Plant Physiol. (1986) 82, 1031–1035
0032-0889/86/82/1031/05/$01.00/0

Effect of Heat Shock on the Metabolism of Glutathione in Maize Roots[1]

Received for publication April 1, 1986

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ABSTRACT

High performance liquid chromatography analyses revealed that glutathione (GSH) and cysteine are two of the major low molecular weight thiol compounds in maize root extracts. Treatment of maize roots to heat shock temperatures of 40°C resulted in a decrease of cysteine levels and an increase of GSH levels. Pulse labeling of maize roots with [^{35}S]cysteine showed that the rate of incorporation of ^{35}S into GSH or glutathione disulfide (GSSG) in heat shocked tissues was twice that in nonheat shocked tissues. In addition, extracts from heat shocked maize, barley, and soybean tissues contained an unidentified low molecular weight compound that increased from 1.2- to 8-fold within 2 hours of heat shock treatment depending on the tissue and plant involved. Our results indicate that during heat shock there is an increase in the activity of the GSH synthetizing capacity in maize root cells. The elevated synthesis of GSH may be related to the cells capacity to cope with heat stress conditions.

Organisms respond to heat stress by inducing or enhancing the expression of a set of genes encoding heat shock proteins (29). A correlation between the induction of hsp[3] synthesis and increased resistance to supraoptimal temperatures has been found in various organisms such as yeast, *Dictyostelium*, insects, cultured mammalian cells and plants (for review, see Atkinson and Walden [2]). Although the function for some of the hsp has been determined in some organisms: lysyl tRNA synthetase in *Escherichia coli* (12), ATP-dependent protease in *E. coli* (25), ubiquitin in chicken embryo fibroblasts (4), an isoprotein of enolase in yeast (14), and a poly(A)-binding protein in HeLa cells (30); the identity of most of the hsp remains unknown. In maize there are at least 10 new polypeptides synthesized at heat shock temperatures as revealed in one-dimensional gel electrophoresis. These can be further resolved in 20 to 30 spots by two-dimensional gel electrophoresis (3, 5, 6).

Another feature of the heat shock response is that it can also be induced by chemicals such as arsenite, arsenate, mercury ions, and other sulfhydryl reagents (1, 19, 28). These observations suggest that thiols may play a significant role during the heat shock response. Recently, Mitchel *et al.* (23) presented evidence that the tripeptide GSH, one of the major small mol wt thiols found in eukaryotic cells (7, 21, 22, 27), increased during heat shock and may be important in the early response to thermal stress. They demonstrated that Chinese hamster VT9 cells exposed to buthionine sulfoximine, a specific inhibitor of GSH synthesis, or diethylmaleate, an agent that binds GSH, reduced the intracellular GSH levels and increased the thermal sensitivity of the cells at heat shock temperatures.

In the present investigation, we have shown that the levels of GSH in maize roots increase during heat stress. Our results confirm the observation by Mitchell *et al.* (23) and provide evidence, based on labeling experiments using [^{35}S]cysteine, that the elevation of GSH levels is due, at least in part, to an increased rate of synthesis of GSH during heat shock, In addition, we report the presence of an unidentified compound in maize, barley, and soybean tissues that increases up to 8-fold within 2 h of heat shock.

MATERIALS AND METHODS

Chemicals. Triton X-100 was obtained from Research Products International Corp., Elk Grove Village, IL. Monobromobimane (Thyolite) was supplied by Calbiochem, La Jolla, CA. [^{35}S] Cysteine was obtained from Amersham, Arlington Heights, IL. HPLC grade methanol was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. HPLC columns were obtained from two different sources: Spherisorb 3-amino-propyl, 5 micron, from Custom LC, Inc., Houston, TX; Altex ultrasphere-ODS, 5 micron, from Beckman, Berkeley, CA. All other chemicals were reagent grade and were obtained from Sigma Chemical Co.

Plant Material and Growth Conditions. Maize (*Zea mays* L.) seeds (hybrid 222, 1983 crop) were purchased from Crow's Hybrid Corn Co. (Milford, IL). Seeds were surface sterilized and germinated in the dark at 28°C under sterile conditions on paper towels moistened with 0.1 mM $CaCl_2$ in glass trays as described by Cooper and Ho (5).

