GLUTATHIONE TRANSFERASES — STRUCTURE AND CATALYTIC ACTIVITY

Authors: **Bengt Mannervik**
U. Helena Danielson
Department of Biochemistry
University of Uppsala
Uppsala, Sweden

Referee: Brian Ketterer
Department of Biochemistry
University of London
London, England

I. INTRODUCTION

The glutathione transferases (E.C. 2.5.1.18) are known as enzymes that catalyze the nucleophilic attack of the sulfur atom of glutathione on electrophilic groups in a second substrate. The enzymes occur abundantly in most forms of life investigated and are generally considered to serve in the intracellular detoxication of mutagens, carcinogens, and other noxious chemical substances. Glutathione, γ-glutamylcysteinylglycine, appears to be limited essentially to aerobic organisms.[1] Consequently, glutathione transferases cannot be expected to occur in other forms of life. Multiple forms of glutathione transferase have been discovered in virtually every organism in which glutathione transferase activity has been found (for a survey and for references to earlier reviews, see Reference 2). In most cases, the variant forms are distinguished by differences in catalytic properties, suggesting separate or complementary activities.

In addition to their enzymatic function, it has been suggested that glutathione transferases may serve as intracellular carrier proteins of certain organic molecules, acting as an intracellular equivalent to albumin in blood plasma.[3] In this assumed capacity of reversible binding and transport of various ligands, the corresponding protein was named ligandin.[4] The covalent binding of certain reactive electrophilic molecules,[5] with consequent inactivation and immobilization, has been proposed as an additional protective role of the glutathione transferases.[6]

The expression of the multiple forms of glutathione transferase differs from one tissue to another and may be altered by inducers of drug metabolism (cf. Reference 2). The occurrence of the different forms of transferase changes dramatically in an organ-specific manner during the transition from the fetal to the adult state.[7-9] In the mouse, a sex-related difference in the hepatic expression of a specific enzyme form, apparently under testosterone control, has been noted.[10]

The structure and control of the genes of glutathione transferase are currently under study.[11-17]

It recently has been found that certain forms of glutathione transferase are expressed at high levels in mammalian tumor cells (cf. Reference 18). This opens the possibility of using the expression in clinical diagnosis of neoplastic tissues and raises the question of the possible role of glutathione transferase in the development of drug resistance often encountered in the chemotherapy of cancer.

Evidently, a number of significant biological and medical questions regarding the glutathione transferases have to be answered.

II. NOMENCLATURE AND CLASSIFICATION

A. Multiple Enzyme Forms and Nomenclature

Even though it is generally assumed that a major biological role of the glutathione transferases is the detoxication of reactive electrophiles, a full understanding of their function is lacking. The "natural" substrates of the enzymes are essentially unknown, consequently the multiple forms of the enzyme cannot be clearly distinguished by the usual criteria based on substrate specificities.

An early attempt to classify different forms of glutathione transferase was made by Boyland and Chasseaud,[19] who introduced the terms aryltransferase, epoxide transferase, alkyltransferase, aralkyltransferase, and alkenetransferase. At the time, available evidence suggested that such glutathione transferases could be distinguished on the basis of their specificities towards their electrophilic substrates. However, separation and extensive purification of several forms of the enzyme demonstrated conclusively that they displayed overlapping substrate specificities and that their activities were not limited to a single functional group of the second substrate. For example, the protein isolated as "epoxide transferase" was also active with alkyl and aralkyl halogenides,[20] and two isoforms of "aryltransferase" were both active with an epoxide substrate.[21] Consequently, the original nomenclature was replaced by designations based on the physical or structural properties of the proteins rather than on their enzymatic properties. In discarding the substrate-based nomenclature, it should be borne in mind that almost all investigations have been made with relatively poor substrates that lack biological relevance, such as chloronitrobenzenes and halogenated hydrocarbons. The recent identification of a glutathione transferase in rat tissues with unusually high activity with 4-hydroxyalkenals,[22] a class of substrates which may be formed in vivo, suggests the discovery of a true "alkenetransferase" and points to a possible future reintroduction of names indicating the function of the enzymes. However, at present, such a nomenclature has no solid foundation.

Jakoby and co-workers suggested that the six forms of glutathione transferase, which they had identified in rat liver, should be named empirically as glutathione transferases E, D, C, B, A, and AA, in the order of their elution from a carboxymethylcellulose ion-exchange matrix.[23-25] A seventh form, earlier identified by Gillham[26] as particularly active with menaphthylsulfate, was named transferase M. The two isoenzymes of glutathione "aryltransferase", forms I and II, were identified as transferases C and A, respectively.[21]

Glutathione transferases in the cytosol are dimeric proteins, and it was shown by Mannervik and Jensson[27] that six major enzyme forms in rat liver can be regarded as homo- and heterodimeric combinations of four different subunits with distinct substrate specificities. The finding that the enzymatic properties of a protein dimer reflect the subunit composition led to the suggestion that an enzyme should be named on the basis of its constituent subunits.[27] The participants in a workshop on glutathione transferases agreed to adopt this nomenclature and decided to denote each distinct protein subunit by an Arabic numeral.[28] Accordingly, transferase A in Jakoby's nomenclature, consisting of two identical subunits named "3", was renamed "rat glutathione transferase 3-3". Transferase C, a heterodimer of subunits 3 and 4, was called "rat glutathione transferase 3-4". So far, eight enzymatically distinct rat subunits have been named. Table 1 shows the rat enzymes designated by Arabic numerals and their previous designations, where applicable.

An additional system for naming the subunits of rat glutathione transferase is based on their relative mobilities in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Originally, subunits Y_a, Y_b, and Y_c, in order of decreasing mobility, were distinguished.[32] Estimates of M_r for the subunits range between 21,000 and 29,000, but amino acid sequence determinations show that the true M_r values are close to 25,000. A caveat to the use of the electrophoretically determined M_r values is that the relative mobilities of the different subunits may depend on the degree of cross-linking in the polyacrylamide gel.[34]

Table 1
NOMENCLATURE FOR THE CYTOSOLIC RAT GLUTATHIONE TRANSFERASES

New nomenclature	Previous nomenclature: References 4, 25, 29—31	Previous nomenclature: Reference 27	Alternative subunit designations: References 29, 31—33
Glutathione transferase 1-1	B (ligandin)	L_2	Y_a
Glutathione transferase 1-2		BL	Y_a, Y_c
Glutathione transferase 2-2	AA	B_2	Y_c
Glutathione transferase 3-3	A	A_2	Y_b^1
Glutathione transferase 3-4	C	AC	Y_b^1, Y_b^2
Glutathione transferase 3-6	P		
Glutathione transferase 4-4	D	C_2	Y_b^2
Glutathione transferase 4-6	S		
Glutathione transferase 5-5	E		
Glutathione transferase 6-6	M_T		Y_n
Glutathione transferase 7-7	P		Y_p or Y_f
Glutathione transferase 8-8	K		Y_k

Note: Modified and updated from Reference 28.

Table 2
NOMENCLATURE FOR THE CYTOSOLIC MOUSE GLUTATHIONE TRANSFERASES

New nomenclature	Previous nomenclature	Mouse strain	Ref.
Mouse glutathione transferase C 1-1	GT-8.7	CD-1	36
Mouse glutathione transferase D 1-1	F3	DBA/2J	37
Mouse glutathione transferase N 1-1	MIII	NMRI	35
Mouse glutathione transferase C 2-2	GT-9.3	CD-1	36
Mouse glutathione transferase N 3-3	MII	NMRI	35
Mouse glutathione transferase N 4-4	MI	NMRI	35

Note: Transferases C 1-1, D 1-1, and N 1-1 are identical or closely related enzymes from three different strains of mice.

In the discussion of the nomenclature, several important principles were adopted.[28] The name of the enzyme should specify the biological species, but not the organ, from which the enzyme was obtained. Only such forms that have been characterized by enzymatic and other functional properties should be named in order to make identification possible by different groups of investigators. Subunits should be numbered sequentially in the order that they are being discovered and characterized.

In the extension of the nomenclature to other mammalian species, it was decided that glutathione transferases in other species should be named by the same principle, but independent of the rat enzymes (cf. Reference 35). The reason is that present knowledge is not sufficient for matching enzymes from different species and a one-to-one correspondence is far from obvious. Table 2 shows the mouse enzymes identified and named thus far. The mouse strain from which the enzyme was obtained may be indicated by a letter.[35] Consequently, the enzyme form GT-8.7 from the CD-1 strain[36] has been designated mouse glu-

tathione transferase C 1-1, F3 from the DBA/2J strain[37] transferase D 1-1, and MIII from the NMRI strain[35] transferase N 1-1. Mouse transferase 1-1 is believed to be the same protein in all three strains, and the letter only serves to distinguish possible strain variants. Such variant proteins might be related to the known strain differences in the sensitivity of mice to certain toxic compounds.

Additional, less abundant, forms of glutathione transferase have been isolated, but not yet named. For example, rat[38] and mouse lung[39] contain several minor forms with near-neutral or acidic isoelectric points.

The human glutathione transferases were originally designated by Greek letters.[40] Five basic proteins purified from human liver cytosol were named glutathione transferases α, β, γ, δ and ϵ, in the order of increasing isoelectric points. In view of their similarities in enzymatic and immunological properties[40] and the marked polymorphism in the expression of the basic transferases (cf. References 41 and 42), the original isoenzymes cannot be rigorously identified with enzymes later purified. For this reason, they have sometimes simply been referred to collectively as transferase (α-ϵ)[43] or "basic transferases".[44] However, three basic human enzymes, two homodimers and a corresponding heterodimer, recently have been isolated and characterized.[45-47] A distinct enzyme with a near-neutral isoelectric point was named glutathione transferase μ.[48] A third type, glutathione transferase π, has been isolated from placenta.[49,50] This enzyme is probably identical[51] to the acidic protein first isolated from erythrocytes and designated transferase ρ.[52] Acidic transferases from human organs, including lung, kidney, and lens, also appear to correspond to transferase π (cf. Reference 2). The designation glutathione transferase π will be used throughout since the placental form is the most well-characterized protein and is the one used as a reference for comparisons.

The three major types of human glutathione transferase have been classified as "basic", "near-neutral", and "acidic" on the basis of their isoelectric points.[2,44] However, some reports suggest that acidic enzyme forms are structurally related to the "basic" transferases.[45,53] Furthermore, the rat (transferase 7-7) and mouse (transferase MII or N3-3) enzymes equivalent to the human "acidic" transferase are basic proteins. Consequently, it is more rational to refer the various cytosolic mammalian transferases to the three structurally defined classes Alpha, Mu, and Pi.[54]

Arabic numerals have not yet been adopted for naming the human glutathione transferases since the polymorphism of the human enzymes has made definitive identifications of the multiple forms difficult. Neither has a similar unifying nomenclature been proposed for the transferases from maize or other biological species referred to in this review.

In addition to the cytosolic glutathione transferases discussed previously, a distinct membrane-bound enzyme has been identified.[55] This protein was first isolated from the microsome fraction of rat liver and is generally referred to as microsomal glutathione transferase, even though it is also present in other subcellular fractions.[56] The corresponding enzyme has also been purified from mouse liver[57] and human liver[58].

B. Criteria for Distinction of the Multiple Forms of Glutathione Transferase

The multiplicity of glutathione transferases in an organism creates problems in the identification of individual forms. In the rat, for example, evidence for the occurrence of more than 12 cytosolic and at least 1 microsomal transferase exists. It is virtually impossible to identify a particular form by a single criterion, unless a full structural characterization is carried out. The problem of identification may arise when new sources of the enzyme are investigated or in studies of changes in enzyme profiles during development, induction, or cell transformation. Consequently, it has been emphasized that a set of characteristics involving substrate specificities, sensitivities to inhibitors, reactions with specific antisera, and physicochemical properties should be used for identification of an enzyme form. Tables 3

Table 3
PHYSICOCHEMICAL CHARACTERISTICS OF RAT GLUTATHIONE TRANSFERASES

	Property					
Isoenzyme	Class	Apparent subunit M_r (kdalton)[a]	Subunit M_r[b]	No. amino acids per subunit[b]	Isoelectric point	Ref.
1-1	Alpha	25	25 434	221	10	59, 60
1-2	Alpha	25 + 28	—	—	9.9	59
2-2	Alpha	28	25 209	220	9.8	59, 61
3-3	Mu	26.5	25 806	217	8.9	59, 62
3-4	Mu	26.5	—	—	8	59
3-6	Mu	26.5 + 26	—	—	7.4	63
4-4	Mu	26.5	25 592	217	6.9	59, 64, 65
4-6	Mu	26.5 + 26	—	—	6.1	63
5-5	—[c]	26.5	—	—	7.3	66
6-6[d]	Mu	26	—	—	5.8	68
7-7	Pi	24	23 307	209	7.0	69, 70
8-8	Alpha	24.5	—	—	6.0	22
Microsomal	—	17	17 237	154	10.1	71, 72

[a] Relative values estimated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.
[b] N-terminal methionine residue not included.
[c] Not yet classified.
[d] Recent work suggests that the major testicular enzyme, designated transferase 6-6, is a heterodimer.[232]

Table 4
PHYSICOCHEMICAL CHARACTERISTICS OF MOUSE GLUTATHIONE TRANSFERASES

	Property			
Isoenzyme	Class	Apparent subunit M_r (kdalton)[a]	Isoelectric point	Ref.
N 1-1	Mu	26.5	8.5	35
N 3-3	Pi	23	8.7	35
N 4-4	Alpha	25	9.7	35
Microsomal	—	17	8.8	57

[a] Relative values estimated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

to 11 summarize some of the properties that may help to identify individual transferases in rat, mouse, and man. Several of the characteristics may be evaluated by use of catalytic amounts of enzyme. For a definitive identification, a determination of the amino acid sequence may be necessary.

C. Classification

The individual forms of cytosolic glutathione transferase as identified in different species have no obvious relationship to one another. For example, rat liver cytosol contains at least

Table 5
PHYSICOCHEMICAL CHARACTERISTICS OF CYTOSOLIC HUMAN GLUTATHIONE TRANSFERASES

	Property					
Isoenzyme	Class	Apparent subunit M_r (kdalton)[a]	Subunit M_r[b]	No. amino acids per subunit[b]	Isoelectric point	Ref.
B_1B_1	Alpha	25	25 516[c]	221	8.9	47
B_1B_2	Alpha	25	—	—	8.75	47
B_2B_2	Alpha	25	—	—	8.4	47
μ	Mu	26.5	—	—	6.6	43
ψ	Mu	26.5	—	—	5.5	74
π	Pi	23	—	209	4.8	51, 75
Skin "9.9"	Alpha	27.5	—	—	9.9	76

[a] Relative values estimated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.
[b] N-terminal methionine not included.
[c] Values determined for subunit H_a, the most abundant class Alpha subunit in liver,[73] which we assume is identical to subunit B_1. A second class Alpha subunit H_b (apparent M_r = 27.5 kdalton)[73] cannot yet be identified with a subunit of any of the purified and characterized enzymes.

six major basic transferases with distinct enzymatic properties.[27,81] Mouse liver contains three major basic transferases,[35] and human liver contains one to several basic forms, depending on the individual from which the sample was obtained.[40-42,82] In only a few cases may a single form in one species be identified directly with an enzyme form in another species by use of isoelectric point, chromatographic properties, or specific activities with different substrates. Consequently, major species differences in the occurrence of the multiple forms of glutathione transferase appeared to be the general opinion (cf. Reference 37) until a few years ago.

In the case of the rat enzymes, it had been established that at least two discrete families of isoenzymes exist.[27] Subunits within a family could form hybrids in the form of heterodimers, in addition to homodimers. For example, rat subunits 1 and 2 (in the present nomenclature) occur in the three binary combinations of transferases 1-1, 1-2, and 2-2. Similarly, rat transferases 3-3, 3-4, and 4-4 are members of a second family.

The human glutathione transferases were found to represent three distinct types, distinguishable by enzymatic, immunological, and physicochemical properties.[43,80,83] The cytosolic mouse transferases were similarly found to represent three different types.[35]

The availability of cytosolic glutathione transferases from three mammalian species (rat, mouse, and man) in the same laboratory made more extensive characterizations and comparisons possible.[84] For example, each species contained at least one transferase acting as a "nonselenium-containing" glutathione peroxidase (cf. Reference 85) with cumene hydroperoxide as the substrate. Likewise, certain traits in the sensitivities to inhibitors appeared common to enzymes from each of the three species. Finally, antisera raised against rat or human transferases cross-reacted in certain cases with enzymes from other species, in spite of the fact that they did not react with members of other enzyme families in the species from which the antigen was derived.

