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BIOSYNTHESIS OF *S*-(4-ETHYLAMINO-6-ISOPROPYLAMINO-2-*s*-TRIAZINO) GLUTATHIONE: PARTIAL PURIFICATION AND PROPERTIES OF A GLUTATHIONE *S*-TRANSFERASE FROM CORN*

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Abstract—A soluble glutathione *S*-transferase from corn leaves was purified 7·6-fold by differential centrifugation, ammonium sulfate fractionation and gel filtration. Active enzyme preparations were also isolated from leaves of sorghum, sugarcane, Johnson grass and Sudan grass. The enzyme catalyzed the conjugation of several substituted 2-chloro-*s*-triazine herbicides with reduced glutathione. The formation of 1 mole of chloride ion for every mole of glutathione conjugate produced was demonstrated with ^{14}C and ^{36}Cl-labeled 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine. A rapid ion exchange assay system for following the rate of ^{14}C-labeled *s*-triazine conjugate formation was developed. The pH optimum for 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine conjugate formation was between 6·6 and 6·8. Enzyme specificity for reduced glutathione was demonstrated. Specificity and inhibition studies with substituted *s*-triazines indicated that a chlorine atom in the 2-position and *N*-alkyl side-chains in the 4 and 6 positions were required for enzyme activity. The apparent K_m values for 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine and reduced glutathione were $3{\cdot}7 \times 10^{-5}$ M and $2{\cdot}4 \times 10^{-3}$ M, respectively. Inhibition studies demonstrated a competitive inhibition with 2-methylmercapto-4,6-bis-isopropylamino-*s*-triazine ($K_i = 2{\cdot}8 \times 10^{-5}$ M) and with sulfobromophthalein ($K_i = 2{\cdot}7 \times 10^{-5}$ M). Since this appears to be the first report of glutathione *S*-transferase activity in plants, the similarities and differences between the corn leaf enzyme and previously reported animal glutathione *S*-transferase systems were discussed. The role of this enzyme system in the rapid *in vivo* detoxification and selectivity of substituted 2-chloro-*s*-triazine herbicides was also discussed.

INTRODUCTION

THE RAPID formation of several water-soluble metabolites in corn or sorghum leaf tissues treated with the selective herbicide, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, was reported by Shimabukuro and Swanson.[1] Lamoureux *et al.*[2,3] have recently isolated and identified two of these metabolites as *S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino) glutathione and γ-L-glutamyl-*S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino)-L-cysteine. These authors[3] also reported the presence of a soluble enzyme in corn and sorghum that catalyzed the formation of *S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino) glutathione. The present report describes the partial purification and characterization of a soluble glutathione *S*-transferase from corn leaves which catalyzes the conjugation of substituted 2-chloro-*s*-triazines with glutathione.

* Use of trade names is for the purpose of identification and does not constitute endorsement by the U.S. Department of Agriculture.

[1] R. H. SHIMABUKURO and H. R. SWANSON, *J. Agri. Food Chem.* **17**, 199 (1969).
[2] G. L. LAMOUREUX, R. H. SHIMABUKURO, H. R. SWANSON and D. S. FREAR, *J. Agri. Food Chem.* **18**, 81 (1970).
[3] G. L. LAMOUREUX, R. H. SHIMABUKURO, H. R. SWANSON and D. S. FREAR, Abstr. 16, 157th Meeting ACS, Minneapolis, Minnesota (1969).

RESULTS AND DISCUSSION

Partial Purification of Glutathione S-Transferase from Corn

Table 1 shows the results of a typical purification experiment in which the enzyme was purified 7·6-fold with better than 100 per cent recovery of enzyme activity. The recovery of more than 100 per cent of the enzyme units found in the crude extract may be due to the presence of small molecular weight endogeneous inhibitors that are removed by gel filtration on Sephadex G-25. The apparent low yield, together with a 3·0-fold purification of the $(NH_4)_2SO_4$ fraction, may indicate an inhibition of this fraction by both high concentrations of $(NH_4)_2SO_4$ and by small molecular weight endogeneous inhibitors present in the resuspended, unwashed $(NH_4)_2SO_4$ pellet. Dialysis against dilute phosphate buffer restored the yield of this fraction to approximately the same value as the Sephadex G-25 fraction.

