc. Am. Assoc. Cancer Res.,* **24,** 90 (1983).

55) Eriksson, L. C., Sharma, R. N., Roomi, M. W., Ho, R. K., Farber, E. and Murray, R. K. A characteristic electrophoretic pattern of cytosolic polypeptides from hepatocyte nodules generated during liver carcinogenesis in several models. *Biochem. Biophys. Res. Commun.,* **117,** 740–745 (1983).

56) Roomi, M. W., Satoh, K., Sato, K. and Farber, E. Identification of P-21 polypeptide from rat hepatocyte nodule as placental glutathione S-transferase. *Proc. Am. Assoc. Cancer Res.,* **26,** 77 (1985).

57) Rushmore, T. H., Sharma, R. N. S., Roomi, M. W., Harris, L., Satoh, K., Sato, K., Murray, R. K. and Farber, E. Identification of a characteristic cytosolic polypeptide of rat preneoplastic hepatocyte nodules as placental glutathione S-transferase. *Biochem. Biophys. Res. Commun.,* **143,** 98–103 (1987).

58) Sternberger, L. A., Hardy, P. H., Cuculis, J. J. and Meyer, H. G. The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.,* **18,** 315–333 (1970).

59) Hsu, S-M., Raine, L. and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.,* **29,** 577–580 (1981).

60) Tatematsu, M., Tsuda, H., Shirai, T., Masui, T. and Ito, N. Placental glutathione S-transferase (GST-P) as a new marker for hepatocarcinogenesis: *in vivo* short-term screening for hepatocarcinogens. *Toxicol. Pathol.,* **15,** 60–68 (1987).

61) Reddy, J. K., Azarnoff, D. L. and Hignite, C. E. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature,* **283,** 397–398 (1980).

62) Rao, M. S., Lalwani, N. D. and Reddy, J. K. Sequential histologic study of rat liver during peroxisome proliferator [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid (Wy-14,643)-induced carcinogenesis. *J. Natl. Cancer Inst.,* **73,** 983–990 (1984).

63) Greaves, P., Irisarri, E. and Monro, A. M. Hepatic foci of cellular and enzymatic alteration and nodules in rats treated with clofibrate or diethylnitrosamine followed by phenobarbital: their rate of onset and their reversibility. *J. Natl. Cancer Inst.,* **76,** 475–484 (1986).

64) Rao, M. S., Subbarao, V. and Reddy, J. K. Peroxisome proliferator-induced hepatocarcinogenesis: histochemical analysis of ciprofibrate-induced preneoplastic and neoplastic lesions for γ-glutamyl transpeptidase activity. *J. Natl. Cancer Inst.,* **77,** 951–956 (1986).

65) Rao, M. S., Tatematsu, M., Subbarao, V., Ito, N. and Reddy, J. K. Analysis of peroxisome proliferator-induced preneoplastic and neoplastic lesions of rat liver for placental form of glutathione S-transferase and γ-glutamyltranspeptidase. *Cancer Res.,* **46,** 5287–5290 (1986).

66) Wirth, P. J., Rao, M. S. and Evarts, R. P. Coordinate polypeptide expression during hepatocarcinogenesis in male F-344 rats: comparison of the Solt-Farber and Reddy models. *Cancer Res.,* **47,** 2839–2851 (1987).

67) Glauert, H. P., Beer, D., Rao, M. S.,

Schwarz, M., Xu, Y-D., Goldsworthy, T. L., Coloma, J. and Pitot, H. C. Induction of altered hepatic foci in rats by the administration of hypolipidemic peroxisome proliferators alone or following a single dose of diethylnitrosamine. *Cancer Res.*, **46**, 4601–4606 (1986).

68) Hendrich, S., Campbell, H. A. and Pitot, H. C. Quantitative stereological evaluation of four histochemical markers of altered foci in multistage hepatocarcinogenesis in the rat. *Carcinogenesis*, **8**, 1245–1250 (1987).