On the basis of the total body of data, obtained by use of various substrates, inhibitors, and antisera, it was proposed that the cytosolic glutathione transferases of rat, mouse, and man should be divided into three classes.[84]

Further support for the classification was sought in the primary structures of the enzymes. N-terminal amino acid sequences could be obtained for most of the transferases characterized

Table 6
SPECIFIC ACTIVITIES (μMOL/MIN/MG) OF RAT GLUTATHIONE TRANSFERASES

Substrate	Class: Enzyme:	Alpha 1-1	Alpha 2-2	Alpha 8-8	Mu 3-3	Mu 4-4	Mu 6-6[a]	Pi 7-7	Unknown 5-5	Microsomal (activated)
1-Chloro-2,4-dinitrobenzene		50	17	10	58	17	190	24	<0.15	30
1,2-Dichloro-4-nitrobenzene		<0.04	<0.04	0.12	5.3	0.18	2.85	0.048	Nil	0.06
Bromosulfophthalein		<0.01	<0.01	—	0.94	0.04	—	0.01[b]	—	<0.01
Ethacrynic acid		0.08	1.24	7.0	0.08	0.62	0.057	3.84	Nil	<0.01
trans-4-Phenyl-3-buten-2-one		<0.004	<0.004	0.10	0.05	1.18	0.019	0.22	<0.001	0.001
4-Hydroxynonenal		2.6	0.67	170	2.7	6.9	—	—	—	—
Leukotriene A_4[c]		0.002	0.0005	—	0.002	0.077	—	—	—	—
1,2-Epoxy-3-(*p*-nitrophenoxy)propane		<0.1	<0.1	—	0.53	1.37	—	—	25.5	<0.01
trans-Stilbene oxide[d]		0.001	0.003	0.033	0.10	2.0	0.13	0.005	—	—
Benzo(a)pyrene 7,8-diol-9,10-oxide		—	0.006	0.18	0.012	0.68	—	5.5	—	—
Cumene hydroperoxide		3.1	7.9	1.10	0.35	0.72	0.19	0.048	12.5	0.8
H_2O_2		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	—	<0.04
Δ^5-Androstene-3,17-dione		4.2	0.36	—	0.02	0.002	—	—	—	—
p-Nitrophenyl acetate		0.79	0.20	—	1.01	0.28	0.19	—	—	—

Note: Data compiled from References 27, 38, 59, 66, 69, and 71.

[a] Unpublished work by Guthenberg, C. and Mannervik, B.
[b] Unpublished work by Tahir, M. K. and Mannervik, B.
[c] Unpublished work by Örning, L., Söderström, M., Hammarström, S., and Mannervik, B.
[d] Unpublished work by Seidegård, J., Danielson, U. H., and Mannervik, B.

Table 7
SPECIFIC ACTIVITIES (μMOL/MIN/MG) OF MOUSE GLUTATHIONE TRANSFERASES

Substrate	Class: Enzyme:	Alpha N 4-4	Mu N 1-1	Pi N 3-3	Microsomal (activated)
1-Chloro-2,4-dinitrobenzene		19	148	119	42
1,2-Dichloro-4-nitrobenzene		0.062	4.4	0.14	0.10
Bromosulfophthalein		0.008	0.58	0.007	<0.01
Ethacrynic acid		0.025	0.12	1.4	<0.01
trans-4-Phenyl-3-buten-2-one		0.009	0.044	0.013	—
4-Hydroxynonenal		1.1	6.0	2.6	—
Leukotriene A_4		—	0.01	0.002	<0.0001
1,2-Epoxy-3-(*p*-nitrophenoxy)propane		0.23	0.48	0.77	—
trans-Stilbene oxide[a]		0.045	0.049	0.013	—
Benzo(a)pyrene 4,5-oxide		0.009	0.076	0.033	—
Benzo(a)pyrene 7,8-diol-9,10 oxide[b]		—	0.11	1.1	—
Cumene hydroperoxide		11.6	0.11	0.14	1.9
H_2O_2		<0.03	<0.03	<0.03	—
Δ^5-Androstene-3,17-dione[c]		0.035	0.043	0.14	—
p-Nitrophenyl acetate		0.011	0.59	0.21	—

Note: Data compiled from References 35, 57, and 77.

[a] Unpublished work by Seidegård, J., Danielson, U. H., and Mannervik, B.
[b] Unpublished work by Robertson, I. G. C., Jernström, B., and Mannervik, B.
[c] The activity is measured in the absence of reduced glutathione since this compound inhibits the isomerase activity of the mouse glutathione transferases.

by other criteria, and the available structural data were fully consistent with the classification proposed.[54] The species-independent classes of cytosolic glutathione transferases thus established were designated by the names of Greek letters spelled out in full: class Alpha, class Mu, and class Pi. The individual human enzymes, glutathione transferases α, μ, and π, correspondingly fall into the classes named. Finally, the membrane-linked glutathione transferase, first isolated from rat liver microsomes, was found to be distinct from the cytosolic enzymes[86] and had to be classified separately from all the cytosolic forms of glutathione transferase.[54]

The generality of the classification introduced for the mammalian glutathione transferases has not been explored in any detail. However, an enzyme from the helminth *Schistosoma japonicum* displays obvious sequence homology with the mammalian class Mu transferases.[87] Two amino acid sequences from maize[16,88] also show structural similarities to the mammalian sequences, but the relationships to the different classes are not equally obvious. Consequently, the available data suggest that the classification adopted for the mammalian transferases can be extended to other animal species, but not necessarily to plants. Finally, it remains to be clarified whether only three classes of cytosolic enzymes exist or if additional classes have to be defined.

III. STRUCTURE OF GLUTATHIONE TRANSFERASES

A. Primary Structure

Amino acid sequences of several glutathione transferases have been determined (Figure 1). For two enzymes, rat microsomal transferase[72] and rat transferase 4-4,[65] the entire sequences were elucidated by the techniques of protein chemistry. In about a dozen additional cases, N-terminal primary structures comprising 10 to 40 amino acids have been obtained by Edman degradation (for compilations, see References 2, 54, and 65). However, most

Table 8
SPECIFIC ACTIVITIES (μMOL/MIN/MG) OF HUMAN GSH TRANSFERASES

	Class:	Alpha				Mu	Pi
Substrate	Enzyme:	B_1B_1	B_2B_2	Skin "9.9"	α-ϵ	μ	π
1-Chloro-2,4-dinitrobenzene		82	80	—	64	187	105
1,2-Dichloro-4-nitrobenzene		0.25	0.79	—	0.05[a]	0.032	0.11
Bromosulfophthalein		—	—	0	0.005[a]	<0.002	<0.002
Ethacrynic acid		0.11	0.14	0.31	0.03[a]	0.081	0.86
trans-4-Phenyl-3-buten-2-one		0	0	0	0.0015[a]	0.36	0.01
Leukotriene A_4		—	—	—	0.009	0.044	0.002
1,2-Epoxy-3-(*p*-nitrophenoxy)propane		0	0	—	0	0.11	0.37
trans-Stilbene oxide[b]		0.0006	<0.00001	—	0.019	5.2	0.0024
Benzo(a)pyrene 4,5-oxide		—	—	—	0.047	0.92	0.13
Benzo(a)pyrene 7,8-diol-9,10 epoxide		—	—	—	0.038	0.57	0.83
Cumene hydroperoxide		31	104	4.3	10.6	0.63	0.03
H_2O_2		—	—	—	<0.01	<0.01	<0.01
Δ^5-Androstene-3,17-dione		—	—	—	8.0	0.12	—
p-Nitrophenyl acetate		0.66	0.24	—	0.18	0.22	—

Note: Data compiled from References 43, 47, 76, 78, and 79.

[a] Unpublished work by Guthenberg, C. and Mannervik, B.
[b] Unpublished work by Seidegård, J., Danielson, U. H., and Mannervik, B.

Table 9
INHIBITION CHARACTERISTICS, I_{50} VALUES (μM)[a] FOR RAT GSH TRANSFERASES

	Class:	Alpha			Mu			Pi	Microsomal[d]
Inhibitor	Enzyme:	1-1	2-2	8-8[b]	3-3	4-4	6-6[c]	7-7	(activated)
Cibacron blue		0.6	20	0.2	0.25	0.1	0.05	0.3	—
Tributyltin acetate		2	0.5	>100	1	2	10	—	>100
Triethyltin bromide		500	3	>100	1	100	100	100	>10
Triphenyltin chloride		0.5	30	>100	0.1	0.2	30	10	—
Bromosulfophthalein		2	200	—	10	0.5	1	20	1
Hematin		0.1	>10	—	2	1	0.3	4	0.5
Indomethacin		50	100	—	100	1	—	—	—
S-(*p*-bromobenzyl)glutathione		50	2	—	25	6	25	1	—

Note: Data compiled from References 59 and 69.

[a] The I_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6.5, with 1 m*M* 1-chloro-2,4-dinitrobenzene as substrate.
[b] Unpublished work by Jensson, H. and Mannervik, B.
[c] Unpublished work by Tahir, M. K. and Mannervik, B.
[d] Unpublished work by Yalçin, S., Morgenstern, R., and Mannervik, B.

Table 10
INHIBITION CHARACTERISTICS, I_{50} VALUES (μM),[a] FOR MOUSE LIVER GLUTATHIONE TRANSFERASES

Inhibitor	Class: Enzyme:	Alpha N 4-4	Mu N 1-1	Pi N 3-3	Microsomal (activated)
Cibacron blue		10	0.7	0.1	1
Tributyltin acetate		0.1	0.07	4	1.5
Triethyltin bromide		4	0.1	5	—
Triphenyltin chloride		0.3	0.04	10	0.5
Bromosulfophthalein		100	7	70	15
Hematin		0.5	2	4	—
Rose bengal		15	0.7	5	0.15
Indomethacin		>200	200	>200	3
S-hexylglutathione		7	7	10	>100
S-(*p*-bromobenzyl)glutathione		10	15	5	>100

Note: Data compiled from References 35 and 57.

[a] The I_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6.5, with 1 mM 1-chloro-2,4-dinitrobenzene as substrate.

full-length sequences have been deduced from corresponding cDNA structures by use of the "universal" genetic code. So far, all enzymes analyzed as isolated proteins lack the methionine residue encoded by the initiator codon ATG. The structures in Figure 1 are consequently presented without this residue even though direct N-terminal analyses are lacking for some of the proteins. A complication in the analysis is that most members of class Alpha appear to have chemically blocked α-amino groups at the terminus,[83,98,99] which has prevented direct determination of the N-terminal structure. Obviously, chemical analyses of the mature proteins are necessary in order to establish posttranslational processing of the polypeptide chains. Except for the modifications of the N-termini, such as elimination of methionine and masking of the α-amino group, no posttranslational modifications of the glutathione transferases have been noted. Nor have the deduced sequences been found to contain consensus sequences for a modification such as N-glycosylation (cf. Reference 13).

The N-terminal amino acid sequences, when available, appear to give sufficient evidence for classification of the animal glutathione transferases (Figure 1). Homologies within a class are significant and are more obvious than the similarities between sequences from different classes. Sequences in the N-terminal region as well as between positions 60 and 80 appear to be particularly well conserved. By proper alignment, several positions contain the same amino acids throughout all structures. In an evolutionary perspective, it may be inferred that the three classes of animal cytosolic transferases have arisen by divergent evolution from a common ancestral structure.[2,54,100] The separation into distinct classes took place before the divergence of mammalian species since similarities of structures from different species within a class are significantly greater than the similarities of structures from different classes obtained from the same species. The homologies of the glutathione transferases from animal species with those from maize (Figure 1) are sufficiently clear to warrant the assumption of a common evolutionary origin. However, it is not certain that the maize sequences can be referred to any of the classes defined for the animal enzymes. It is possible that these classes emerged in evolution after divergence of the branches leading to animal and plant species.

The microsomal glutathione transferase shows no obvious homology with any of the cytosolic enzymes. The best alignment was obtained with the maize sequences, which may suggest a distant relationship to the microsomal structure. A survey of possible sequence similarities among some glutathione-linked proteins suggested that the cytosolic glutathione transferases are more similar to glutaredoxin (from calf thymus) than they are to the micro-

Table 11
INHIBITION CHARACTERISTICS, I_{50} VALUES (μM)[a] FOR HUMAN GLUTATHIONE TRANSFERASES

	Class:	Alpha					Mu	Pi
Inhibitor	Enzyme:	B_1B_1	B_2B_2	Skin "9.9"	ϵ[b]	α-ϵ	μ	π
Cibacron blue		2.5	24	13	10	4	0.05	0.5
Gossypol acetic acid		—	—	—	—	50	2	>100
Tributyltin acetate		<0.001	0.98	—	0.03	0.1	0.5	4
Triethyltin bromide		1.55	0.145	—	10	10	5	6
Triphenyltin chloride		0.3	1.5	1	0.1	0.25	0.5	>10
Bromosulfophthalein		10.5	125	120	20	75	2	100
Hematin		1.5	40	1	<0.1	0.5	1	5
S-hexylglutathione		4.6	6.6	—	—	3	10	20
S-(*p*-bromobenzyl)glutathione		—	—	—	—	4	1	4

Note: Data compiled from References 47, 76, and 80.

[a] The I_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 6.5, with 1 mM 1-chloro-2,4-dinitrobenzene as substrate.

[b] Unpublished work by Tahir, M. K. and Mannervik, B.

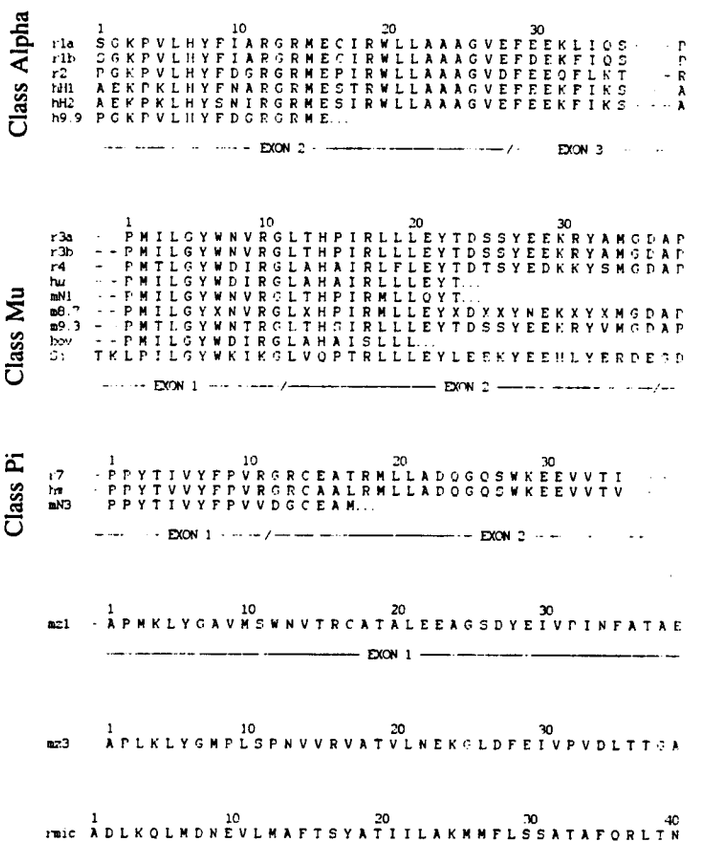


FIGURE 1. Amino acid sequences of glutathione transferases. The primary structures are given without the N-terminal initiator methionine, and full-length sequences are terminated by a colon. The amino acid residues are aligned essentially as described by Persson et al.[89] The classes defined for the cytosolic transferases[54] are indicated to the left. The exons determined for rla,[90] r4,[13] r7,[14,15] and mzl[16] are indicated below the sequences of the class to which the structures have been referred. For mz3, an alternative sequence is given in parenthesis, based on a different ("−1") reading frame for deducing the amino acids in positions 148 to 168, as suggested by Tu et al.[13] Key to sequences (literature references are superscripted): rla = rat subunit 1, Ya, (variant a), pGTB38;[60] rlb = rat subunit 1, Ya, (variant b), pGTR261;[91] r2 = rat subunit 2, Yc, pGTB42;[61] hHl = human subunit H_a-1, pGTH1 or pGST2;[73,92] hH2 = human subunit H_a-2, GTH2;[93] h9.9 = human skin transferase "9.9";[76] r3a = rat subunit 3, Y_b^1, (variant a), pGTA/C44;[62] r3b = rat subunit 3, Y_b^1, (variant b), pGTR200;[94] r4 = rat subunit 4, Y_b^2, pGTA/C48 or pGTR187;[64,65,95] hμ = human transferase μ;[83] mN1 = mouse transferase N 1-1;[54] m8.7 = mouse transferase GT-8.7;[36] m9.3 = mouse transferase GT-9.3;[36] bov = bovine liver transferase;[96] Sj = *Schistosoma japonicum* transferase;[87] r7 = rat subunit 7, Y_p, pGP5;[70] hπ = human transferase π;[75,83,97] mN3 = mouse transferase N 3-3;[54] mz1 = maize transferase I, pMON9000;[16] mz3 = maize transferase III;[88] and rmic = rat microsomal transferase.[72]

somal transferase.[101] The similarities between the cytosolic and the microsomal transferases are found in some of the catalytic properties and in their common specificity for glutathione. Whether these properties are sufficient to define a common ancestry for the two types of enzyme[2] is highly uncertain.