Since most of the larger particulate matter was rapidly removed by centrifugation at 17,500 *g*, this fraction was arbitrarily selected as the crude cell-free extract. Further centrifugation of this fraction at 78,000 *g* for 90 min resulted in no apparent loss of enzyme activity. Booth *et al.*[4] reported that a similar enzyme system in rats is also associated with the soluble fraction of liver homogenates.

TABLE 1. PARTIAL PURIFICATION OF GLUTATHIONE *S*-TRANSFERASE FROM CORN

Fraction	Volume (ml)	Protein (mg/ml)	*Units/ml	Total units	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
17,500 *g* Supernatant	997	3·4	2·3	2283	0·7	100	—
40–60% $(NH_4)_2SO_4$	25	23·0	48·9	1223	2·1	54	3·0
Sephadex G-25	100	5·1	26·9	2685	5·3	118	7·6

* 1 unit = 1 mμmole of *S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino) glutathione/30 min.

Enzyme Distribution

The distribution of enzyme activity in crude, cell-free extracts (17,500 *g* supernatant fractions) of several plant species and tissues was determined. Leaf tissues of tolerant species, corn, sorghum, Johnson grass, Sudan grass and sugarcane were all found to be good sources of the enzyme. Root tissues of corn and sorghum, however, contained very little enzyme activity. Crude extracts of leaves from susceptible species, pea, oats, wheat, barley and pigweed leaves contained no detectable enzyme activity.

Evidence indicates that the glutathione *S*-transferase enzyme is a major factor in determining the selectivity of substituted 2-chloro-*s*-triazine herbicides which are known to be potent inhibitors of photosynthesis. Isolated chloroplasts from tolerant and susceptible species were equally sensitive to the inhibition of the Hill reaction by substituted 2-chloro-*s*-triazine herbicides.[5] However, a recovery of photosynthesis accompanied by rapid formation of substituted 2-chloro-*s*-triazine-glutathione conjugates was observed in leaf tissues of tolerant species only.[1,2] The soluble enzyme, glutathione *S*-transferase, was present in the leaves of tolerant species, but not in susceptible species. Apparently, selectivity is achieved in tolerant species by a rapid detoxification of the herbicide to the glutathione conjugate before it reaches the target site, the chloroplast.

[4] J. BOOTH, E. BOYLAND and P. SIMS, *Biochem. J.* **79**, 516 (1961).
[5] D. E. MORELAND and K. L. HILL, *Weeds* **10**, 229 (1962).

Identification of Reaction Products

The products of the reaction were identified by using both ^{14}C ring and ^{36}Cl-labeled 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine as substrates. Methanol extracts from several enzyme assays with ^{14}C ring labeled 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine were pooled. The water-soluble conjugate formed was separated from the remaining unreacted ^{14}C-labeled substrate by thin-layer chromatography and eluted with methanol. The resulting methanol eluate was further purified and identified as *S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino) glutathione by the procedures of Lamoureux *et al.*[2,3] The tentative identification of glutathione conjugates formed with other ^{14}C-labeled substrates was based on their water solubility and similar behavior during thin-layer chromatography.

The identification of the chloride ion as the other product of the reaction was based on differences in the behavior of the radioactive ^{36}Cl and ^{14}C reaction products produced. The results of a typical experiment are shown in Table 2. The triazine substrate and the glutathione conjugate are quantitatively adsorbed on strong cation exchange columns.[2,3,6,7] The unreacted ^{14}C substrate can also be separated from the water-soluble reaction products

TABLE 2. DISTRIBUTION OF ^{14}C AND ^{36}Cl-LABELED REACTION PRODUCTS FROM GLUTATHIONE *S*-TRANSFERASE ASSAY*

Treatment	Cation exchange column eluant† (dis/min)	Aq. phase of $CHCl_3$ water partition† (dis/min)	Cl-ppt of aq. phase (dis/min)
^{14}C	106	6210	180
^{14}C control	0	260	—
^{36}Cl	6955	6140	5055
^{36}Cl control	850	170	—

* Standard reaction mixture incubated for 2 hr at 25° with 39,625 dis/min/assay, of either ^{14}C- or ^{36}Cl-labeled atrazine (specific activity = 0·08 $\mu c/\mu$mole) and 0·8 mg of G-25 enzyme fraction.
† Refs. 4 and 5.

by partitioning between $CHCl_3$ and water.[6,7] Essentially, all of the ^{14}C-labeled products were adsorbed on the ion-exchange resin while the anionic chloride ion from the ^{36}Cl experiment was not adsorbed and passed through the column. Over 80 per cent of the water-soluble products formed with ^{36}Cl-labeled substrate were precipitated with $AgNO_3$ while the water-soluble products from the ^{14}C-labeled substrate were not affected.