69) Ogiso, T., Tatematsu, M., Tamano, S., Tsuda, H. and Ito, N. Comparative effects of carcinogens on the induction of placental glutathione S-transferase-positive liver nodules in a short-term assay and of hepatocellular carcinomas in a long-term assay. *Toxicol. Pathol.*, **13**, 257–273 (1985).

70) Moore, M. A., Nakamura, T. and Ito, N. Immunohistochemically demonstrated glucose-6-phosphate dehydrogenase, γ-glutamyl transpeptidase, ornithine decarboxylase and glutathione S-transferase enzymes: absence of direct correlation with cell proliferation in rat liver putative preneoplastic lesions. *Carcinogenesis*, **7**, 1419–1424 (1986).

71) Moore, M. A., Nakagawa, K., Satoh, K., Ishikawa, T. and Sato, K. Single GST-P positive liver cells — putative initiated hepatocytes. *Carcinogenesis*, **8**, 483–486 (1987).

72) Takahashi, S., Tsutsumi, M., Nakae, D., Denda, A., Kinugasa, T. and Konishi, Y. Persistent effect of a low dose of preadministered diethylnitrosamine on the induction of enzyme-altered foci in rat liver. *Carcinogenesis*, **8**, 509–513 (1987).

73) Kitagawa, T., Moore, M. A., Nomura, K., Sato, K. and Ishikawa, T. Failure in demonstrating the initiating activity *in vivo* of hepatocarcinogens lacking mitogenicity *in vitro*. *Proc. Jpn. Cancer Assoc., 45th Annu. Meet.*, 84 (1986) (in Japanese).

74) Garvey, L. K., Swenberg, J. A., Hamm, T. E., Jr, and Popp, J. A. Di(2-ethylhexyl)-phthalate: lack of initiating activity in the liver of female F-344 rats. *Carcinogenesis*, **8**, 285–290 (1987).

75) Williams, G. M., Maruyama, H. and Tanaka, K. Lack of rapid initiating, promoting or sequential syncarcinogenic effects of di(2-ethylhexyl)phthalate in rat liver carcinogenesis. *Carcinogenesis*, **8**, 875–880 (1987).

76) Roomi, M. W., Columbano, A., Ledda-Columbano, G. M. and Sarma, D. S. R. Lead nitrate induces certain biochemical properties characteristic of hepatocyte nodules. *Carcinogenesis*, **7**, 1643–1646 (1986).

77) Hiratsuka, A., Kawashima, K., Watabe, T., Satoh, K., Hatayama, I. and Sato, K. Inversion of enantioselectivity in GSH conjugation of 9,10-dihydrobenzo [*a*] pyrene 7,8-oxide in hepatic cytosol of rats following induction of hepatic hyperplastic nodules by chemical carcinogens. *Cancer Lett.*, **38**, 31–39 (1987).

78) Benedetti, A., Comporti, M. and Esterbauer, H. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim. Biophys. Acta*, **620**, 281–296 (1980).

79) Kitahara, A., Yamazaki, T., Ishikawa, T., Camba, E. A. and Sato, K. Changes in activities of glutathione peroxidase and glutathione reductase during chemical hepatocarcinogenesis in the rat. *Gann*, **74**, 649–655 (1983).

80) Sato, K., Takaya, S., Imai, F., Hatayama, I. and Ito, N. Different deviation patterns of carbohydrate-metabolizing enzymes in primary rat hepatomas induced by different chemical carcinogens. *Cancer Res.*, **38**, 3086–3093 (1978).

81) Hacker, H-J., Moore, M. A., Mayer, D. and Bannasch, P. Correlative histochemistry of some enzymes of carbohydrate metabolism in preneoplastic and neoplastic lesions in the rat liver. *Carcinogenesis*, **3**, 1265–1272 (1982).

82) Moore, M. A., Hacker, H-J. and Bannasch, P. Phenotypic instability in focal and nodular lesions induced in a short term system in the rat liver. *Carcinogenesis*, **4**, 595–603 (1983).