It has been suggested that, in the course of evolution, genetic events that involve recombination of exons have afforded differential catalytic properties to a glutathione-binding

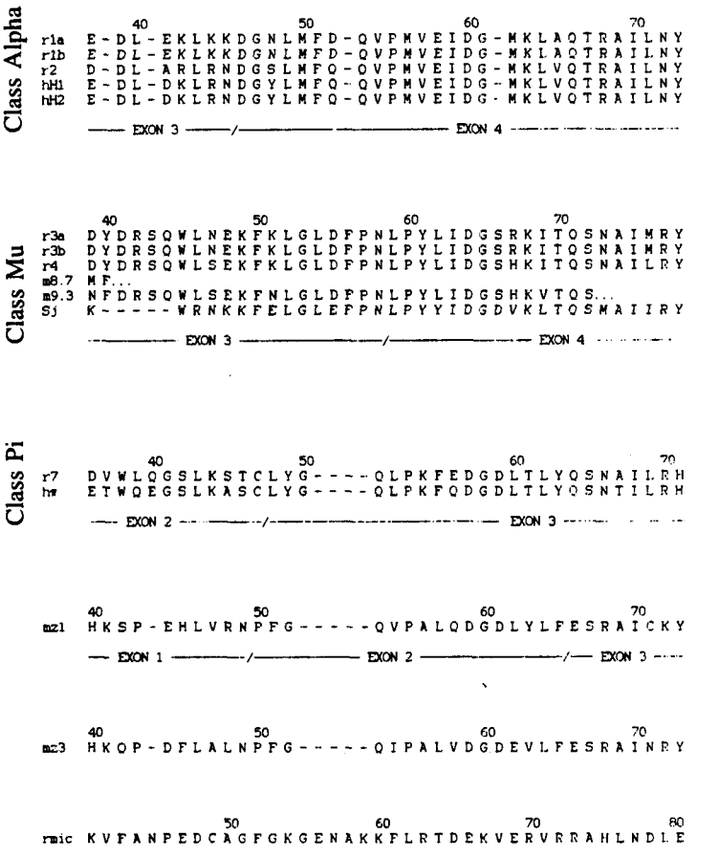


FIGURE 1. (continued)

parent protein.[2] At present, the exon/intron junctions are known for rat glutathione transferase subunit 1a,[12,90] subunit 4,[13] and subunit 7,[14,15] as well as for maize transferase I.[16] The exons established, marked in Figure 1, indicate that certain regions of the primary structures (e.g., those corresponding to exons 2 and 4 of rat subunit 1a in class Alpha) show a higher degree of homology than do others. This differential homology is consistent with the earlier proposal, but the differences in number of exons (a minimum of three and a maximum of eight) as well as the differences in the positions of the splicing sites for the different sequences present difficulties in the inferences about the mechanisms of molecular evolution.

B. Secondary and Tertiary Structures

Little is known about the folding of the polypeptide chain of the glutathione transferases. Crystals of a bovine class Pi enzyme which are suitable for X-ray diffraction analysis have been obtained, but the structure has not yet been solved.[228] In our laboratory, rat transferase 4-4 has been crystallized, but the crystals have not yet been suitable for diffraction studies.

In the absence of definitive data, the three-dimensional structure has been probed by indirect methods. Circular dichroism spectroscopy of "ligandin" has provided estimates of secondary structure.[102] However, the ligandin preparations used are now known to contain

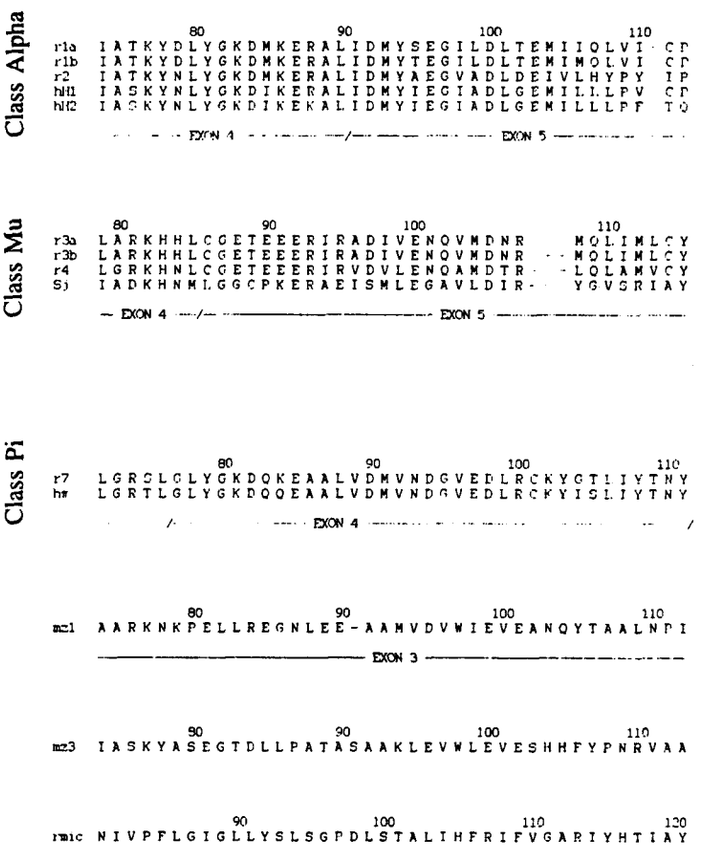


FIGURE 1. (continued)

more than one subunit,[103] a condition which makes the evaluation somewhat obscure. In the case of human glutathione transferase μ, the circular dichroism spectrum indicated 23% α-helix and 25% β-structure.[43] These values are similar to those previously calculated for ligandin. However, it is well known that such spectroscopic data may yield inaccurate estimates of secondary structure, and the values must therefore be regarded with great caution.

A completely theoretical approach is the prediction of secondary structures from the known amino acid sequence. In a recent study,[89] the five rat subunits 1, 2, 3, 4, and 7, which represent all three classes of cytosolic glutathione transferases, were subjected to such an analysis. The predictions of α-helix and β-structures were similar for all sequences and were not significantly different from the estimates obtained by circular dichroism spectroscopy. In a more detailed comparison, it was found that the class Alpha subunits 1 and 2 were mutually more similar in the predicted secondary structure than pairs of subunits from different classes. The same conclusion could be drawn for the class Mu subunits 3 and 4.

In general, proteins have been divided into four classes on the basis of their secondary structures.[104] The predicted secondary structures show that all glutathione transferases should be referred to as α/β proteins, characterized by an alteration of α-helixes and β-strands along the polypeptide chain. It appears possible that the transferases, like many other α/β proteins, have an active-site cavity, which is formed in the region where the C-terminal ends of two adjacent β-strands join α-helixes on opposite sides of a β-sheet.

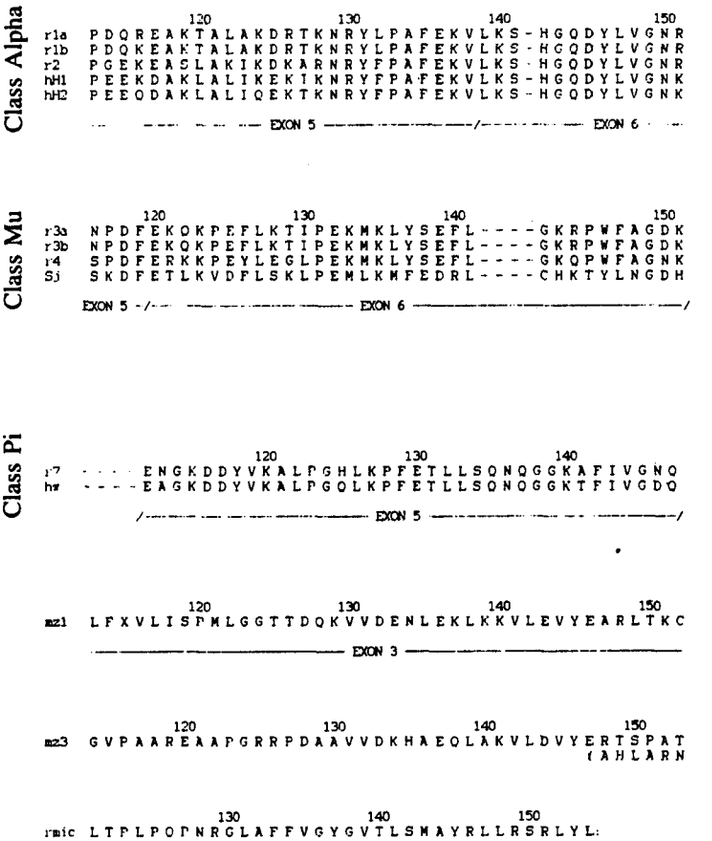


FIGURE 1. (continued)

C. Quaternary Structure

The mammalian cytosolic glutathione transferases all appear to be dimers of identical or similar protein subunits.[2,81] The mammalian microsomal enzyme has been proposed to be a trimer or a tetramer.[72,105] The dimeric structure of the cytosolic transferases is well established, but the quaternary structure of the microsomal enzyme is more difficult to elucidate. *In situ*, the structure of the membrane-associated transferase has to be approached by indirect methods and, in solubilized form, the protein interacts with detergent molecules rather than with the constituents of the membrane.

The interactions between the subunits of the cytosolic glutathione transferases allow stable heterodimeric structures to be formed. Originally, it was found that rat transferase 1-2 (then referred to as ligandin) consists of two nonidentical subunits, which could be separated by SDS-PAGE and which appeared to differ in M_r.[103] It was later found that other heterodimeric structures exist in the rat.[27,33,106-108] It was generalized that corresponding hybrids should be found among, e.g., the human enzymes.[44] More recently, evidence for such heterodimeric human transferases has been obtained.[45-47] The families containing the hybridizable subunits can now be equated with the three classes of cytosolic transferases.[54] Indeed, it has been found that interspecies hybrid forms can be produced in vitro, e.g., between the subunits of human transferase μ and mouse transferase N 1-1.[229] It would seem that subunits belonging to the same class of transferases might be recognized by their ability to hybridize with one another.

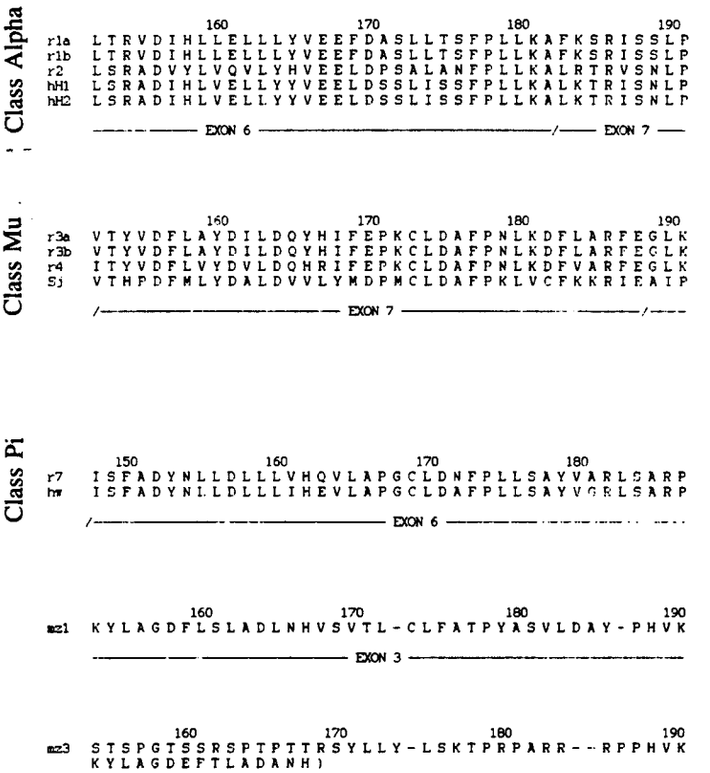


FIGURE 1. (continued)

The functional implications of quaternary structure is a classial problem in protein chemistry.[109] In the case of the various forms of cytosolic glutathione transferase, it was proposed that the active site may be situated at the subunit-subunit interface and that the specificity for the electrophilic substrate would be determined by the particular combination of two protein subunits.[110] Accordingly, the substrate specificity would be altered by recombination of subunits. However, detailed studies of the kinetics of the homodimeric rat transferases 1-1, 2-2, 3-3, and 4-4, as well as the corresponding heterodimeric transferases 1-2 and 3-4, demonstrated that the activities measured with several substrates at different degrees of saturation were additive.[111] Likewise, inhibition studies of homo- and heterodimeric glutathione transferases demonstrated that the kinetic properties of a heterodimer could be accurately predicted from those of the corresponding homodimers.[112,113] Therefore, the available data strongly suggest that the subunits of cytosolic glutathione transferases are kinetically independent and that the subunit-subunit interface does not harbor the active site.

Equilibrium-binding studies involving substrates and products of the catalytic reaction demonstrate that a functional dimeric glutathione transferase has one binding site for glutathione and one for the electrophilic substrate per subunit.[114] The binding studies show simple hyperbolic saturation curves for the substrates and products, suggesting that the significant deviations from Michaelis-Menten kinetics[21,115-117] have to explained by kinetic effects. Hysteretic or "memory" effects in catalysis have been proposed as an explanation for the nonhyperbolic rate behavior (cf. Reference 2).

Several lines of evidence indicate that the cytosolic glutathione transferases may undergo conformational changes. Vander Jagt et al.[118] have reported time-dependent alterations of the kinetic properties caused by bilirubin or proteins (e.g., albumin). Furthermore, in the

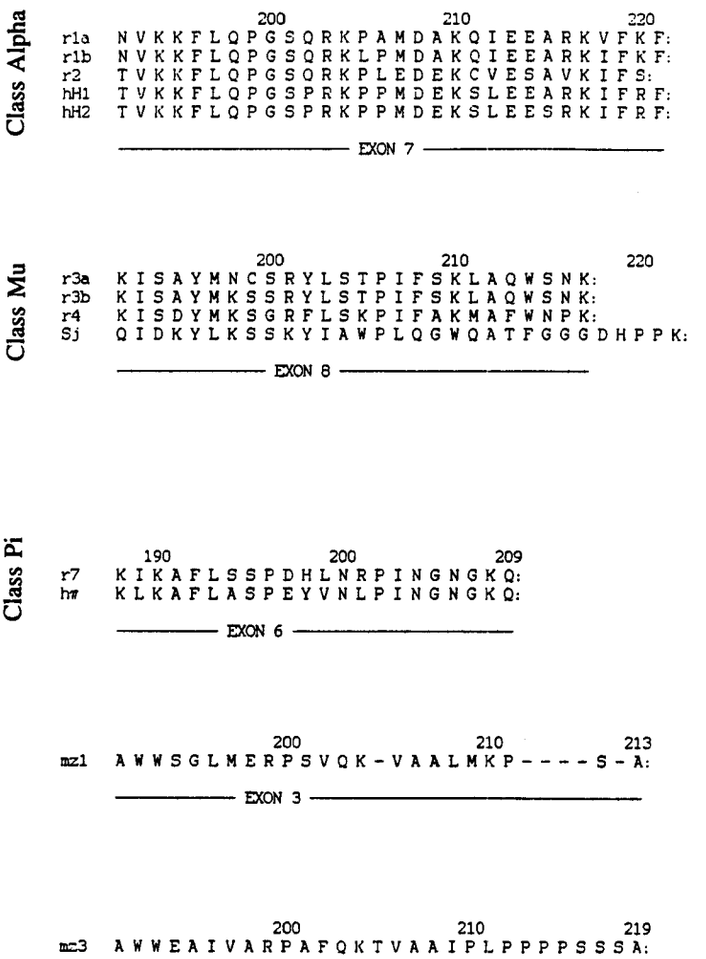


FIGURE 1. (continued)

course of crystallization of transferases, it was noted that the addition of a glutathione derivative markedly influenced the solubility of the protein.[230] All these observations are most readily explained by structural changes in the protein. It remains to be clarified whether such conformational transitions take place on a time scale commensurate with the rate of the reaction catalyzed and thus can be considered as integral events in the catalytic mechanism.

The microsomal glutathione transferase may be activated by *N*-ethylmaleimide[119] or by other sulfhydryl-blocking reagents such as disulfides.[120] Partial proteolysis may also activate the enzyme.[120] In addition, bromosulfophthalein, normally used as an inhibitor, serves as an activator when present in a certain concentration range.[57] A similar activation by sulfhydryl-blocking agents has been reported for a basic transferase from human erythrocytes.[121] The activation of the microsomal glutathione transferase appears to involve a conformational change of the protein which may be effected by diverse chemical means.[120] Whether this structural change is related to that indicated for the cytosolic enzyme forms is not known. In both cases, the nature of the implied conformational changes and the structural level at which they operate are unknown. Further investigations are necessary to find out if the alterations occur primarily at the quaternary or the tertiary levels.