These data indicate that the reaction catalyzed by the partially purified corn glutathione *S*-transferase is as shown in Fig. 1.

Effect of pH and Buffer Systems

The pH optimum for 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine conjugate formation by partially purified corn leaf glutathione *S*-transferase was found to be between 6·6 and 6·8. This optimum is lower than the broad optimum about pH 8·0 reported by Booth *et al.*[4] for rat liver glutathione *S*-transferase.

A non-enzymatic catalysis of *S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino) glutathione formation was observed with tris (hydroxymethyl) aminoethane (tris) buffer systems. The

[6] R. H. SHIMABUKURO, *J. Agri. Food Chem.* **15**, 557 (1967).
[7] R. H. SHIMABUKURO, R. E. KADUNCE and D. S. FREAR, *J. Agri. Food Chem.* **14**, 392 (1966).

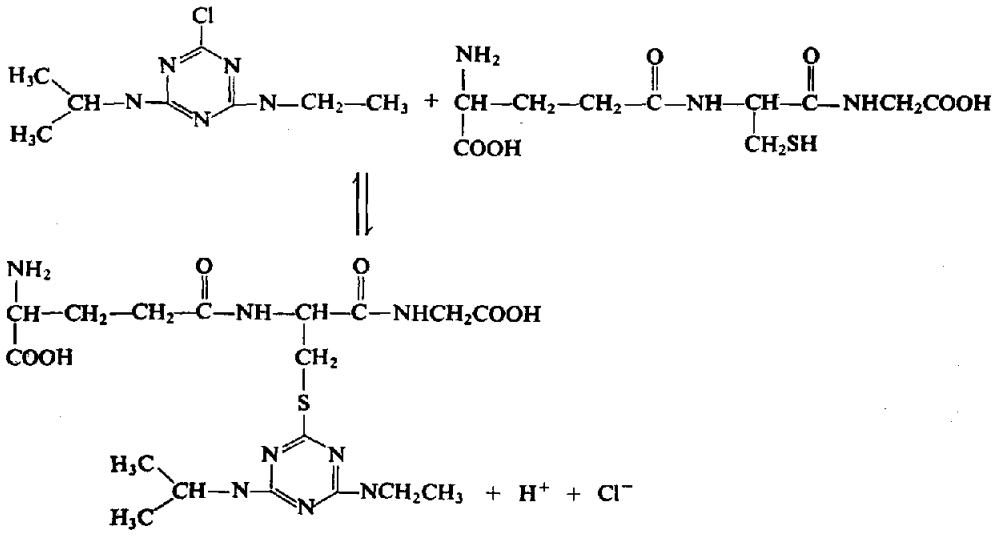

FIG. 1. GLUTATHIONE *S*-TRANSFERASE CATALYZED FORMATION OF *S*-(4-ETHYLAMINO-6-ISOPROPYLAMINO-2-*s*-TRIAZINO) GLUTATHIONE.

rate of this reaction was greater as the pH increased between pH 7·0 and pH 9·0 and resulted in a 10–20 per cent conversion of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine to the glutathione conjugate at pH 8·4 under standard assay conditions either in the absence of enzyme or with boiled enzyme. A similar effect was not observed with *N*-tris (hydroxymethyl) glycine (Tricine) buffer systems under the same conditions and in the same pH range. It appears, therefore, that the free primary amine group of tris may be functioning as a model nucleophilic catalytic agent. Whether or not a similar primary amine group as found in the side chains of lysine and arginine functions at the active site of the enzyme, remains to be determined.

Enzyme Stability

The stability of the enzyme to freezing, lyophilizing and boiling has been determined. Boiling for 15 min reduces enzyme activity 80–90%. A combination of freezing, thawing and lyophilizing results in very little loss of enzyme activity. This is true of the original, the crude 17,500 *g* supernatant and the G-25 Sephadex filtered enzyme fractions. The latter enzyme fraction is stable for at least 3 months either frozen or lyophilized and stored dry. At 4°, the G-25 preparation is stable for 3 days and loses only 20% of its activity when held at room temp. for 3 hr. The G-25 fraction can also be stored at 4° as a 60% NH_4SO_4 suspension which can be dialyzed against 0·1 M phosphate buffer with no loss in activity.