83) Moore, M. A., Satoh, K., Kitahara, A., Sato, K. and Ito, N. A protein cross-reacting immunohistochemically with rat glutathione *S*-transferase placental form as a marker for preneoplasia in Syrian hamster pancreatic and hepatocarcinogenesis. *Jpn. J. Cancer Res. (Gann)*, **76**, 1–4 (1985).

84) Shiratori, Y., Soma, Y., Maruyama, H., Sato, S., Takano, A. and Sato, K. Immunohistochemical detection of the placental form of glutathione S-transferase in dysplastic and neoplastic human uterine cervix lesions. *Cancer Res.*, **47**, 6806–6809 (1987).

85) Hatayama, I., Satoh, K. and Sato, K. Developmental and hormonal regulation of the major form of hepatic glutathione S-transferase in male mice. *Biochem. Biophys.*

Res. Commun., **140**, 581–588 (1986).
86) Tan, K. H., Meyer, D. J., Coles, B. and Ketterer, B. Thymine hydroperoxide, a substrate for rat Se-dependent glutathione peroxidase and glutathione transferase isoenzymes. *FEBS Lett.*, **207**, 231–233 (1986).
87) Muramatsu, M., Okuda, A., Kano, T. and Sakai, M. Structure and regulation of rat glutathione S-transferase P (GST-P) gene. *In* "Glutathione S-Transferase and Carcinogenesis," ed. T. J. Mantle, C. B. Pickett and J. D. Hayes, pp. 111–119 (1987). Taylor & Francis, London, New York and Philadelphia.
88) Sugioka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T. and Muramatsu, M. Cloning and the nucleotide sequence of rat glutathione S-transferase P cDNA. *Nucleic Acids Res.*, **13**, 6049–6057 (1985).
89) Knoll, B. J., Longley, M. and Sell, S. Cloning of a cDNA encoding the rat placental glutathione S-transferase: overproduction of the mRNA in hepatocellular carcinomas. *Tumour Biol.*, 7, 123–135 (1986).
90) Pemble, S. E., Taylor, J. B. and Ketterer, B. Tissue distribution of rat glutathione transferase subunit 7, a hepatoma marker. *Biochem. J.*, **240**, 885–889 (1986).
91) Pemble, S. E., Taylor, J. B., Dixon, K. H., Cowell, I. G. and Ketterer, B. Tissue expression of glutathione transferase subunit 7: a possible multigene family. *In* "Glutathione S-Transferases and Carcinogenesis," ed. T. J. Mantle, C. B. Pickett and J. D. Hayes, pp. 121–123 (1987). Taylor & Francis, London, New York and Philadelphia.
92) Taylor, J. B., Pemble, S. E., Cowell, I. G., Dixon, K. H. and Ketterer, B. Molecular biology of glutathione transferases. *Biochem. Soc. Transact.*, **15**, 578–581 (1987).
93) Okuda, A., Sakai, M. and Muramatsu, M. The structure of the rat glutathione S-transferase P gene and related pseudogenes. *J. Biol. Chem.*, **262**, 3858–3863 (1987).
94) Sakai, M., Okuda, A. and Muramatsu, M. Regulation of the rat glutathione S-transferase P gene. *Proc. Jpn. Cancer Assoc., 46th Annu. Meet.*, 133 (1987) (in Japanese).
95) Masuda, R., Yoshida, M. C., Sasaki, M., Okuda, A., Sakai, A. and Muramatsu, M. Localization of the gene for glutathione *S*-transferase P on rat chromosome 1 at band q43. *Jpn. J. Cancer Res. (Gann)*, **77**, 1055–1058 (1986).
96) Telakowski-Hopkins, C. A., Rothkopf, G. S. and Pickett, C. B. Structural analysis of a rat liver glutathione S-transferase Ya gene. *Proc. Natl. Acad. Sci. USA*, **83**, 9393–9397 (1986).
97) Pickett, C. B., Telakowski-Hopkins, C. A., Ding, G. J-F., Ding, V. D-H. and King, R. G. Regulation of genes encoding glutathione S-transferases in normal and preneoplastic liver. *In* "Glutathione S-Transferases and Carcinogenesis," ed. T. J. Mantle, C. B. Pickett and J. D. Hayes, pp. 75–85 (1987). Taylor & Francis, London, New York and Philadelphia.
98) Pickett, C. B., Telakowski-Hopkins, C. A., Ding, G. J-F., Ding, V. D-H. and King, R. G. Expression of genes encoding glutathione S-transferases in normal and preneoplastic liver. *Biochem. Soc. Transact.*, **15**, 725–727 (1987).
99) Tu, C-P. D., Lai, H-C. J. and Reddy, C. C. The rat glutathione S-transferases supergene family: molecular basis of gene multiplicity. *In* "Glutathione S-Transferases and Carcinogenesis," ed. T. J. Mantle, C. B. Pickett and J.D. Hayes, pp. 87–110 (1987). Taylor & Francis, London, New York and Philadelphia.
100) Tu, C-P, D., Lai, H-C. J., Li, N-q., Weiss, M. J. and Reddy, C. C. The Yc and Ya subunits of rat liver glutathione S-transferases are the products of separate genes. *J. Biol. Chem.*, **259**, 9434–9439 (1984).
101) Obara, T., Makino, T., Ura, H., Yokose, Y., Kinugasa, T., Moore, M. A., Sato, K. and Konishi, Y. Comparative histochemical investigation of the glutathione S-transferase placental form and γ-glutamyltranspeptidase during N-nitrosobis(2-hydroxypropyl)-amine-induced pancreatic carcinogenesis in hamsters. *Carcinogenesis*, **7**, 801–805 (1986).
102) Moore, M. A., Fukushima, S., Ichihara, A., Sato, K. and Ito, N. Intestinal metaplasia and altered enzyme expression in propylnitrosamine-induced Syrian hamster cholangiocellular and gallbladder lesions. *Virchows Arch. B*, **51**, 29–38 (1986).
103) Moore, M. A., Tsuda, H., Hacker, H. J., Sato, K., Bannasch, P. and Ito, N. Altered enzyme expression in propylnitrosamine-induced Syrian hamster lung lesions. *Virchows Arch. B* (1987), in press.
104) Moore, M. A., Makino, T., Tsuchida, S., Sato, K., Ichihara, A., Amelizad, Z., Oesch, F. and Konishi, Y. Altered drug metabolizing potential of acinar cell lesions induced in rat pancreas by hydroxyaminoquinoline 1-oxide. *Carcinogenesis*, **8**, 1089–1094 (1987).