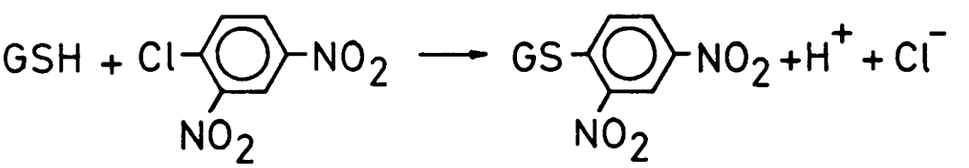
IV. SUBSTRATES

A. Substrates for Characterization of Enzymes

Numerous electrophilic compounds may serve as substrates for glutathione transferases. Comprehensive reviews on the multitude of xenobiotics that may be conjugated with glutathione have been published.[81,122] Many of the substrates are reactive compounds that could react with nucleophilic chemical groups in proteins and nucleic acids and thus cause toxic effects, mutations, and cancer. Originally, the nucleophilic attack catalyzed by the glutathione transferases was considered to be targeted only at an electrophilic carbon atom. Later, it was established that electrophilic nitrogen in nitrate esters, sulfur in organic thiocyanates or disulfides, and oxygen in organic hydroperoxides could serve as alternative targets in the catalyzed reactions.[123]

Most substrates used are products of modern chemical industry and have no biological relevance. Furthermore, most of these compounds give comparatively low enzyme activities. Nevertheless, some of these substrates are valuable tools for the characterization and identification of the different forms of glutathione transferase. The present section is limited to a survey of such substrates.

The single most important substrate used for the demonstration of multiple forms of glutathione transferase in various biological species is 1-chloro-2,4-dinitrobenzene. This compound was originally used as a substrate for the "aryltransferase", but was also recognized as a "general substrate" for the glutathione transferases:[124]

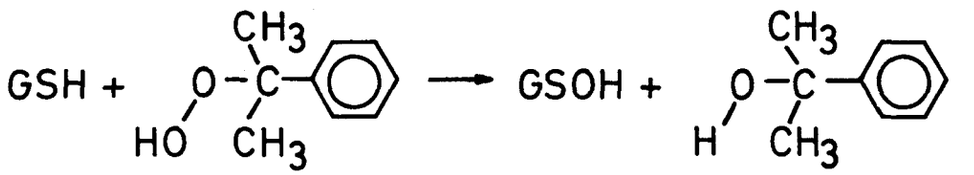


Notwithstanding its significance for detection of glutathione transferase activity, it should be stressed that certain forms of the enzyme express low activity with this substrate. Consequently, some forms may have been overlooked in the samples analyzed exclusively by use of 1-chloro-2,4-dinitrobenzene. Rat glutathione transferase 5-5[66] and two maize transferases[125] are examples of enzymes that display low activity with this substrate. Obviously, several substrates should be used in the screening of new sources of the enzyme.

In some biological species, certain forms of glutathione transferase have been clearly distinguished by their different activities with a given substrate. The maize enzymes mentioned previously, which had low activity with 1-chloro-2,4-dinitrobenzene, were active with the herbicide atrazine.[125] In an earlier study of some animal glutathione transferases, it was similarly found that, although different enzyme forms in the same tissue appeared to show a degree of "cross-specificity" for different substrates, their activity profiles were clearly distinct.[124] For example, sheep liver contains a transferase that displays high activity with methylparathion (dimethyl *p*-nitrophenyl phosphorothionate) and low activity with *S*-crotonyl-*N*-acetylcysteamine as well as another transferase with the opposite relationship between the activities.

More systematic attempts to recognize certain types of mammalian glutathione transferase have been made.[54,84] Even though the original concepts of "aryltransferase", "epoxide-transferase", etc.[19] do not apply as strict descriptors of substrate specificity, it has been found that structurally related glutathione transferases in rat, mouse, and man may display comparatively high specific activities with certain characteristic compounds. Substrates that have proved useful in classifying the mammalian enzymes[54,84] are discussed in the following.

Class Alpha glutathione transferases are highly active with cumene hydroperoxide. The reaction represents the "nonselenium" glutathione peroxidase activity[85,126] and is believed to occur in two steps involving an unstable glutathione sulfenic acid intermediate (GSOH):[127]

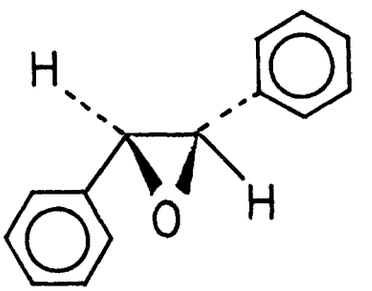


$$GSOH + GSH \rightarrow GSSG + H_2O$$

Reduced glutathione (GSH) is regenerated from the produced glutathione disulfide (GSSG) by the action of glutathione reductase:

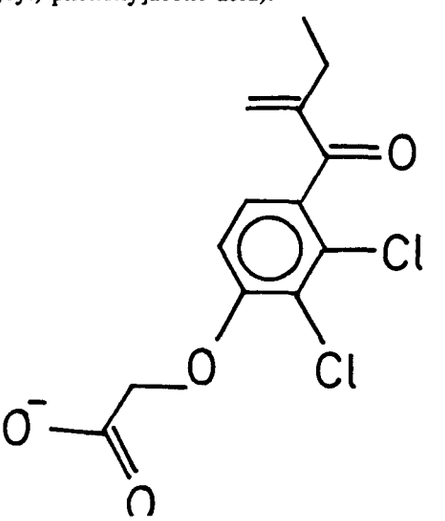
$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Class Mu transferases have been noted to be highly active with epoxides.[43,128] An epoxide that has been found to be particularly useful for identifying human class Mu enzyme is *trans*-stilbene oxide:[129,130]

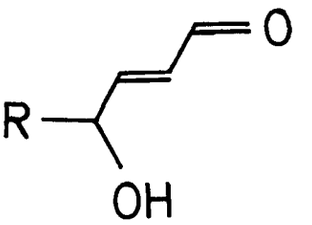


In a recent study, a large number of enzymes from rat, mouse, and man were investigated with this substrate (cf. Tables 6 to 8).[231] The human class Mu transferase, originally referred to as *trans*-stilbene oxide-active glutathione transferase when measured in mononuclear leukocytes,[131] and recently identified with the hepatic transferase μ,[132] has a particularly high activity compared with other enzyme forms tested.

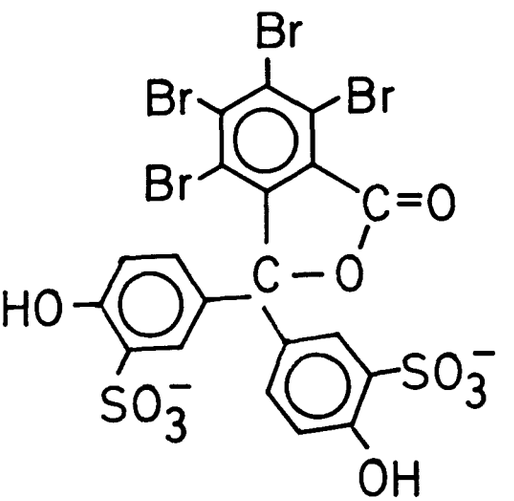
Class Pi transferases display comparatively high activity with ethacrynic acid ([2,3-dichloro-4-(2-methylenebutyryl)-phenoxy]acetic acid):

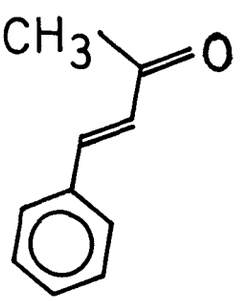


The reaction is a thiol addition to an α,β-unsaturated carbonyl derivative, but this structural feature in itself does not distinguish the class Pi enzymes from other glutathione transferases. The best substrates of this type, so far discovered, are the 4-hydroxyalkenals,[133] but the class Pi enzymes show comparatively low activities with this substrate:[134]



Within a class, different isoenzymes in the same biological species may be further distinguished by the use of additional substrates. For example, the rat class Mu transferases 3-3, 4-4, and 6-6 all express high activities with *trans*-stilbene oxide. However, transferase 3-3 has high activity with bromosulfophthalein and low activity with *trans*-4-phenyl-3-buten-2-one (cf. Table 6):





Transferase 4-4 has low activity with bromosulfophthalein and high activity with *trans*-phenylbutenone, whereas transferase 6-6 has neglibible activity with both of these substrates.

Even if the above-noted class-distinguishing substrates have been useful, they do not give a definitive distinction between transferases of different classes. A single substrate, in most cases, gives specific activities for members of a given class, which fall in a range that partly overlaps the range of the values of another class. Figure 2 shows two-dimensional representations of some of the specific activities obtained with two different substrates. The rectangular areas mark the domains within which values of a particular class have been found. Figure 2A demonstrates the problem of partial overlap, whereas Figure 2B illustrates the more uncommon case of distinct separation of values for different classes.

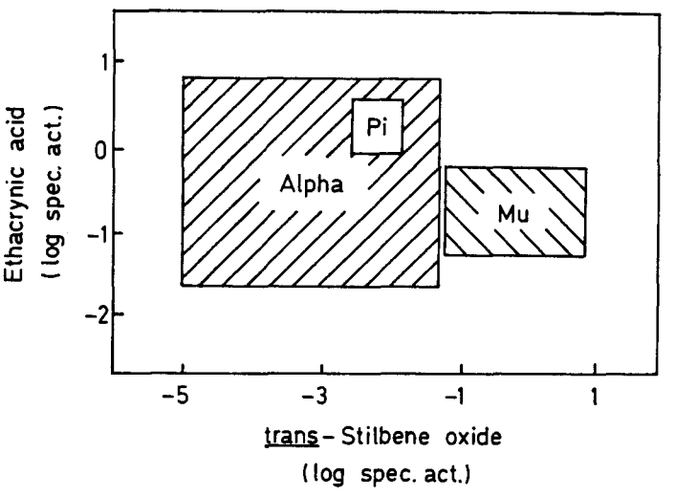
All possible combinations of two substrates, or other distinguishing variables, could be analyzed in the manner shown in Figure 2 in order to find the best descriptors of a given class. However, more powerful results can be obtained by multivariate analysis.[135] In such an analysis, it may be found that a larger number of variables ($n > 2$) may be represented, by a reasonable approximation, by two derived variables in two-dimensional space. For example, specific activities with two different substrates and the I_{50} value for an inhibitor can be plotted as a point in three dimensions for each of a number of enzymes undergoing analysis (Figure 3). A two-dimensional plane is fitted as closely as possible to the set of points. The axes of this plane are the two first principal components of the analysis, and the points corresponding to the different enzymes are projected onto the plane. Such a two-dimensional projection can be made for any finite number of variables and may reveal characteristic features of objects analyzed which do not appear by simpler treatments.

An analysis of this kind was made on the basis of a data set involving specific activities obtained with 9 substrates and I_{50} values for 11 inhibitors determined for 15 different forms of mammalian glutathione transferase.[54] The 15 points in the 20-dimensional space of specific activities and I_{50} values were clustered into 3 regions in the plane defined by the 2 principal components (Figure 4). Each of the three regions contained the members of a class of glutathione transferase as defined by other functional and structural properties of the enzymes.

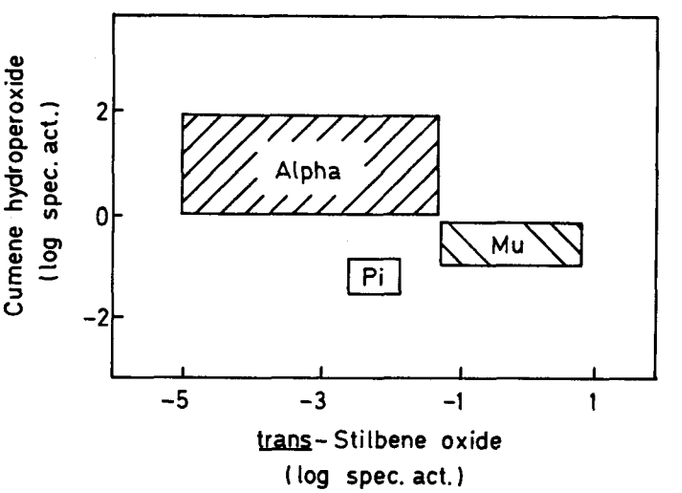
It would appear plausible that the 15 transferases analyzed in the case cited could have been classified on the basis of less than 20 variables. The multivariate analysis may be applied to identify the best descriptors to use for the characterization. Obviously, multivariate analysis may have a general application in the analysis and classification of other families of enzymes or proteins.

B. Structure-Activity Relationships

Only a few of the substrates of glutathione transferases have been used to probe the active site and the reaction mechanism in a more systematic manner. Early studies of some of the purified rat enzymes involved chloronitrobenzene derivatives, organic thiocyanates, and nitrate esters[136] as well as disulfide interchange, *cis-trans* isomerization of maleic acid derivatives, and thiolysis of *p*-nitrophenyl esters.[137] The results supported a model for catalysis involving nucleophilic attack of enzyme-bound glutathione on the electrophilic center of a juxtaposed nonpolar substrate. Hammett plots of the k_{cat} values determined for rat transferases 1-2 and 3-4 acting on six 4-substituted 1-chloro-2-nitrobenzenes were essentially linear when k_{cat} values were correlated with the resonance σ^- values.[136] The k_{cat} values increased with the electron-withdrawing capacity of the *para* substituent of the substrate. Deviations from straight lines observed may be due to the fact that the enzymes used were heterodimeric proteins, which was not known at the time the experiments were performed. Nevertheless, the data indicate that the k_{cat} value is governed principally by the electrophilicity of the substrate reacting with glutathione. This mechanistic feature may be particularly important for aromatic substitution reactions since the attainment of the transition state is expected to involve the introduction of the negatively charged electron pair of the glutathione



A



B

FIGURE 2. Two-dimensional ranges of specific activities for mammalian glutathione transferases of classes Alpha, Mu, and Pi. Each rectangular domain encloses the specific activities determined for members of the class indicated. (Based on data in Tables 6 to 8.) (A) In one dimension, all three ranges are overlapping, but in two dimensions, class Mu is distinct from classes Alpha and Pi. (B) In two dimensions, the values for all three classes are well separated.

thiolate into the aromatic benzene ring. However, the systematic studies involving 4-hydroxyalkenals (see Reference 134, and below) demonstrate that electrophilicity is not the only factor responsible for the considerable differences in activities obtained with homologous substrates.

A limited study of the inhibitory effect of *S*-alkylglutathiones on rat glutathione transferases 3-3 and 3-4 demonstrated that longer alkyl chains provided more potent inhibitors.[21] Since these compounds are competitive with glutathione[114,116,117] and probably act at the active site, these results indicate the importance of hydrophobic interactions for binding.

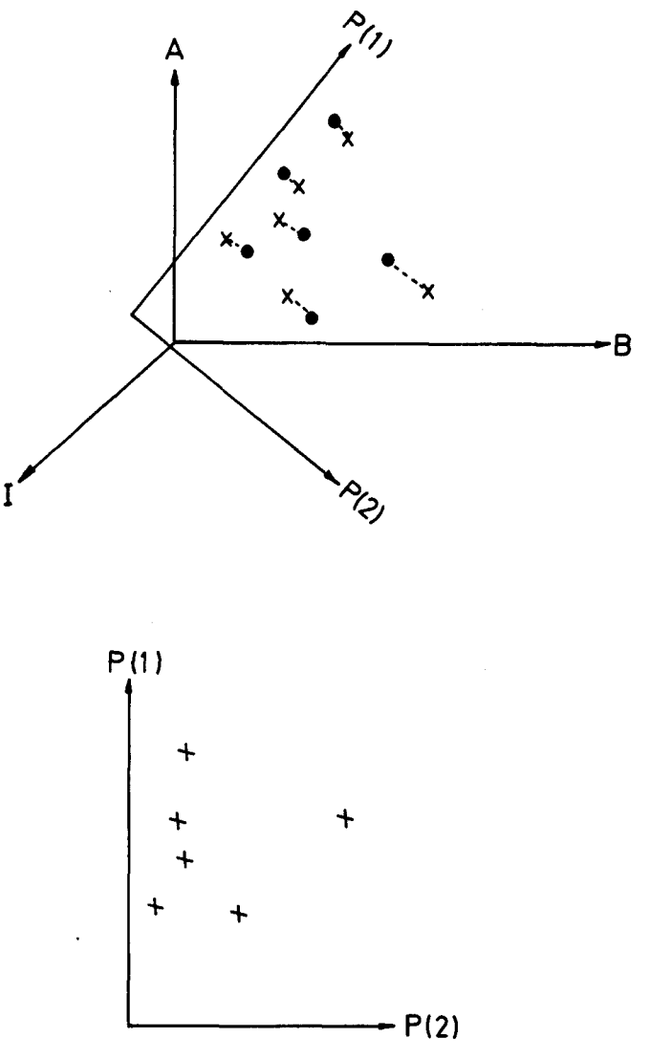


FIGURE 3. Pattern recognition in a set of enzymes by multivariate analysis of catalytic properties. Each enzyme is represented by a point in three-dimensional space, defined by specific activities with two substrates, A and B, and the I_{50} value obtained with an inhibitor, I. A plane spanned by orthogonal axes is fitted to the set of points, and the projections of the points to the plane represent the enzymes in the coordinates of the principal components P(1) and P(2) in the two-dimensional subspace.