Substrate Specificity

Enzyme activity was not observed when reduced glutathione was replaced with dithiothreitol (2,3-dihydroxy-1,4-dithiolbutane), mercaptoethanol, 2,3-dimercaptopropanol or L-cysteine, and no inhibition of enzyme activity occurred when 1×10^{-3} M oxidized glutathione was added to the standard reaction mixture. Thus, the enzyme appears to be specific for reduced glutathione. A similar specificity for reduced glutathione has been reported for animal enzyme systems by Booth *et al.*[4] These investigators observed no activity

with rat liver glutathione *S*-transferase when oxidized glutathione, L-cysteine and *N*-acetyl-L-cysteine were used as substrates.

The isolation of γ-L-glutamyl-*S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino)-L-cysteine as the major metabolite of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine in sorghum leaves[2] raises the possibility that γ-L-glutamyl-L-cysteine may also function as a substrate. Unfortunately, this dipeptide, as well as the other possible dipeptide, L-cysteinyl-L-glycine, were not available and could not be tested as substrates. Another possibility would be that the major metabolite found in sorghum leaves is the result of the action of a carboxypeptidase in these tissues. Carboxypeptidases with apparently rather broad specificities have recently been isolated from beans by Wells[8] and from barley by Visuri *et al.*[9]

The specificity of the enzyme for several substituted *s*-triazines is shown in Table 3. Appreciable enzyme activity was found only with substituted 2-chloro-*s*-triazines. Substitution of a methoxy, methylmercapto or hydroxy group in the 2-position resulted in almost complete loss of activity. The very low specific activity observed with 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine correlates very well with the *in vivo* studies reported by

TABLE 3. TRIAZINE SPECIFICITY OF GLUTATHIONE *S*-TRANSFERASE FROM CORN

Substrate*	Specific activity†
2-Chloro-4-*tert*-butylamino-6-ethylamino-*s*-triazine	6·58
2-Chloro-4-ethylamino-6-isopropylamino-*s*-triazine	4·50
2-Chloro-4-isopropylamino-6-cyclopropylamino-*s*-triazine	3·40
2-Chloro-4,6-bis-isopropylamino-*s*-triazine	2·78
2-Chloro-4,6-bis-ethylamino-*s*-triazine	0·36
2-Chloro-4-amino-6-isopropylamino-*s*-triazine	0·09
2-Hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine	0·07

* The following *s*-triazine compounds did not react to form glutathione conjugates; 2-methylmercapto-4,6-bis-isopropylamino-*s*-triazine, 2-methylmercapto-4-ethylamino-6-isopropylamino-*s*-triazine, and 2-methoxy-4,6-bis(isopropylamino)-*s*-triazine.

† Specific activity = mμmoles of glutathione-*s*-triazine conjugate formed/mg protein/2 hr.

Shimabukuro.[10] In these studies, Shimabukuro found that corn and sorghum plants treated with 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine did not produce water-soluble metabolites but when these same species were treated with 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, water-soluble metabolites were formed.

The relative activities of the substituted 2-chloro-*s*-triazines do not appear to be correlated either with their partitioning coefficients between polar and non-polar solvents as reported by Ward and Holly,[11] or with their water solubilities at pH 7·0 as reported by Ward and Weber.[12] Other chlorosubstituted aromatic pesticides tested as substrates included the following: isopropyl *N*-(3-chlorophenyl) carbamate, 4-amino-3,5,6-trichloropicolinic acid, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 3-(4-chlorophenyl)-1,1-dimethylurea, and 3-(3-trifluoromethylphenyl)-1,1-dimethylurea. All of these compounds failed to react under the standard assay conditions.

[8] J. R. E. WELLS, *Biochem. J.* **97**, 228 (1965).
[9] K. VISURI, J. MIKOLA and T. M. ENARI, *European J. Biochem.* **7**, 193 (1969).
[10] R. H. SHIMABUKURO, *Plant Physiol.* **43**, 1925 (1968).
[11] T. M. WARD and K. HOLLY, *J. Colloid Interface Sci.* **22**, 221 (1966).
[12] T. M. WARD and J. B. WEBER, *J. Agri. Food Chem.* **16**, 959 (1968).