105) Guthenberg, C. and Mannervik, B. Glutathione S-transferase (transferase π) from human placenta is identical or closely related to glutathione S-transferase (transferase ρ) from erythrocytes. *Biochim. Biophys. Acta,* **661,** 255–260 (1981).

106) Soma, Y., Satoh, K. and Sato, K. Purification and subunit-structural and immunological characterization of five glutathione S-transferases in human liver, and the acidic form as a hepatic tumor marker. *Biochim. Biophys. Acta,* **869,** 247–258 (1986).

107) Kodate, C., Fukushi, A., Narita, T., Kudo, H., Soma, Y. and Sato, K. Human placental form of glutathione S-transferase (GST-π) as a new immunohistochemical marker for human colonic carcinoma. *Jpn. J. Cancer Res. (Gann),* **77,** 226–229 (1986).

108) Tsutsumi, M., Sugisaki, T., Makino, T., Miyagi, N., Nakatani, K., Shiratori, T., Takahashi, S. and Konishi, Y. Oncofetal expression of glutathione S-transferase placental form in human stomach carcinomas. *Jpn. J. Cancer Res. (Gann),* **78,** 631–633 (1987).

109) Kano, T., Sakai, M. and Muramatsu, M. Structure and expression of a human class pi glutathione S-transferase (GST-π) messenger RNA. *Cancer Res.,* **47,** 5626–5630 (1987).