The significance of hydrophobic interactions was explored recently by detailed studies of a homologous series (C_5 to C_{15}) of 4-hydroxyalkenals.[134] Some 15 cytosolic glutathione transferases from rat, mouse, and man were characterized by means of their respective k_{cat}/K_m values, which were determined for each of the 10 substrates.

Interpreted according to transition-state theory, k_{cat}/K_m is related to the activation energy, ΔG_T, required to bring free enzyme and free substrate to the transition state. In a homologous series of substrates, the incremental Gibbs free energy:

$$\Delta\Delta G_b = -RT \ln \frac{(k_{cat}/K_m)_A}{(k_{cat}/K_m)_B} \quad (1)$$

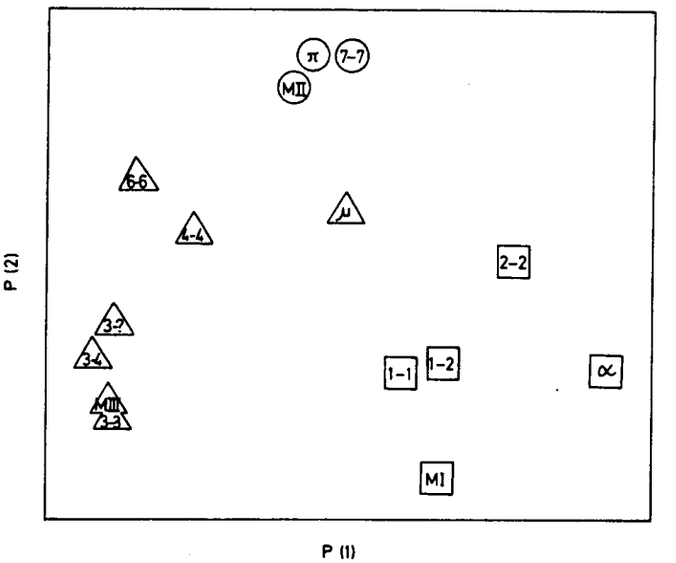


FIGURE 4. Pattern recognition analysis of mammalian glutathione transferases. Each point represents an enzyme in the plane of the principal components P(1) and P(2) obtained by multivariate analysis. The coordinates were computed from specific activities determined with 9 different substrates and I_{50} values for 11 inhibitors for a set of 15 enzymes from rat, mouse, and man. The shapes of the symbols indicate classes Alpha, (□), Mu (△), and Pi (○). (From Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M., and Jörnvall, H., *Proc. Natl. Acad. Sci. U.S.A.*, 82, 7202, 1985. With permission.)

is a measure of the energy difference change involved in transferring substrate A rather than substrate B from enzyme to water. In other words, $\Delta\Delta G_b$ measures the change in binding energy between enzyme and substrate in the transition-state complex if A is substituted for B.[138] Figure 5 displays some of the results obtained with glutathione transferases and 4-hydroxyalkenals.

In general, glutathione transferases display increased binding energies in response to increased hydrophobicity of the 4-hydroxyalkenal substrate.[134] Each CH_2 group of the substrate contributes approximately 2.9 kJ/mol at 30°C. However, for most of the enzymes, steric limitations of the active site appear to offset the increased binding energy expected for the higher homologs in the series.

The structure-activity relationships determined with the 4-hydroxyalkenals divided the 15 mammalian glutathione tranferases into 3 groups according to their responses to increased chain length of the substrate.[134] However, these three groups, each containing enzymes from rat, mouse, and man, did not correspond to the three classes of glutathione transferase rigorously defined by primary structures.[54] Whether these groups as such have a functional significance in vivo remains to be established.

C. Endogenous Substrates

Most of the chemical compounds studied as substrates for glutathione transferases do not occur naturally and have no significance in relation to the true biological function of the enzymes, even if some of them may be relevant to toxicology. However, it has been stressed that oxidative metabolism of a variety of endogenous substances gives rise to reactive electrophiles that should be considered possible "natural" substrates.[100,128,139] In an evo-

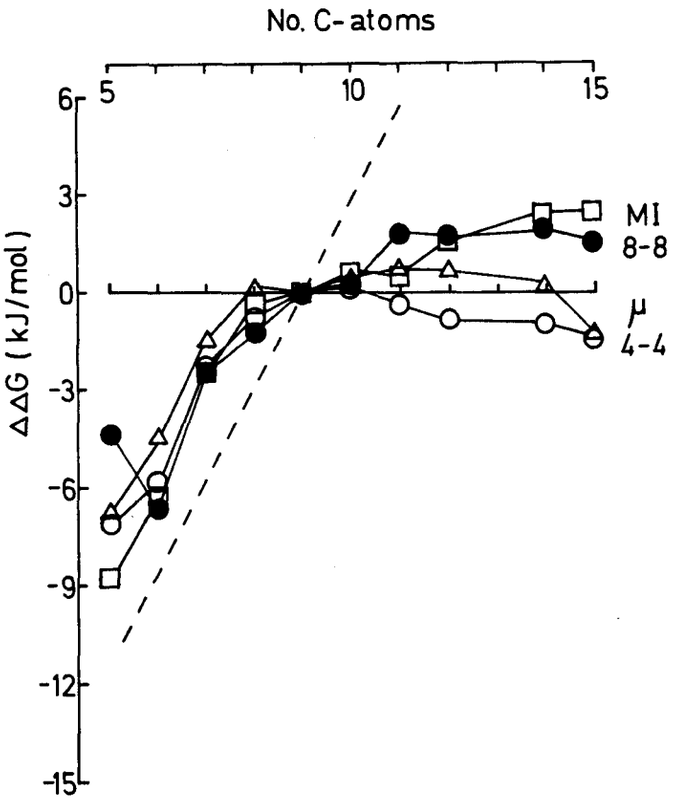


FIGURE 5. Incremental Gibbs free energy for the binding of homologous 4-hydroxyalkenals to glutathione transferases. The values are calculated from Equation 1 with 4-hydroxynonenal as the reference compound. The dashed line represents an incremental binding energy of 2.9 kJ/mol for each CH_2. (From Danielson, U. H., Esterbauer, H., and Mannervik, B., *Biochem. J.*, 247, 707, 1987. With permission.)

lutionary perspective, it appears that glutathione emerged as an important biomolecule when oxygen became an abundant component of the atmosphere.[1] Consequently, it has been proposed that glutathione-dependent enzymes, including the transferases, evolved in aerobic organisms in response to the requirements of inactivation of toxic products of oxygen metabolism.[100] Substances containing carbon-carbon double bonds may be particularly prone to yield reactive oxidation products. Aromatic compounds and polyunsaturated fatty acids are abundant biomolecules of this kind. In addition to its endogenous compounds, an organism is also exposed to a wide range of potentially toxic compounds produced by other species in the biosphere.[140] Some of the possible types of substrates that may be biologically important are given in the following.

Quinones represent one class of reactive compounds which may be detoxified by glutathione conjugation. For example, the major phenolic product in grape juice is 2-*S*-glutathionylcaftaric acid.[141] Menadione (2-methyl-1,4-napthoquinone) has been shown to undergo enzymatic glutathione conjugation.[142] A possible endogenous substrate is dopaquinone, which has been shown to be conjugated with glutathione in human malignant melanoma.[143] This tumor has been found to contain a high concentration of class Pi glutathione transferase.[144] Purified rat glutathione transferase 1-2 (but not tranferase 3-3 or 3-4) has been shown to catalyze the further metabolism of the benzo(a)pyrene 1,6- and 3,6-quinones produced by

microsomal oxidation of benzo(a)pyrene.[145,146] However, the chemistry of the reaction of quinones with thiols involves redox processes as well as thioether formation,[147-149] and further investigations are required to clarify the transformations catalyzed by glutathione transferases.

Organic hydroperoxides, but not the less hydrophobic H_2O_2, are substrates for glutathione transferases.[85,126] A study involving seven cytosolic homodimeric rat transferases demonstrated that linoleate hydroperoxide and arachidonate hydroperoxide in most cases gave activities comparable to the model substrate cumene hydroperoxide.[150] The enzyme displaying the highest specific activity was glutathione transferase 5-5. Thymine hydroperoxides and "DNA hydroperoxides" have been shown to react with glutathione in the presence of rat liver cytosol.[151] One of the thymine derivatives, 5-hydroperoxymethyl uracil, and peroxidized DNA were shown to be substrates for purified rat transferases.[150] In the case of peroxidized DNA, the class Mu enzymes, especially transferase 4-4, were shown to have the highest activities. With hydroperoxides previously used, class Alpha enzymes have been found to show the highest values.[54,128]

Epoxides is a third group of substrates that can be formed by oxidation of carbon-carbon double bonds in vivo. Epoxides may be derivatives of naturally occurring compounds as well as of xenobiotics and are known as mutagenic and carcinogenic substances. Nearly 50 arene oxides or aliphatic epoxides were tested as substrates with purified sheep glutathione transferase.[152] Unfortunately, the number and nature of the enzyme forms present in the enzyme preparation are unknown, but the study shows that almost all epoxides give some activity. Rat glutathione transferase 5-5 was the first pure enzyme shown to have significant activitity with epoxides.[153]

Arachidonic acid is an important polyunsaturated fatty acid that gives rise to several epoxide derivatives. Arachidonic acid oxides[154] as well as leukotriene A_4 and its nonphysiological methylester[78,155] have been shown to serve as substrates for the well-characterized rat and human glutathione transferases. Cholesterol α-epoxide (5α, 6α-epoxycholestan-3β-ol) is another naturally occurring substrate for the enzymes.[156,157]

Alkenes — in many cases α,β-unsaturated carbonyl compounds — are produced during lipid peroxidation. In this group, 4-hydroxyalkenals have been shown to be excellent substrates for glutathione transferases.[22,133,134,158] In the case of rat transferase 8-8, these compounds are by far the best substrates found.[22,134]

These examples lead to the conclusion that several types of toxic electrophiles which are produced intracellularly may function as "natural" substrates for the glutathione transferases. The variety of functional groups and the carbon skeletons to which they are attached may be one of the causes why so many different forms of glutathione transferase have evolved.

D. Substrates Related to Cancer and Drug Resistance

Many chemical carcinogens are electrophilic compounds that may be inactivated by reaction with glutathione.[122] Such detoxication reactions may inhibit binding of carcinogens to DNA and thereby prevent initiation of cancer. It is also possible that compounds acting as tumor promoters are inactivated by reactions involving glutathione.

Epoxides represent an important group of mutagenic and carcinogenic compounds which illustrate important aspects of the role of glutathione transferases in detoxication. In general, class Mu enzymes are the transferases that show the highest activities with most epoxides.[54,128] The human enzymes show very clear differences in their specific activities, e.g., using styrene 7,8-oxide as substrate. This compound is a metabolite of styrene, to which workers in chemical industries may be exposed.[43] Transferase μ has an activity more than 100 times higher than the class Alpha enzymes and approximately 20 times the activity of transferase π. Similar relationships between the activities have been noted for benzo(a)pyrene 4,5-oxide[43] and pyrene 4,5-oxide.[159] Even larger differences appear when *trans*-stilbene oxide is used (cf. Table 8). The physiological significance and the toxicological implications of

the differences are not completely understood, but it has been proposed that transferase μ may have an especially important role in the detoxication of geno- and cytotoxic epoxides.[43] Particularly noteworthy is the discovery that only 60% of the human population express transferase μ,[41,43] and a survey of smokers demonstrated[130] that individuals lacking transferase μ had a significantly higher incidence of lung cancer than those who display transferase μ activity (*trans*-stilbene oxide activity).

In relation to the carcinogenicity of benzo(a)pyrene, its metabolite benzo(a)pyrene-7,8-diol-9,10-oxide is considered an ultimate carcinogen. This diolepoxide, like other epoxides, is a substrate for the class Mu glutathione transferases, but is an even more efficient substrate for the class Pi transferases.[79] Class Alpha transferases have low activities with this substrate.

In the discussion of the relationship of glutathione conjugation of DNA binding, mutagenesis, and cancer, it also should be noted that some chemicals are activated by the conjugation reaction.[160] *vic*-Dihaloalkenes are such compounds that have been shown to give mutagenic glutathione derivatives in the presence of glutathione transferase.[160-162] A reactive episulfonium derivative, arising from the primary *S*-(2-haloethyl)glutathione conjugate, is assumed to be the mutagenic alkylating species and a corresponding S-[2-(N^7-guanyl)ethyl]glutathione has been isolated from modified DNA.[163] These findings show that a general protective effect of glutathione conjugation is not always true.

Another undesired effect of glutathione-dependent reactions appears to be the inactivation of drugs used in cancer chemotherapy. Several lines of evidence suggest that glutathione and glutathione transferases are involved in drug resistance.[164-166] The cellular resistance acquired by extended drug exposure has in many cases been found to be associated with elevated levels of glutathione transferase, especially the class Pi enzyme.[164,167-170] Furthermore, glutathione transferase-dependent conjugates of the alkylating drug melphalan have been demonstrated.[171]

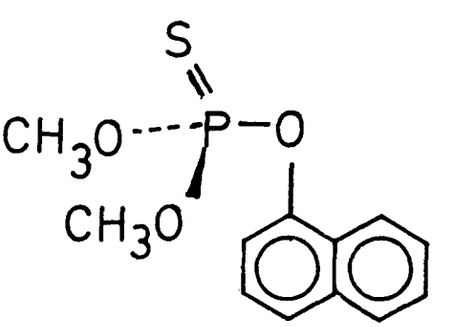
Little is known about the specificities of the different types of glutathione transferase for chemotherapeutic compounds. However, recent studies have demonstrated that class Mu enzymes are particularly effective in the inactivation of *bis*-(2-chloroethyl)-1-nitrosourea.[172]

Consequently, it would appear that the investigation of antineoplastic drugs as substrates for the different types of glutathione transferase would be an important area of future research.

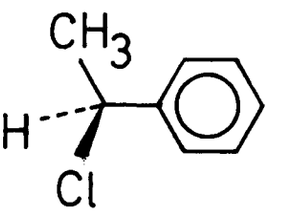
E. Stereoselectivity in Catalysis

Some of the substrates of glutathione transferase contain chiral or prochiral centers, which are involved in the enzymatic reaction. Like most enzymes, the glutathione transferases display stereoselectivity in the catalysis.

Phosphate ester demethylation catalyzed by glutathione transferases from pig liver was shown to have a high stereoselectivity.[173] For example, a 90% stereoselective removal of a methyl group from dimethyl 1-naphthylphosphorothionate was noted. In this nucleophilic displacement, the prochiral phosphorus atom is one atom removed from the methyl group attacked:



A second type of displacement reaction involves the benzylic carbon of phenethyl halides:



Partially purified enzymes from rat liver catalyzed the conjugation of glutathione with racemic or enantiomerically enriched 1-chloro-1-phenylethane.[174,175] Transferase 3-3 was the most active enzyme and also the one demonstrating the highest enantioselectivity of the enzyme forms tested. For all enzymes, the preferred enantiomer had the *S* absolute stereochemical configuration at the benzylic carbon. In a study involving administration of styrene and styrene oxide to rats, the stereochemistry of the metabolites pointed to a similar preference of the glutathione transferases for the *S*-configured benzyl position.[176] An unresolved mixture of rat transferases showed the opposite enantioselectivity in a series of single aliphatic oxiranes, i.e., the *R*-enantiomer reacted more rapidly than the *S*-antipode.[177] However, different glutathione transferases may have opposite stereoselectivities, which explains why the cytosol fractions of various rat tissues, known to differ in enzyme compositions, display distinct stereoselectivities with styrene oxide as well as with some arene oxides.[178]

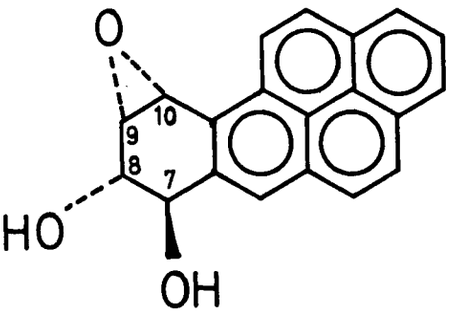
The human glutathione transferases μ and π showed a slight (1.3- to 1.8-fold) enantiopreference for (7*S*)-styrene oxide.[159] Transferase π selectively catalyzed an attack on the benzylic oxirane carbon (C-7), whereas the poorly active class Alpha enzymes catalyzed the reaction with the terminal epoxide carbon (C-8). Transferase μ catalyzed glutathione conjugation to both of these positions.