Two aliphatic chloro-substituted herbicides, 2-chloro-*N*-isopropylacetanilide and 4-chloro-2-butynyl-3′-chlorocarbanilate, reacted very rapidly with reduced glutathione either in the presence or absence of enzyme. In 4-chloro-2-butynyl-3′-chlorocarbanilate studies, the reaction rate of the boiled control was significantly less than in the presence of the enzyme. This may indicate an enzymatic conjugation of reduced glutathione with these aliphatic chloro-substituted herbicides in addition to the fairly rapid conjugation that apparently takes place non-enzymatically. The non-enzymatic reaction of several animal mercapturic acid precursors with reduced glutathione to form conjugates has also been noted by Boyland and Chasseaud.[13] Several aliphatic chloro-substituted herbicides are presently being used for the control of weeds in corn, sorghum and sugarcane. It is not known, however, whether or not these compounds react to form glutathione conjugates *in vivo*. The results reported here indicate that 2-chloro-*N*-isopropylacetanilide and possibly other related herbicides may react non-enzymatically and/or enzymatically to form glutathione conjugates when applied to the foliar tissues of corn, sorghum, sugarcane, and other species resistant to these specific compounds.

Double reciprocal plots of initial velocities and variable concentrations of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine at non-saturating concentrations of reduced glutathione yielded a family of straight lines which intersected at a point to the left of the ordinate and slightly above the abscissa. According to the report of Morrison[14] such results show that the glutathione *S*-transferase mechanism is sequential. Kinetic data from several experiments with the corn leaf G-25 enzyme fraction were processed on an IBM 360 computer with the sequential bireactant mechanism program of Cleland.[15,16] The apparent K_m constants for 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine and reduced glutathione were $3{\cdot}7 \times 10^{-5}$ M and $2{\cdot}4 \times 10^{-3}$ M, respectively. During these kinetic studies, at least 1 μmole of reduced glutathione was necessary before a measurable initial velocity could be determined. The rate of the enzyme reaction was proportional to enzyme concentration up to only 1·00 mg of protein. It appears, therefore, that some of the reduced glutathione may not be available for enzymatic reaction and may be bound by other proteins in the partially purified enzyme preparation. Consequently, the reported apparent K_m value for reduced glutathione may be high.

Enzyme Inhibition

The results of experiments on the inhibition of partially purified glutathione *S*-transferase are shown in Table 4.

S-Methylglutathione, an analog of reduced glutathione, was a weak inhibitor, and the disulfide compounds, cystine and oxidized glutathione, were not inhibitory at the 1·0 mM level. Enzyme activity was inhibited by 1·0 mM dithiothreitol and 2,3-dimercapto propanol, but not by cysteine and 2-mercaptoethanol. The reasons for the differences in the per cent inhibition between 2,3-dimercaptoethanol and 1,4-dithiothreitol as well as the inhibition by dithiol compounds, but not by monothiol compounds, are not apparent and must await further purification of the enzyme.

[13] E. BOYLAND and L. F. CHASSEAUD, in *Advances in Enzymology*, Vol. 32, pp. 173–219, Interscience, New York (1969).
[14] J. F. MORRISON, *Australian J. Science* **27**, 317 (1965).
[15] W. W. CLELAND, *Nature* **198**, 463 (1963).
[16] W. W. CLELAND, in *Advances in Enzymology*, Vol. 29, pp. 1–32, Interscience, New York (1967).

TABLE 4. INHIBITION OF GLUTATHIONE *S*-TRANSFERASE FROM CORN

Inhibitor	Conc. (mM)	Inhibition (%)
S-Methyl glutathione	1·0	11
Dithiothreitol	1·0	40
2,3-Dimercaptopropanol	1·0	72
2-Ethylamino-4-isopropylamino-6-methylmercapto-*s*-triazine	0·06	38
2,4-bis(Isopropylamino)-6-methylmercapto-*s*-triazine	0·06	61
2-Methoxy-4,6-bis(isopropylamino)-*s*-triazine	0·06	26
2,4-Dichloro-6-(2-chloroanilino)-*s*-triazine	0·05	29
2-Amino-4-nitrophenol	1·0	22
1,2-Dichloro-4-nitrobenzene	1·0	54
Sulfobromophthalein	0·1, 0·01	41, 13
2,3,6-Trichlorophenylacetic acid	0·1, 0·01	29, 19
4-Chloro-2-butynyl-*m*-chlorocarbanilate	0·1	34
2-Chloro-*N*-isopropyl acetanilide	0·1	29
2-Chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide	0·1	22

Inhibition of enzyme activity by 0·1 mM *N*-ethylmaleimide, *p*-chloro-mercuribenzoate, $HgCl_2$, sodium arsenite and iodoacetamide was not very pronounced even when the enzyme was preincubated with inhibitor prior to the addition of substrates. This may indicate a competitive displacement of the thiol inhibitor by the excess glutathione present in the reaction mixture, with little inactivation during the course of the experiment.