110) Moore, M. A., Hacker, H-J. and Bannasch, P. Phenotypic instability in focal and nodular lesions induced in a short term system in the rat liver. *Carcinogenesis,* **4,** 595–603 (1983).

111) Goldsworthy, T. L. and Pitot, H. C. The quantitative analysis and stability of histochemical markers of altered hepatic foci in rat liver following initiation by diethylnitrosamine administration and promotion with phenobarbital. *Carcinogenesis,* **6,** 1261–1269 (1985).

112) Tatematsu, M., Mera, Y., Inoue, T., Satoh, K., Sato, K. and Ito, N. Stable phenotypic expression of glutathione S-transferase placental type and unstable phenotypic expression of γ-glutamyltransferase in rat liver preneoplastic and neoplastic lesions. *Carcinogenesis,* **9,** 215–220 (1988).

113) Tsuda, H., Fukushima, S., Imaida, K., Ito, N., Sato, K., Nakamura, T. and Oesch, F. Analyses of the effects of various promoters and inhibitors on altered enzyme phenotypes in preneoplastic cell liver foci in rapid bioassay models in rats. *Proc. 4th Sardinian Int. Meet.* (1988), in press.

114) Ito, N., Tsuda, H., Tatematsu, M., Inoue, T., Tagawa, Y., Aoki, T., Uwagawa, S., Ogiso, T., Masui, T., Imaida, K., Fukushima, S. and Asamoto, M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase-P form positive foci in rats. An approach for a new medium-term bioassay system. *Carcinogenesis,* in press.

115) Mitaka, T. and Tsukada, H. Sexual difference in the histochemical characteristics of "altered cell foci" in the liver of aged Fischer 344 rats. *Jpn. J. Cancer Res. (Gann),* **78,** 785–790 (1987).

116) Oyamada, M., Dempo, K., Fujimoto, Y., Takahashi, H., Satoh, M. I., Mori, M., Masuda, R., Yoshida, M. C., Satoh, K. and Sato, K. Spontaneous occurrence of placental glutathione S-transferase-positive foci in the livers of LEC rats. *Jpn. J. Cancer Res. (Gann),* **79,** 5–8 (1988).

117) Power, C. Sinha, S., Webber, C., Manson, M. M. and Neal, G. E. Transformation related expression of glutathione-S-transferase P in rat liver cells. *Carcinogenesis,* **8,** 797–801 (1987).

118) Cowan, K. H., Batist, G., Tulpule, A., Sinha, B. K., and Myers, C. E. Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. *Proc. Natl. Acad. Sci. USA,* **83,** 9328–9332 (1986).

119) Wolf, C. R., Lewis, A. D., Carmichael, J., Adams, D. J., Allan, S. G. and Ansell, D. J. The role of glutathione in determining the response of normal and tumour cells to anticancer drugs. *Biochem. Soc. Transact.,* **15,** 728–730 (1987).

120) Thorgeirsson, S. S., Huber, B. E., Sorrell, S., Fojo, A., Pastan, I. and Gottesman, M. M. Expression of the multidrug-resistant gene in hepatocarcinogenesis and regenerating rat liver. *Science,* **236,** 1120–1122 (1987).

121) Fairchild, C. R., Ivy, S. P., Rushmore, T., Lee, G., Koo, P., Goldsmith, M. E., Myers, C. E., Farber, E. and Cowan, K. H. Carcinogen-induced *mdr* overexpression is associated with xenobiotic resistance in rat preneoplastic liver nodules and hepatocellular carcinomas. *Proc. Natl. Acad. Sci. USA,* **84,** 7701–7705 (1987).

122) Tsuchida, S., Hoshino, K., Sato, T., Ito, N. and Sato, K. Purification of γ-glutamyltransferases from rat hepatomas and hyperplastic hepatic nodules, and comparison with the enzyme from rat kidney. *Cancer Res.,* **39,** 4200–4205 (1979).