The stereochemistry of rat glutathione transferases in the conjugation of arene oxides has been studied by Armstrong and co-workers.[179,180] Transferase 4-4 was stereospecific in attacking the oxirane carbon of *R* absolute configuration in a series of K-region arene oxides, including phenanthrene 9,10-oxide. Substitution of nitrogen for carbon in various positions in the biphenyl ring system caused decreased hydrophobicity and a loss in stereospecificity, suggesting that hydrophobic interactions between substrate and the active site are important for maintaining the stereoselectivity. In contrast, the homologous transferases 3-3 and the heterodimeric transferase 1-2 showed little stereoselectivity toward both arene and aza-arene oxides.

In agreement with the results obtained with the rat enzymes,[179,180] glutathione transferase from little skate was found to be selective for glutathione conjugation with *R*-configured carbon of K-region arene oxides, such as benzo(a)pyrene 4,5-oxide and pyrene 4,5-oxide.[181]

The human glutathione transferase μ showed a similar high selectivity for *R*-configured oxirane carbon as the homologous rat transferase 4-4.[159] The opposite stereoselectivity (*S* preference) was demonstrated for human transferase π, whereas the stereopreference of the class Alpha enzymes was low.

From the toxicological point of view, the stereochemistry of the bay-region diolepoxide of benzo(a)pyrene is even more interesting than that of the K-region arene oxides. Several rat transferases were tested with (±)-*anti*-benzo(a)pyrene 7,8-diol 9,10-oxide, and the most active enzymes were found to react almost exclusively with the tumorigenic (+)-enantiomer of *R,S,S,R*, absolute stereochemical configuration.[182,183] The most active enzyme was the class Pi transferase 7-7.[38] The position of glutathione attachment is not known, and the stereoselectivity would be different for a reaction with C-9 (*S*-configuration) than that with C-10 (*R*-configuration):



In the case of the human enzymes, it was also demonstrated that the class Pi transferase π was the most efficient catalyst with *anti*-benzo(a)pyrene 7,8-diol 9,10-oxide.[79] Tranferase μ was the second most active human enzyme, like the class Mu transferase 4-4 in the rat, but in distinction from the other active enzymes, transferase μ was equally efficient with both the (+)- and the (−)-enantiomers.

Obviously, marked differences in substrate stereo- and regioselectivities exist between the glutathione transferases. Stereoselectivity in product inhibition of rat transferases has also been demonstrated with several glutathione conjugates.[175,184] Such studies may help to probe the chirality and topography of the active site.

F. Catalytic Mechanism

Detailed information about the structure and function of the active site of the glutathione transferases is lacking, but some general conclusions can be drawn. Each subunit has a complete active site, which does not appear to contribute to the catalytic properties of the active site of the neighboring subunit.[111-113,180] Even though the steady-state kinetics deviate from Michaelis-Menten behavior, equilibrium-binding experiments do not show any significant deviations from hyperbolic binding isotherms for substrates and products of the enzymatic reaction.[114]

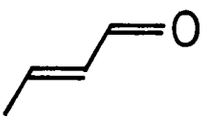
Each active site has a binding site for glutathione and an adjacent, partly hydrophobic binding site for the electrophilic substrate. These subsites of the active-site cavity have been referred to as the G- and H-site, respectively.[185] The specificity of the G-site is high, even if some derivatives of glutathione, such as homoglutathione[23] and γ-glutamylcysteine,[186] have been shown to serve as alternative thiol substrates. The binding of glutathione appears to involve ionic bonds; evidence for arginine residues interacting with the carboxyl groups of glutathione has been obtained.[187]

The high specificity for the thiol substrate glutathione has been puzzling in view of the broad specificity for the electrophilic substrate. It would seem possible that a small molecule such as 2-mercaptoethanol should be capable of serving as an alternative substrate by occupying the active-site position that accommodates the thiol group of glutathione. The suggested glutathione-induced conformational change (see Section III.C) would be a mechanism affording the specificity observed, if it is assumed that only thiols bound to the protein conformation stabilized by glutathione could acquire the proper orientation for catalysis to occur. Only glutathione and structurally related molecules are assumed to induce the necessary conformational change. This proposal would explain the thiol specificity as well as the deviations from Michaelis-Menten kinetics.

One essential component of the catalytic mechanism is the activation of the thiol group of glutathione, presumably by a base-assisted deprotonation. Another concerns the activation of the electrophilic substrate. The latter component has been more difficult to approach since the substrates used have often been nonphysiological and not always very active as substrates in comparison to the relevant substrates for enzymes in general. The finding that 4-hydrox-

yalkenals give very high specific activities with the glutathione transferases and the notion that they may represent biologically important substrates provide a foundation for a more meaningful analysis of the catalytic mechanism.

The glutathione conjugation of 4-hydroxyalkenals is fundamentally a thiol addition to an α,β-unsaturated carbonyl compound similar to the classical Michaelis reaction.[188] The basic structural elements of the alkenal are the carbon-carbon double bond and the adjacent carbonyl group, which activates the double bond:

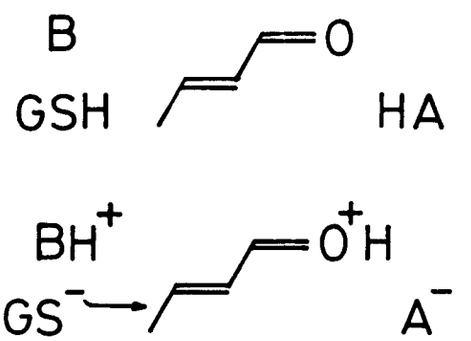


This system of conjugated double bonds can be recognized in several of the established substrates for glutathione transferases and may be one of the structural determinants for which the enzyme specificity has evolved.[133,134,189] In addition to the alkenals, *trans*-phenylbutenone, ethacrynic acid, and quinones are substrates containing this electrophilic structural element.

The α,β-unsaturated carbonyl configuration is found in numerous natural products, few of which have been tested as substrates. Some examples are piperine in black pepper, piperitone in peppermint oil, and citral in citrus fruit oil.

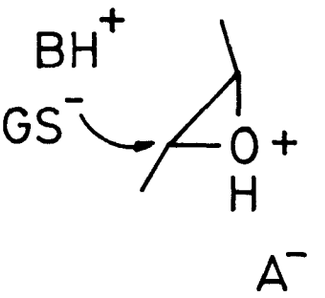
Conjugate addition reactions of α,β-unsaturated carbonyl compounds normally involve nucleophilic attack on the β alkene carbon. Bases are expected to increase the reactivity of the attacking nucleophile. Furthermore, the reaction is facilitated by protonation of the carbonyl oxygen, which increases the electrophilicity of the β carbon.

Consequently, it may be proposed that the active site of glutathione transferases catalyzing this type of reaction should contain a base (B), facilitating the deprotonation of the thiol group of glutathione, and an acid (HA), polarizing the carbonyl function:

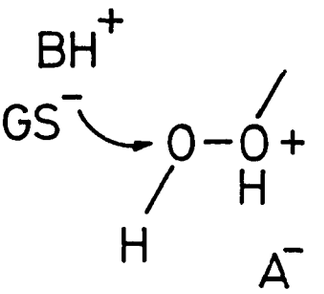


In principle, a Lewis acid such as a metal ion could serve the second purpose, but there is no evidence for essential metal ions in the transferases. Finally, a proton should be delivered to the α carbon of the alkene. This final step in the mechanism requires a proton donor, which could be the conjugate acid of the original base, an additional group of the enzyme, or simply a hydronium ion of the aqueous reaction medium.

It should be noted that the minimal requirement of a base and an acid for the most efficient catalysis also applies to the reaction between glutathione and epoxides. Protonation of the oxygen of the oxirane structure increases the electrophilicity of the adjacent carbon atom:



Similarly, the reaction between glutathione and organic hydroperoxides would be facilitated by a corresponding mechanism involving protonation of an oxygen atom:



The chemical nature of the groups proposed in the active site is not known, but an imidazole of a histidine residue is a possible base and a carboxyl group of a glutamic or an aspartic acid residue is a possible Brønsted acid. Both groups would be expected to be uncharged at neutral pH if positioned in a hydrophobic environment. It may be significant that all primary structures elucidated have a histidine in the vicinity of position No. 165 (Nos. 154 to 168).[233] An Asp-Gly sequence near position No. 65 is conserved through all amino acid sequences analyzed, except for the microsomal enzyme, but several other positions are possible for the proposed acidic group of the mechanism. Nevertheless, the formulation of possible requirements for the catalytic mechanism should be helpful in the design of decisive chemical modification and site-directed mutagenesis experiments.

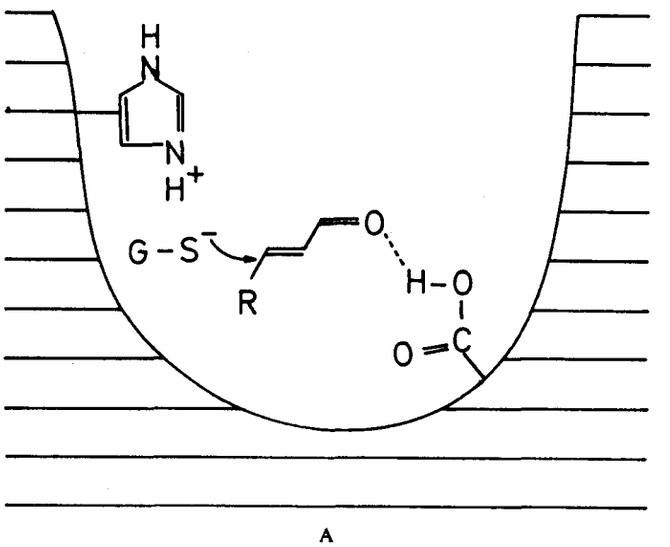
A hypothetical H-site with 4-hydroxyalkenal, an epoxide, or a hydroperoxide as the bound electrophilic substrate in presented in Figure 6. Knowledge about the true steric relationships between the proposed catalytic groups of the enzyme, the thiol group of glutathione, and the substrate molecule would explain the observed stereo- and regioselectivities.

V. INHIBITION

A. General Aspects

Inhibition studies are classic approaches to the study of enzymes in vitro as well as in vivo.[190-192] Inhibitors that are substrate analogs may be used to probe the active site and the catalytic mechanism. Some inhibitors may be suitable as ligands in affinity chromatography. Others may act in vivo and be involved in normal cellular control mechanisms or be pharmacologically useful substances. In the case of isoenzymes or other multiple forms of an enzyme with the same catalytic activity, inhibition studies may help to distinguish the different enzyme species.

Consequently, a number of reasonably effective inhibitors for glutathione transferase have



A

FIGURE 6. Hypothetical mechanisms for the glutathione transferase-catalyzed reactions with (A) 4-hydroxyalkenals, (B) epoxides, and (C) organic hydroperoxides.

been listed in Table 12 with an approximate ranking of their inhibition strengths. Absolute values are not directly comparable since the original data cited include I_{50} values, true inhibition constants (K_i), or simply percent inhibition at a given inhibitor concentration. Furthermore, the use of enzymes from different biological species in some cases makes it impossible to compare the relative effectiveness of different compounds. Table 12 may serve as a source of information about the type of compounds that have been tested and may be of use in the design of other substances for the various applications previously indicated. Detailed information about the experimental protocols, the enzyme preparations used, and the evaluation of the inhibitory effect may be found in the original references cited in the table.

Glutathione derivatives are substrate and product analogs and consequently are potential inhibitors for glutathione transferases. The use of such compounds as affinity ligands and as mechanistic probes has been reviewed previously.[2] For some inhibitors, such as steroids and porphyrin derivatives, a second binding site, distinct from the active site, appears to exist on the protein.[221-223] The finding that steroids such as bile acids and steroid hormone derivatives are relatively strong inhibitors indicates a binding site suited for these structures and suggests that the glutathione transferases have a role in steroid metabolism. Similarly, the tight binding of hematin and other porphyrin derivatives is instrumental in the suggested intracellular transport function of some enzyme forms.[3,224]

In the group of pharmacologically active drugs, the strong inhibition of a rat liver transferase by indomethacin[199,200] has been ascribed to transferase 4-4.[59] The selective effect of this anti-inflammatory drug seemed to be of special significance in view of the finding that transferase 4-4 is the most active cytosolic enzyme in the conjugation of leukotriene A_4 to give leukotriene C_4.[155] However, the selective inhibitory effect is only observed at high concentrations of the substrate 1-chloro-2,4-dinitrobenzene, and not with other substrates.[225] Furthermore, the main responsibility for the biosynthesis of leukotriene C_4 appears to be associated with a distinct membrane-bound enzyme, leukotriene C synthase, and not with the cytosolic or the microsomal forms of glutathione transferase previously studied.[77] The

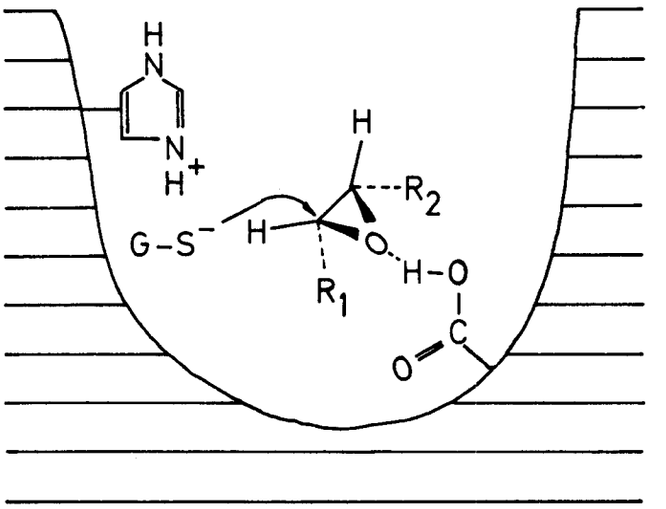


FIGURE 6B.

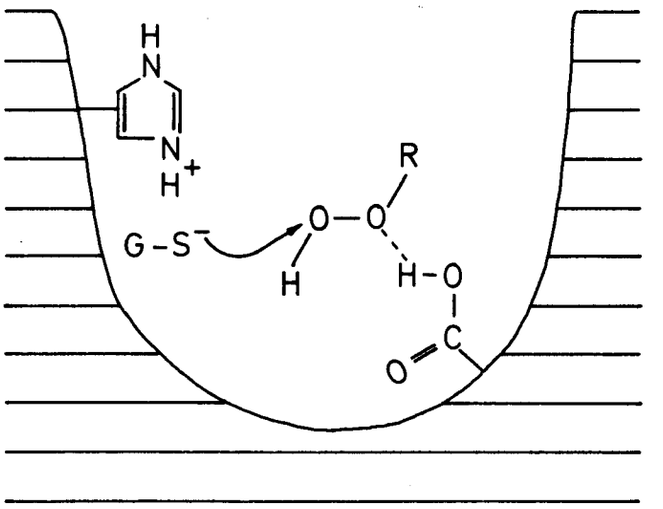


FIGURE 6C.

paradoxical inhibition of glutathione transferase 4-4 by indomethacin has been attributed to preferential binding of the inhibitor to enzyme/1-chloro-2,4-dinitrobenzene complexes[225] in an otherwise regular random-order mechanism (cf. Reference 2). Under the presumed intracellular conditions, inhibition by indomethacin is not specific for rat glutathione transferase 4-4, but affects all forms of mammalian (including human) glutathione transferase investigated.