Several substituted *s*-triazine compounds were tested as enzyme inhibitors. Inhibition was observed with the compounds listed in Table 4 while essentially no inhibition was observed with 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine, 2-chloro-4-amino-6-isopropylamino-*s*-triazine and 2-chloro-4-ethylamino-6-amino-*s*-triazine at the same concentration levels. The fungicide, 2,4-dichloro-6-(2-chloroanilino)-*s*-triazine was an effective inhibitor of glutathione conjugation with 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, but it is not known whether or not it also functions as a substrate because ^{14}C-labeled material was not available for enzyme assays. Lineweaver–Burk plots of kinetic data from several inhibition studies have shown that 2-methylmercapto-4,6-bis(isopropylamino)-*s*-triazine is a strong competitive inhibitor of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine conjugation. When these data were processed in an IBM 360 computer with the linear competitive inhibition program of Cleland,[15,16] an apparent K_i value of $2{\cdot}8 \times 10^{-5}$ M was calculated for 2-methylmercapto-4,6-bis(isopropylamino)-*s*-triazine. The results of the substituted *s*-triazine inhibition and specificity studies indicate that substituted 2-methylmercapto and probably 2-methoxy-*s*-triazines are able to react competitively at the active site of the enzyme, but do not function as substrates. The substituted 2-hydroxy and *N*-dealkylated *s*-triazines, on the other hand, do not function effectively either as substrates or as inhibitors and probably are not readily bound at or near the enzyme active site.

Inhibition of enzyme activity by 2-amino-4-nitrophenol, 1,2-dichloro-4-nitrobenzene and sulfobromophthalein may indicate similarities between animal glutathione *S*-aryltransferase systems and the substituted 2-chloro-*s*-triazine conjugating system in corn. These compounds have been reported as substrates and inhibitors of glutathione *S*-aryltransferases from animals.[4,13,17–19] Grover and Sims[19] have reported that sulfobromophthalein is a

[17] J. G. WIT and P. LEEUWANGH, *Biochem. Biophys. Acta* **177**, 329 (1969).
[18] A. J. COHEN, J. N. SMITH and H. TURBERT, *Biochem. J.* **90**, 457 (1964).
[19] P. L. GROVER and P. SIMS, *Biochem. J.* **90**, 603 (1964).

competitive inhibitor of 1,2-dichloro-4-nitrobenzene conjugation in rat liver (apparent $K_i = 3 \times 10^{-6}$ M). Lineweaver–Burk plots and computer analysis of kinetic data from inhibition studies with corn leaf G-25 fractions have shown that sulfobromophthalein is also a strong competitive inhibitor of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine conjugation (apparent $K_i = 2{\cdot}7 \times 10^{-5}$ M). The corn leaf enzyme was also inhibited by 2,3,6-trichloro-phenylacetic acid, a reported synergist of substituted 2-chloro-*s*-triazine herbicides.[20] These results suggest that a number of substituted chloro and nitro aryl compounds, and a variety of animal glutathione *S*-transferase inhibitors and substrates may also function as synergists in tolerant species such as corn, sorghum, Sudan grass, sugarcane and Johnson grass by the inhibition of a major substituted 2-chloro-*s*-triazine detoxification pathway, glutathione conjugation.

The alkyl chlorinated carbanilate and anilide herbicides, 4-chloro-2-butynyl-*m*-chlorocarbanilate, 2-chloro-*N*-isopropyl acetanilide and 2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide also inhibited the conjugation of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine. In the previously described substrate specificity studies, it was noted that the first two of these herbicides reacted with glutathione to form conjugates. It is not surprising, therefore, that the three herbicides shown at the bottom of Table 4 are also inhibitors of substituted 2-chloro-*s*-triazine conjugation.