B. Discrimination between Multiple Forms of Enzyme by Inhibition

It has been emphasized that several sets of data may be needed for distinction of different forms of an enzyme.[2] In several studies involving glutathione transferases, inhibition data

Table 12
SELECTED INHIBITORS OF GLUTATHIONE TRANSFERASE

Inhibitor	Effect[a]	Enzyme source	Ref.
Glutathione derivatives			
S-sulfoglutathione	+ +	Rat liver and lung cytosol	193
S-methylglutathione	+	Human and rat isoenzymes, maize, giant foxtail	21, 43, 194
S-ethylglutathione	+	Rat isoenzymes	21
S-*n*-propylglutathione	+	Rat isoenzymes	21
S-*n*-butylglutathione	+	Rat isoenzymes	21
S-*n*-pentylglutathione	+	Rat isoenzymes	21
S-*n*-hexylglutathione	+ + +	Human, rat, and mouse isoenzymes, maize, giant foxtail	21, 43, 80, 194, 195
S-*n*-heptylglutathione	+ +	Rat isoenzymes	21
S-*n*-octylglutathione	+ +	Human and rat isoenzymes	21, 43
S-*n*-phenylpropylglutathione	+	Rat isoenzymes	21
S-benzylglutathione	+ +	Rat isoenzymes	21, 195
S-*p*-bromobenzylglutathione	+ + +	Human, rat, and mouse isoenzymes	21, 35, 43, 59, 69, 80
S-(2,4-dinitrophenyl)glutathione	+	Rat liver cytosol, maize	194, 195
S-(2-chloro-4-nitrophenyl)glutathione	+ +	Human and rat isoenzymes	43, 116
S-(bromosulphophthaleinyl)-glutathione	+ +	Human isoenzymes	43
S-(propachlor)glutathione	+	Giant foxtail, maize	194
S-(atrazine)glutathione	+	Giant foxtail, maize	194
γ-L-glutamyl-L-serylglycine	+ +	Rat isoenzymes	196
γ-L-glutamyl-L-alanylglycine	+	Rat isoenzymes	196
Bile acids			
Cholate	+	Human and rat isoenzymes	29, 43, 197
Glycocholate	+	Rat liver cytosol	197
Taurocholate	+	Rat liver cytosol	197
Deoxycholate	+ +	Human and rat liver cytosol	43, 197
Taurodeoxycholate	+	Rat liver cytosol	197
Chenodeoxycholate	+ +	Rat isoenzymes	29, 197
Lithocholate	+ + +	Rat isoenzymes	29
Lithocholate 3-sulfate	+ + +	Rat isoenzymes	29
Steroid hormone derivatives			
17β-Estradiol disulfate	+ + +	Rat isoenzymes	198
Estradiol-3-sulfate	+ + +	Rat isoenzymes	198
Estradiol-17-sulfate	+ +	Rat isoenzymes	198
Cortisol monosulfate	+ +	Rat isoenzymes	198
Pregnenolone monosulfate	+ +	Rat isoenzymes	198
Dehydroisoandrosterone monosulfate	+ +	Rat isoenzymes	198
Anti-inflammatory drugs and related compounds			
Indomethacin	+ + +	Human, rat, and mouse isoenzymes, maize, giant foxtail	35, 59, 80, 194, 199, 200
Meclofenamic acid	+	Rat liver cytosol	199
Piriprost	+ + +	Rat isoenzymes	201
Sulfasalazine	+ + +	Rat isoenzymes	202
Diuretic drugs			
Ethacrynic acid	+ + +	Rat isoenzymes	203
Tienilic acid	+ + +	Rat isoenzymes	203
Indacrynic acid	+ + +	Rat isoenzymes	203
Furosemide	+ +	Rat isoenzymes	203
Bumetanide	+ +	Rat isoenzymes	203

Table 12 (continued)
SELECTED INHIBITORS OF GLUTATHIONE TRANSFERASE

Inhibitor	Effect[a]	Enzyme source	Ref.
Other drugs and related compounds			
Penicillic acid	+	Rat isoenzymes	204
6-Propyl-4-hydroxypyrimidine-2-sulfinate	+	Rat liver cytosol	205
6-Propyl-4-hydroxypyrimidine-2-sulfonate	+	Rat liver cytosol	205
Ciprofibrate	+	Rat iosenzymes	206
Gossypol acetic acid	+ + +	Human isoenzymes	80
Porphyrins and related compounds			
OO′-diacetylhematoporphyrin	+ + +	Rat liver cytosol	207
Monohydroxyethylmonovinyldeutero-porphyrin	+ + +	Rat liver cytosol	207
Mesohaem	+ + +	Rat liver cytosol	207
Hematoporphyrin	+ +	Rat liver cytosol	207
Protoporphyrin IX	+ +	Rat liver cytosol	207
Coproporphyrin I	+ +	Rat liver cytosol	207
Uroporphyrin I	+	Rat liver cytosol	207
Hematin	+ + +	Human, rat, and mouse isoenzymes	35, 59, 69, 80, 112
Bilirubin	+ +	Human and rat isoenzymes	43, 208
Plant phenols			
Purpurogallin	+ + +	Human tissues and rat isoenzymes	209
Alizarin	+ +	Human tissues and rat isoenzymes	209
Quercetin	+ + +	Human tissues and rat isoenzymes	209
Ellagic acid	+ + +	Human tissues and rat isoenzymes	209
Herbicides			
S-(tridiphane)glutathione	+ +	Maize, giant foxtail	194
2,4-Dichlorophenoxyacetate	+ + +	Human and rat isoenzymes, erythrocytes	210, 211
2,4,5-Trichlorophenoxyacetate	+ +	Human and rat isoenzymes, erythrocytes	210, 211
Metal compounds			
Cadmium iodide	+ +	Rat liver cytosol	212
Mercuric chloride	+ +	Rat isoenzymes and calf liver cytosol	213—215
Mercuric acetate	+ +	Rat isoenzymes	212, 214
Methylmercuric chloride	+ +	Calf liver cytosol	215
Copper chloride	+	Rat isoenzymes	213
Cadmium chloride	+	Rat isoenzymes and calf liver cytosol	213, 215
Phenylmercuric acid	+ +	Rat isoenzymes	214
Sodium ethylmercurithiosalicylate	+ +	Rat isoenzymes	214
Lead acetate	+	Calf liver cytosol	215
Di-*n*-butyltin dichloride	+ + +	Rat liver cytosol	216
Di-*n*-butyltin sulfide	+ + +	Rat liver cytosol	216
Tributyltin acetate	+ + +	Human, rat, and mouse isoenzymes	35, 59, 69, 80
Triethyltin bromide	+ + +	Human, rat, and mouse isoenzymes	35, 59, 69, 80, 112, 217
Tributyltin chloride	+ + +	Rat liver cytosol	217
Triphenyltin chloride	+ + +	Human, rat, and mouse isoenzymes, maize, giant foxtail	35, 59, 69, 80, 194, 217

Table 12 (continued)
SELECTED INHIBITORS OF GLUTATHIONE TRANSFERASE

Inhibitor	Effect[a]	Enzyme source	Ref.
Trimethyltin chloride	+ + +	Rat liver cytosol	217
Tricyclohexyltin chloride	+ + +	Rat liver cytosol	217
Diphenyltin chloride	+ +	Rat liver cytosol	217
Dibutyltin acetate	+ +	Rat liver cytosol	217
Diethyltin bromide	+ +	Rat liver cytosol	217
Triethyllead chloride	+ + +	Rat liver cytosol	217
Triethylgermanium chloride	+ +	Rat liver cytosol	217
Triphenyltin hydroxide	+ + +	Rat liver cytosol	217
Triphenyltin acetate	+ + +	Rat liver cytosol	217
Triphenyltin isothiocyanate	+ + +	Rat liver cytosol	217
Triphenyltin sulfide	+ + +	Rat liver cytosol	217
Triphenyltin oxide	+ +	Rat liver cytosol	217
Dyes			
Bis-(3,5-dibromo-4-hydroxyphenyl)methane	+ + +	Grass-grub	218
Bis-(3,5-dibromo-4-hydroxyphenyl)methanol	+ + +	Grass-grub	218
3,3′5,5′-Tetrabromo-4,4′-dihydroyxbenzophenone	+ + +	Grass-grub	218
3,5-Dibromo-4-hydroxyphenylphthalide	+ + +	Grass-grub	218
Rhodamine B	+ +	Grass-grub	218
Bromosulfophthalein	+ + +	Human, rat, and mouse isoenzymes, grass-grub, maize, giant foxtail	35, 43, 59, 69, 80, 112, 194, 219
3,6-Dibromosulfophthalein	+ +	Rat isoenzymes	208
Phenolphthalein	+ +	Grass-grub	218
Tetrabromophenolphthalein	+ + +	Grass-grub, housefly	218, 219
Tetrabromophenolphthalein ethyl ester	+ + +	Grass-grub	218, 219
Tetrabromophenolphthalein isopropyl ester	+ + +	Grass-grub	218
Tetrabromophenolphthalein	+ + +	Grass-grub, housefly	218
Leucobenzaurin	+ +	Grass-grub	218
Phenoltetrabromophthalein	+ +	Grass-grub	218
Phenoltetrabromophthalein disulfonate	+ +	Housefly	218
Bromothymol blue	+ +	Grass-grub, housefly	218, 219
Bromocresol purple	+ + +	Grass-grub, housefly	218, 219
Bromocresol green	+ + +	Grass-grub, housefly, maize, giant foxtail	194, 218, 219
Chlorophenol red	+ + +	Grass-grub, housefly	218, 219
Bromophenol blue	+ + +	Grass-grub, housefly	218, 219
Tetrabromophenol blue	+ + +	Grass-grub	218
Phenol red	+ +	Grass-grub, housefly	218, 219
Fluorescein	+ + +	Housefly, rat isoenzymes	208, 218
Eosin	+ + +	Grass-grub, housefly	218, 219
Rose bengal	+ +	Human, rat, and mouse isoenzymes, grass-grub	35, 59, 69, 80, 218, 219
Diiododimethylfluoroscein	+ +	Grass-grub	219
Dichlorofluorescein	+	Grass-grub	219
Cibacron-blue	+ + +	Human, rat, and mouse isoenzymes	35, 59, 69, 80
8-Anilinonaphthalene sulfonate	+ +	Rat isoenzymes	208

Table 12 (continued)
SELECTED INHIBITORS OF GLUTATHIONE TRANSFERASE

Inhibitor	Effect[a]	Enzyme source	Ref.
Dicarboxylic acids			
Azelaic acid	+	Grass-grub	219
Sebaic acid	+ +	Grass-grub	219
Traumatic acid	+ +	Grass-grub	219
Tetradecanedidic acid	+ +	Grass-grub	219
Thapsic acid	+	Grass-grub	219
Chalcone derivatives[b]			
Chalcone[c]	+ +	Mouse and rat liver cytosol	220
4-Methoxychalcone	+ + +	Mouse and rat liver cytosol	220
4′-Phenylchalcone[d]	+ + +	Mouse and rat liver cytosol	220
4′-Phenylchalcone oxide	+	Mouse and rat liver cytosol	220

[a] The effect of the inhibitor is indicated as the strongest inhibition for any isoenzyme or enzyme preparation, estimated using the standard assay with CDNB as substrate or under comparable conditions. The inhibitory effect was scored approximately as: + + +, $I_{50} \leq 5$ μM or $K_i \leq 5$ μM; + +, $I_{50} \leq 100$ μM or $K_i \leq 100$ μM; +, $I_{50} \leq 1$ mM or $K_i \leq 1$ mM.

[b] Activity measured with *cis*-stilbene oxide.

[c] 4-Nitro- and 4′-methoxychalcones were less efficient.

[d] Derivatives with fluoro-, bromo-, methoxy-, and trifluoromethyl groups in the 2-, 3-, and 4-positions and with 2-chloro-, 2-methyl-, 3-hydroxy-, and 4-phenyl-substitutions of 4′-phenylchalcone also were tested. The inhibitory effect of substituents in the 4-position is weak. Compounds with substituents in the 3-position or with electronegative substituents in the 2-position are strong inhibitors.

have proved useful when substrate specificities, immunochemical data, or other criteria have been insufficient. Inhibition studies have the advantage that they require only catalytic amounts of enzyme and can be performed with the most active substrate (or the substrate giving highest sensitivity in the assay if different analytical methods are used). With a limited amount of enzyme, a fairly extensive characterization involving many inhibitors may be possible when a characterization based on alternative (less active) substrates would have been impossible.

For the purpose of distinguishing the different forms of glutathione transferase, inhibition characteristics in the form of I_{50} values have been compiled (cf. Tables 9 to 11). The I_{50} values determined with a standard assay system can easily be estimated by interpolation from a limited number of measurements. This value is simply the value of inhibition giving 50% inhibition at the assay conditions defined for the experiment and, in general, does not have a mechanistic interpretation.

However, for the simple cases of linear inhibition and Michaelis-Menten kinetics, it can be shown how the I_{50} value is related to the inhibition constants (K_i).

Competitive inhibition:

$$v = \frac{V[S]}{K_m(1 + [I]/K_{is}) + [S]} \tag{2}$$

and

$$I_{50} = K_{is}(1 + [S]/K_m) \tag{3}$$

Uncompetitive inhibition:

$$v = \frac{V[S]}{K_m + [S](1 + [I]/K_{ii})} \tag{4}$$

and

$$I_{50} = K_{ii}(1 + K_m/[S]) \tag{5}$$

Noncompetitive inhibition:

$$v = \frac{V[S]}{K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \tag{6}$$

and

$$I_{50} = \frac{(K_m + [S])K_{is}K_{ii}}{K_mK_{ii} + K_{is}[S]} \tag{7}$$

Thus, competitive inhibition at low substrate saturation ($[S]/K_m \ll 1$) gives $I_{50} \approx K_{is}$, and uncompetitive inhibition at substrate concentrations approaching saturation ($[S]/K_m \gg 1$) gives $I_{50} \approx K_{ii}$. These inhibition constants can be obtained as the points of intersection with the y-axis if I_{50} is plotted as a function of [S] and 1/[S], respectively. Competitive inhibition yields a straight line in the first case, and uncompetitive inhibition yields a straight line in the second case. Noncompetitive inhibition gives nonlinear graphs in both plots, but the points of intersection with the y-axis are K_{is} and K_{ii} in the two plots, respectively (Figure 7).

Data not obeying Michaelis-Menten kinetics are more difficult to analyze, even though they would asymptotically approach the behavior of the I_{50} value of the simple Michaelis-Menten equation at high or low substrate concentrations.

C. Distinction between Homo- and Heterodimers

An important problem in the identification and characterization of the multiple forms of cytosolic glutathione transferase is the distinction between homo- and heterodimers. The discrimination is sometimes difficult because of the overlapping substrate specificities, the similarities in physical properties, and the simultaneous occurrence of many forms in the same tissue. A similar problem occurs in other enzyme families, e.g., that of the human alcohol dehydrogenases.[226,227]

It has been demonstrated for the glutathione transferases that simple inhibition studies, involving measurements under standard conditions of residual enzyme activity as a function of inhibitor concentration, can be used to distinguish heterodimeric enzymes from the corresponding homodimeric forms.[113] The basis for the analysis is the additivity of the activities of different subunits, which allows prediction of the kinetic properties of a heterodimer from those of the homodimers. The additivity applies to the effects of both substrates[111] and inhibitors.[112,113]

In the simplest case, involving a reversible linear inhibition (competitive, noncompetitive, or uncompetitive) and Michaelis-Menten kinetics, the fractional velocity, y, can be expressed:

$$y = \frac{I_{50}}{I_{50} + [I]} \tag{8}$$

where y is the ratio of initial velocities in the presence and absence of inhibitor (I) and I_{50} is a constant equal to the inhibitor concentration giving 50% inhibition. A plot of y vs. log [I] is symmetrical with a point of inflexion and a maximal slope of $-(\ln 10)/4 \approx -0.58$ at $[I] = I_{50}$.[113] Oligomeric enzymes with identical and noncooperative subunits are indistinguishable from monomeric enzymes in their kinetic behavior.

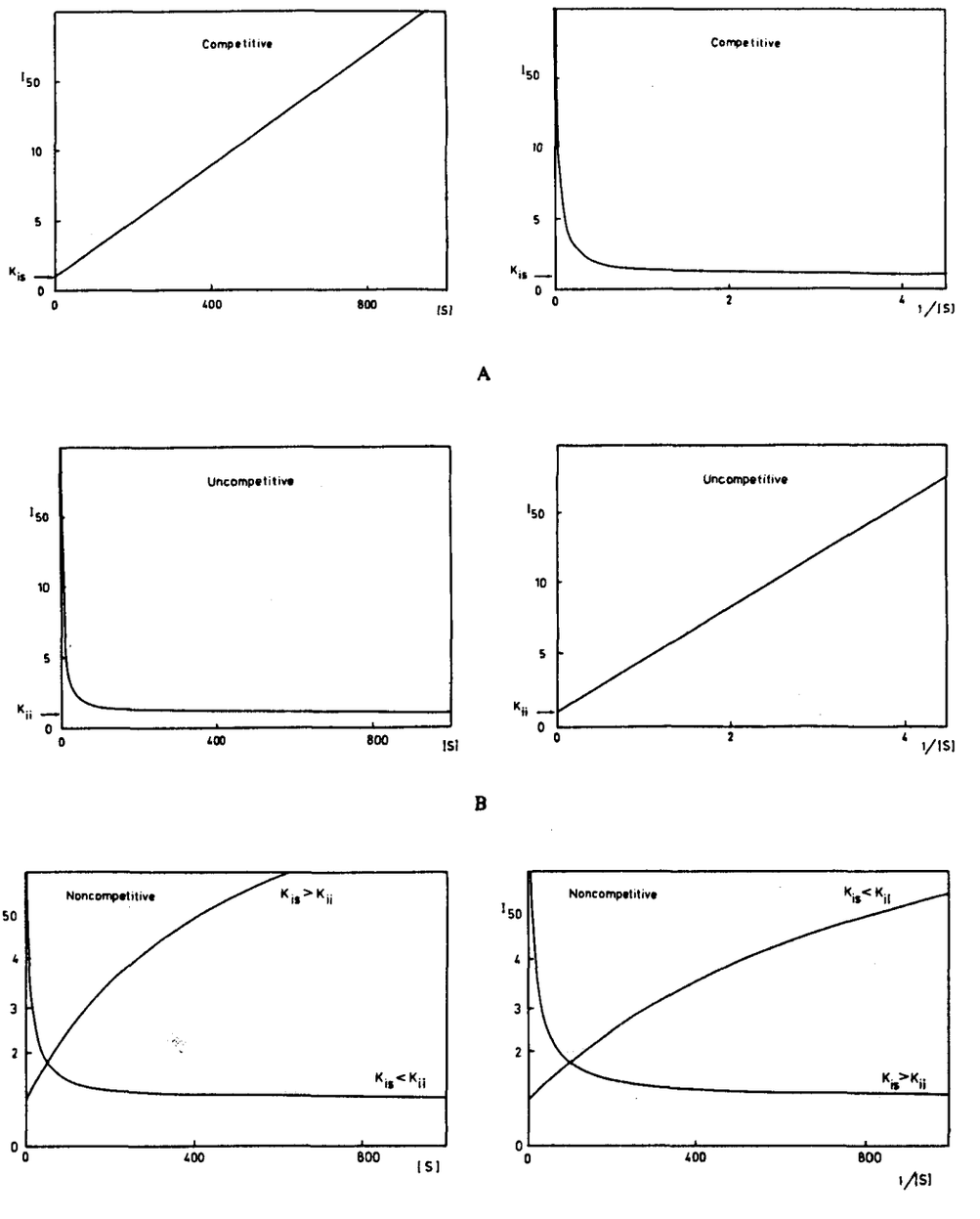


FIGURE 7. Theoretical curves of I_{50} vs. [S] or 1/[S] for the classical types of linear inhibition. The calculations are based on Michaelis-Menten kinetics and Equations 2 to 7, using $K_m = 50$ and $0.1 < [S] < 1000$ (arbitrary units). The intercepts with the y-axis correspond to the values of the inhibition constants. (A) Competitive inhibition ($K_{is} = 1$); (B) uncompetitive inhibition ($K_{ii} = 1$); (C) noncompetitive inhibition ($K_{is} = 1$, $K_{ii} = 10$; $K_{is} = 10$, $K_{ii} = 1$).

The simultaneous action of two nonidentical subunits, A and B, can be described by a similar expression of the fractional velocity:

$$y = \frac{I_{50}^{A}(1 - x)}{I_{50}^{A} + [I]} + \frac{x\, I_{50}^{B}}{I_{50}^{B} + [I]} \qquad (9)$$

where I^{A}_{50} and I^{B}_{50} are the corresponding inhibition parameters for the two subunits and x is the fraction of the total enzymatic activity that is contributed by subunit B. The corresponding graph has a negative slope <0.58 at 50% inhibition (assuming $I^{A}_{50} \neq I^{B}_{50}$). Therefore, a heterodimeric enzyme is expected to have a curve shape distinct from that of a homodimer. If the two values of I^{A}_{50} and I^{B}_{50} are sufficiently different, the plot will show an intermediary plateau, whereas closely spaced values only result in a decreased negative slope of the graph. It also should be noted that x must not be too small if the contribution of the second subunit is to be noticeable. In other words, both subunits must contribute significantly to the enzymatic activity measured.

Figure 8 shows simulated y vs. log [I] curves for some representative combinations of I_{50} values, ranging from a homodimer ($I^{A}_{50} = I^{B}_{50}$) to a heterodimer with various degrees of differential sensitivities of the subunits.

Figure 9 demonstrates to what degree the most active subunit governs the curve shape. A value of x = 0.05 gives a graph that is almost indistinguishable from that of a homodimeric (or monomeric) enzyme (i.e., x = 0). Optimal conditions for detection of the altered curve shape are obtained when x = 0.5 and the difference between I^{A}_{50} and I^{B}_{50} is as large as possible.

In cases where the inhibition curve is a higher degree function of inhibitor concentration, it may be more difficult to distinguish homo- from heterodimers. Under these circumstances, a heterodimer cannot simply be identified by a deviation from symmetrical sigmoid curve of y vs. log [I] and a maximal negative slope <0.58. Nonetheless, the fractional velocity curve of a heterodimer should still be expected to represent the sum of the curves of the corresponding homodimers. If the graph of one of the subunits is known, that of the second subunit can be deduced by subtraction of the first curve from that of the putative heterodimer.

The analysis of inhibition curves by the above approach has proved simple and useful for discrimination between homo- and heterodimeric forms of glutathione transferases. Figures 10 and 11 show some results from experimental studies. Only catalytic amounts of enzyme are required, and the method can even be applied to impure preparations, provided that the enzyme is free from other isoenzymes or other activities interfering with the measurements. In the search for suitable inhibitors that give simple inhibition curves for the homodimeric glutathione transferases, it has been found that trialkyltin derivatives may often serve the purpose (cf. Reference 113).

Finally, it should be noted that experiments involving alternative substrates in combination with inhibitors may be even more powerful than studies with a single substrate. The use of a substrate specific for one of the subunits of a heterodimer may shift the entire inhibition curve in a predictable manner.[112]

VI. AREAS OF FUTURE INVESTIGATIONS

In recent years, our knowledge of the structure and function of the glutathione transferases has advanced significantly. Primary structures of the three major classes of cytosolic mammalian transferases, the microsomal enzyme, as well as amino acid sequences of invertebrate and plant enzymes have been elucidated. Numerous additional sequences may be obtained in the near future by means of recombinant DNA technology. An important problem remaining is the posttranslational modification that blocks the N-terminus of most class Alpha transferases. The nature of this modification should be approached by the techniques of protein chemistry. Furthermore, the biological significance of the processing of the protein should be elucidated. For example, is the N-terminal modification a mechanism regulating the biological half-life of the protein?

Crystallographic studies of the glutathione transferases should increase our knowledge of the folding of the polypeptide chain and facilitate comparisons with other glutathione-

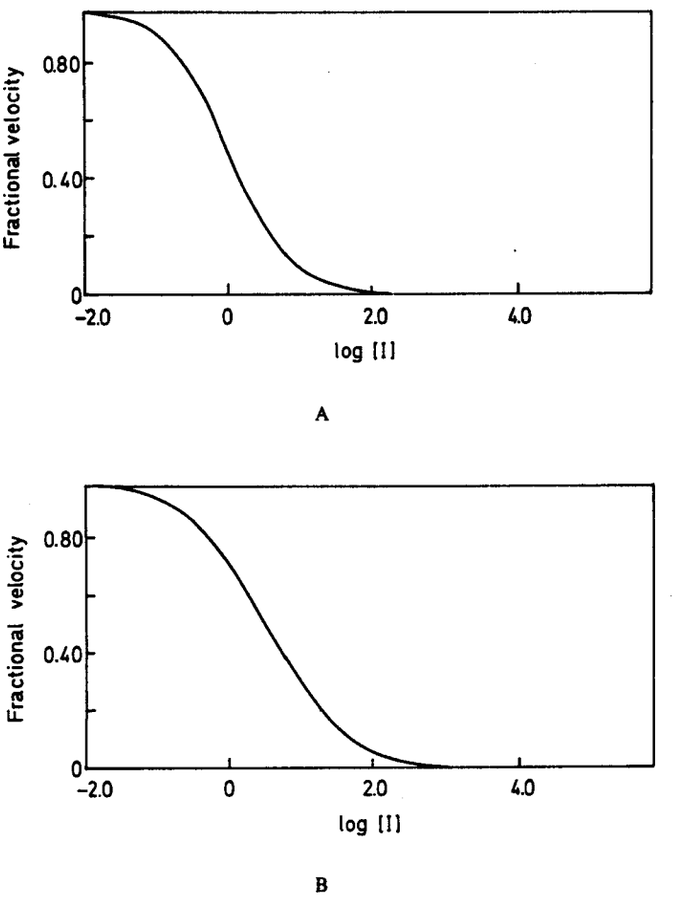


FIGURE 8. Theoretical inhibition curves for linear inhibition of noncooperative homo- and heterodimeric enzymes. Effect of differences in I_{50} values of the subunits. Fractional velocity plotted vs. log [I] according to Equation 9 for an enzyme containing two subunits, A and B. The subunits are assumed to be equally active ($x = 0.5$). (A) $I_{50}^{A} = I_{50}^{B} = 1$ (arbitrary units); (B) $I_{50}^{A} = 1$; $I_{50}^{B} = 10$; (C) $I_{50}^{A} = 1$; $I_{50}^{B} = 100$; (D) $I_{50}^{A} = 1$; $I_{50}^{B} = 10{,}000$.

dependent proteins. Conjectures about evolutionary relationships may thus receive support from structural data or be rejected. Obviously, the three-dimensional structure has to be elucidated in order to arrive at a detailed understanding about the binding properties, the catalytic mechanism, and the conformational changes of the transferases. Further insight into the catalytic process and the binding function can be obtained by the combination of three-dimensional structural data and protein modification by classical chemical means or by site-directed mutagenesis.

From the chemical point of view, the design of specific and effective inhibitors of the glutathione transferases may have several important applications. In vitro, rationally designed inhibitors would be valuable for incisive structure-activity studies. In vivo, selective inhibitors may help to define more accurately the biological functions of the proteins. Clinically, inhibitors might be administered to counteract the inactivation of cancer drugs which is effected by glutathione transferases.

In addition to the alleged role of glutathione transferases in the drug resistance of cancer cells, the enzymes, e.g., in insects and plants, are implied in certain resistance mechanisms against pesticides and herbicides. This topic of toxicology is currently the subject of investigations in several laboratories.

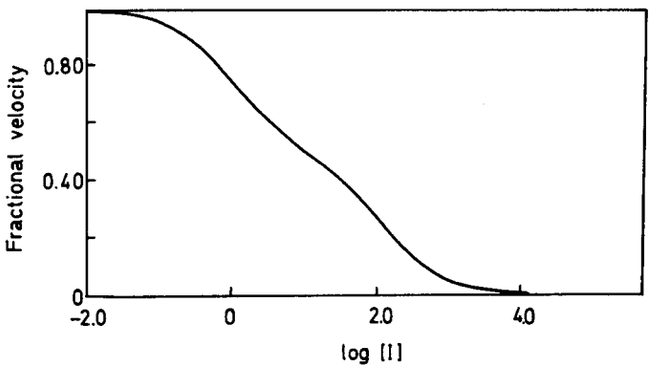


FIGURE 8C.

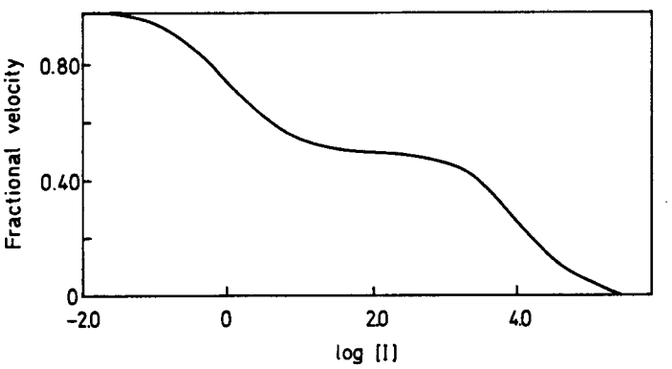
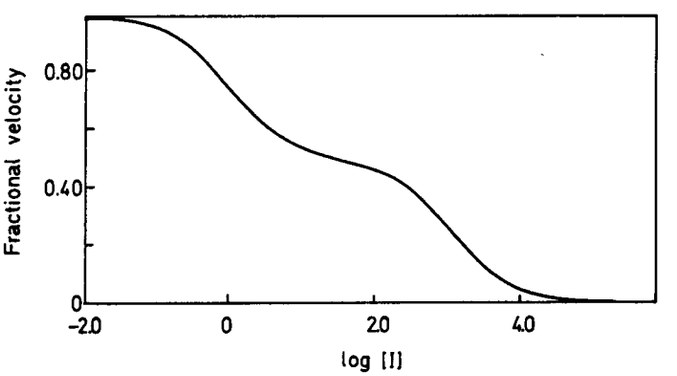


FIGURE 8D.



A

FIGURE 9. Theoretical inhibition curves for linear inhibition of noncooperative homo- and heterodimeric enzymes. Effect of differences in relative catalytic activities of the subunits. Fractional velocity plotted vs. log [I] according to Equation 9 for an enzyme containing two subunits, A and B, with $I^A_{50} = 1$ and $I^B_{50} = 1000$ (arbitrary units). (A) x = 0.5; (B) x = 0.25; (C) x = 0.05.

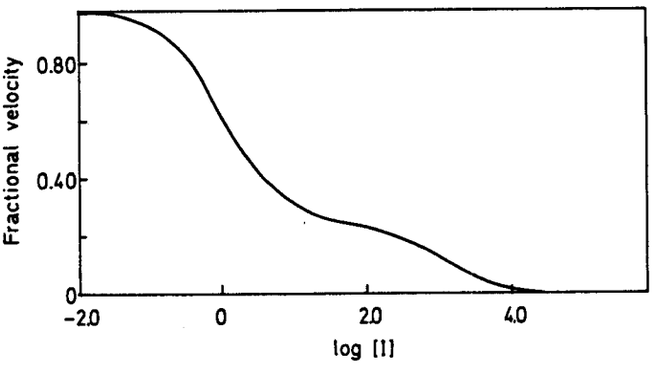


FIGURE 9B.

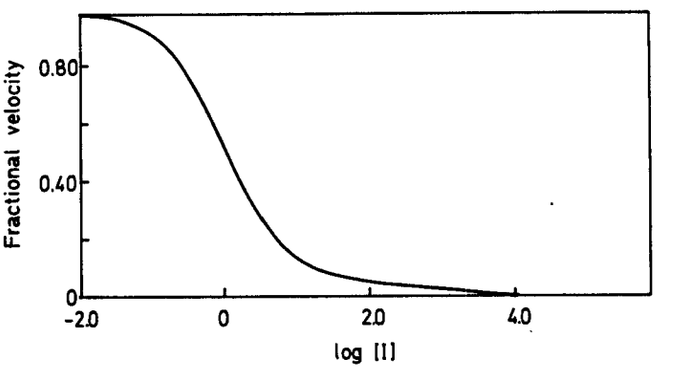


FIGURE 9C.

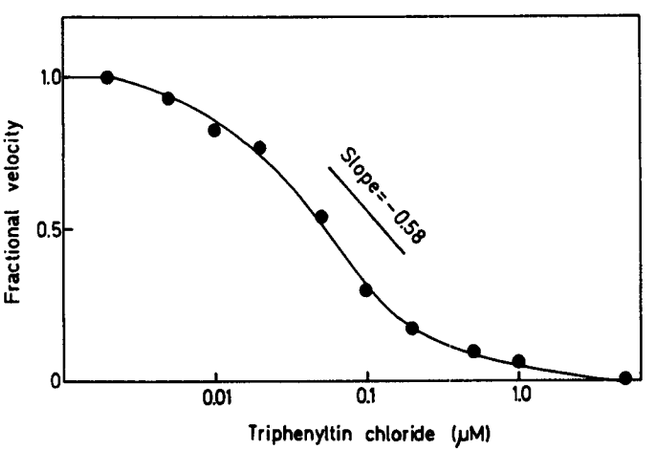


FIGURE 10. Experimental inhibition curve for homodimeric rat glutathione transferase 1-1. (From Tahir, M. K. and Mannervik, B., *J. Biol. Chem.*, 261, 1048, 1986. With permission.)

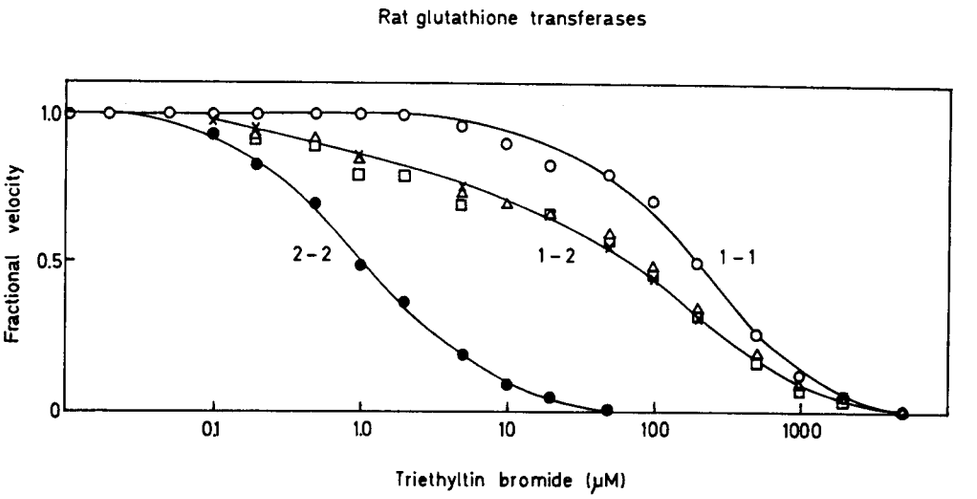


FIGURE 11. Experimental inhibition curves for homodimeric rat glutathione transferases 1-1 and 2-2, as well as the heterodimeric transferase 1-2. (From Tahir, M. K. and Mannervik, B., *J. Biol. Chem.*, 261, 1048, 1986. With permission.)

Finally, genetic studies are required in order to elucidate whether glutathione transferase deficiency can be linked to disease in humans or other biological species. Such findings would advance our understanding of the biological importance of the glutathione transferases.

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