EXPERIMENTAL

Plant Materials

Leaf tissues were excised from young plants grown in the greenhouse in one-half strength Hoagland's nutrient solution and with supplementary light to give at least a 12-hr photoperiod. Species studied included: corn (*Zea mays* L.), sorghum (*Sorghum vulgare* L.), Sudan grass (*Sorghum sudanense*), Johnson grass (*Sorghum halepense* L.), sugarcane (*Saccharum officianarum*), pea (*Pisum sativum* L.), oats (*Avena sativa* L.), wheat (*Triticum aestirum* L.), barley (*Hordeum vulgare* L.), pigweed (*Amaranthus retroflexus* L.) and Bermuda grass (*Cynodon dactylon*).

Enzyme Preparation

Excised leaf tissue was rinsed with H_2O, blotted dry and ground to a fine powder with liquid N_2 in a vented stainless-steel Waring Blendor assembly. The frozen powder was slurried with 5 vol. of 0·1 M pK phosphate buffer, pH 6·8, and one-half weight of Polyclar AT (insoluble polyvinylpyrolidione). In some experiments, 1×10^{-3} M $Na_2S_2O_5$ was added to the slurry mixture. After standing 15 min, the slurry was squeezed through four layers of cheesecloth and centrifuged at 17,500 *g* for 30 min. The supernatant was then fractionated between 30–60% saturation with ammonium sulfate.

The 30–60% saturated $(NH_4)_2SO_4$ precipitate was suspended in a minimal volume of 0·1 M pK phosphate buffer, pH 6·8, placed on a 2·5 × 30 cm Sephadex G-25 column and eluted with the same buffer system. The G-25 eluate fraction was lyophilized in test tubes and stored dry at −12°. Enzyme stored in this manner was reconstituted by adding H_2O and was used for most routine assays. Assays were also made using the original 17,500 *g* supernatant, the 30–60% $(NH_4)_2SO_4$ precipitate suspension and the G-25 eluate.

Optimum pH studies were made with enzyme eluted from G-25 Sephadex with 0·01 M PO_4, pH 7·5. Citrate, phosphate and Tricine buffers, 0·2 M, with a pH range of 4·5–9·0 were added to the assay reaction mixture and the actual pH determined with a micro electrode assembly.

Enzyme Assays

The enzyme assay was based on the rate of glutathione conjugate formation. The standard reaction mixture contained 100 μmoles of phosphate buffer, pH 6·86, 10 μmoles of reduced glutathione, 25–35 mμmoles of ^{14}C ring-labeled 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, 0·3–0·9 mg of enzyme and H_2O to a final volume of 1·0 ml. The reaction was initiated by the addition of substrates and incubated at 25° for 30 min. Enzyme activity was linear for 2 hr and proportional to protein concentration under these assay condi-

[20] E. D. WEIL, J. S. NEWCOMER and E. DORFMAN, *U.S. Pat. Office Gaz.* **807**, 989 (1964).

tions. Controls were heated in a boiling-water bath for 15 min and cooled to room temp. prior to the addition of substrates. No significant increase in enzyme activity was observed when the reaction was run under N_2 and assays were routinely made under aerobic conditions. The reaction was terminated by rapid freezing in a dry ice–acetone bath followed by lyophilization.

The lyophilized reaction mixture was extracted with 0·6 ml of MeOH and the ^{14}C-labeled glutathione conjugate was separated from the remaining ^{14}C-labeled substrate by TLC using a 150 μl aliquot of the MeOH extract on 250 μ SiO_2 HF plates, followed by development to a height of 15 cm with two ascending solvent systems. The first solvent was benzene–acetic acid–H_2O (60:40:3). This was followed, after air drying for 30 min at room temp., by the second solvent, *n*-BuOH–acetic acid–H_2O (120:30:50). A spot of nonradioactive 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine (10 μg) was placed on each plate as a reference compound that could be readily detected as a fluorescent quenching spot under u.v. light. By this two-step development system, the R_f of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine was 0·90–0·96, the R_f of 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine was 0·50–0·65 and the R_f of *S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino) glutathione was 0·22–0·28. The area between the u.v.-visible reference marker, 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine, and the origin was scraped off the dry developed chromatogram for the quantitative determination of the ^{14}C-labeled glutathione conjugate by liquid scintillation counting.[6,21]

An alternate procedure for the separation and quantitative determination of *S*-(4-ethylamino-6-isopropylamino-2-*s*-triazino) glutathione was also developed. This procedure was based on the ion-exchange adsorption chromatography of the glutathione conjugate formed under standard assay conditions. Enzyme activity was terminated by placing the reaction tubes in an ice bath and adding 1·0 ml of MeOH. 1 ml of the resulting 50% MeOH-reaction mixture was placed directly on a 0·5 × 5 cm column of 200–400 mesh AG1-X8 anion-exchange resin in the acetate form. The unreacted atrazine was eluted from the column with 10 ml of MeOH. The adsorbed glutathione conjugate was eluated with 5·0 ml of 3 N acetic acid and a 0·50 ml aliquot of this eluate was quantitatively assayed for ^{14}C.[6,7] The ion-exchange procedure results in better than a 90% recovery of glutathione conjugate and in less than a 2% difference in the data obtained when compared to the more laborious and time-consuming TLC procedure.

Inhibition studies were carried out by incubating the inhibitor with the enzyme and buffer at 25° for 15 min before initiating the reaction by the addition of substrates. Several inhibitors were added as 95% EtOH solutions to a maximum level of 1%, v/v of EtOH. Previous studies demonstrated that this level of ethanol did not affect enzyme activity.

The enzyme unit (U) was defined as that amount of enzyme required for the biosynthesis of 1 mμmole of glutathione conjugate per 30 min under standard assay conditions.

Protein was determined by the method of Lowry *et al.*[22] with crystalline bovine serum albumin as the standard.

Reagents

Geigy Chemical Corporation, Yonkers, New York, provided ^{14}C-labeled 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine, 2-chloro-4,6-bis-isopropylamino-*s*-triazine, 2-chloro-4-*tert*-butylamino-6-ethylamino-*s*-triazine, 2-chloro-4,6-bis-ethylamino-*s*-triazine, 2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine, 2-methylmercapto-4,6-bis-isopropylamino-*s*-triazine, 2-methylmercapto-4-ethylamino-6-isopropylamino-*s*-triazine, 2-methoxy-4,6-bis(isopropylamino)-*s*-triazine and ^{36}Cl-labeled 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine. Gulf Research and Development Co., Kansas City, Kansas, supplied ^{14}C-labeled 2-chloro-4-isopropylamino-6-cyclopropylamino-*s*-triazine and 4-chloro-2-butynyl-*m*-chlorocarbanilate. Other ^{14}C-labeled compounds used in these studies were generously provided as follows: 2-chloro-*N*-isopropyl acetanilide from Monsanto Company, St. Louis, Missouri; 3-(3-trifluoromethylphenyl)-1,1-dimethylurea from CIBA Corporation, Vero Beach, Florida; 3-(3,4-dichlorophenyl)-1,-1dimethylurea and 3-(4-chlorophenyl)-1,1-dimethylurea from E. I. duPont deNemours and Co., Welmington, Delaware; 4-amino-3,5,6-trichloropicolinic acid from Dow Chemical Co., Midland, Michigan; isopropyl *N*-(3-chlorophenyl) carbamate from Pittsburgh Plate Glass Company, Pittsburgh, Pennsylvania; and 2-chloro-4-amino-6-isopropylamino-*s*-triazine from Dr. R. H. Shimabukuro of this laboratory.

Non-radioactive compounds were provided as follows: 2-methylmercapto-4-ethylamino-6-isopropylamino-*s*-triazine, 2-methylmercapto-4,6-bis(isopropylamino) *s*-triazine, and 2-methoxy-4,6-bis(isopropylamino)-*s*-triazine from Geigy Chemical Corp., Yonkers, New York; 2,4-dichloro-6-(2-chloroanilino)-*s*-triazine from Chemagro Corporation, Kansas City, Missouri; 2-chloro-2-butynyl-*m*-chlorocarbanilate from Gulf Research and Development Co., Kansas City, Kansas; 2-chloro-*N*-isopropylacetanilide and 2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide from Monsanto Company, St. Louis, Missouri; 2,3,6-trichlorophenylacetic acid from Heyden Newport Chemical Corporation, Garfield, New Jersey; and 2-chloro-4-amino-6-isopropylamino-*s*-triazine and 2-chloro-4-ethylamino-6-amino-*s*-triazine from Dr. R. E. Kadunce and Dr. F. S. Tanaka of this laboratory. Polyclar AT (research powder grade) was purchased from

[21] F. SNYDER and N. STEPHEN, *Anal. Biochem.* **4**, 128 (1962).
[22] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).

General Aniline and Film Corporation, Melrose Park, Illinois. All other reagents used were obtained commercially and were of the highest purity available.

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