Analysis of Small Molecular Weight Thiols by HPLC. For intact seedling experiments, 10 two-d old seedlings were transferred to 250 ml Erlenmeyer flasks containing 10 ml of 0.1 mM $CaCl_2$ and incubated at 28°C for 3 to 4 h prior to a further incubation at either 28 or 40°C (20 seedlings were used per time point). For excised tissue experiments, the same conditions were used except that the explants (ten 1.5 cm root tips) were incubated in 25 ml Erlenmeyer flasks containing 1 ml of 20 mM Na succinate (pH 5.0), 0.1 mM $CaCl_2$. The tissue was homogenized in 1 N $HClO_4$, 2 mM EDTA, 0.1% Triton X-100 (4 ml/g fresh weight). The homogenate was centrifuged in a microfuge for 5 min. *N*-Ethylmorpholine was added to the supernatant to a final concentration of 10 mM and then one-half vol of 2 M KOH, 0.3 M MOPS was added. The precipitate was removed by centrifugation and the supernatant was derivatized by reacting with the fluorescent compound monobromobimane and analyzed by HPLC as described by Newton *et al.* (24) (method A) or derivatized by reacting with iodoacetate and DNFB followed by HPLC

[1] Supported by National Science Foundation grant DCB-8316319.

[2] J. N-S. was supported by a fellowship from the Division of Biology and Biomedical Sciences at Washington University and a program training grant from the Monsanto Company, St Louis, MO.

[3] Abbreviations: hsp, heat shock proteins; MOPS, (3-[*N*-morpholino] propanesulfonic acid); NEM, *N*-ethylmaleimide; DNFB, 2,4-dinitrofluorobenzene; MB, monobromobimane; γ-L-glu-glu, γ-L-glutamyl-glutamic acid.

analysis as described by Reed *et al.* (26) with the modifications by Livesey and Reed (20) (method B). Alternatively, the tissue was homogenized in 0.1 N HCl (4 ml/g fresh weight) and centrifuged in a microfuge for 5 min. To the supernatant was added one volume of 4 M methanesulfonic acid and the mixture was frozen at −80°C for 15 min. After thawing, the solution was centrifuged and the supernatant derivatized and analyzed by HPLC by either of the methods described above.

To identify thiol compounds, aliquots of the homogenates were brought to 10 mM NEM and pH adjusted to 8.0 immediately prior to derivatization with 1 M NaOH. HPLC profiles of these samples were compared with the profiles of samples not treated with NEM. Further identification was accomplished by comparing elution times of unknowns with known thiols and amino acids used as internal or external standards. To calculate thiol concentrations, standard curves were made by plotting peak areas against concentrations.

Labeling *in Vivo* and Analysis of Metabolites. Ten 1.5 cm root tips from 2-d-old seedlings were incubated for 3 h in 2 ml of 20 mM Na succinate (pH 5.0), 0.1 mM $CaCl_2$ at 28°C prior to pulse labeling with [^{35}S]cysteine (1050 Ci/mmol). Samples were further incubated at 28 or 40°C. The tissue was labeled with 30 to 50 μCi/ml of [^{35}S]cysteine for different lengths of time. After labeling, the medium was removed and tissues were washed twice with 5 ml of incubation medium containing 1 mM nonradioactive cysteine.

For pulse-chase experiments, roots were pulse labeled for 30 min at 28°C, the medium was removed and roots washed twice with 5 ml of incubation medium containing 1 mM nonradioactive cysteine and further incubated at 28 or 40°C in 2 ml of incubation medium containing 1 mM nonradioactive cysteine. After the incubation the medium was removed and the tissue washed twice with 5 ml of 1 mM nonradioactive cysteine.

Homogenization was done in 0.1% Triton X-100 by the $HClO_4$ procedure described in the previous section. HPLC analysis was performed by method B (26) with further modifications (20). The column gradient was run at a flow rate of 1 ml/min, and 1 ml fractions were collected in scintillation vials. Four ml of ACS (Aqueous Counting Scintillant, from Amersham) was added and the fractions were counted in a Packard (model 3030) liquid scintillation counter.

RESULTS

Analysis of Thiol Compounds in Maize Roots. A variety of sulfhydryl reagents have been reported to be inducers of the heat shock response (1, 19, 28). To gain insight into the role that small mol wt thiols may play during heat shock, we first determined the endogenous thiol compounds present in maize roots. Maize root extracts from seedlings grown at the optimum temperature of 28°C were analyzed for the presence of small mol wt thiol compounds using two HPLC methods: method A, based on the use of the sulfhydryl reagent MB that forms fluorescent derivatives (24) and method B, based on the detection of amino-containing thiol compounds after reaction with iodoacetate and DNFB (26). Method B can also detect disulfides providing they contain at least one amino group. Consistently, peaks that comigrated with authentic cysteine and GSH were found independent of the procedure utilized (Fig. 1, A and B). One set of HPLC controls was prepared in which samples have undergone partial derivatization (dinitrobenzylation step only, as in the case of method B) resulting in reduction of elution times in cysteine and GSH peaks. When no derivatization was carried out (both methods) GSH and cysteine peaks disappeared. Another set of HPLC controls were prepared by reacting samples with NEM prior to derivatization. Similarly, GSH and cysteine peaks disappeared in both cases (method A and B).

In addition to cysteine and GSH, nonthiols like glutamate and an unidentified compound were detected by method B (Fig. 1B). No detectable levels of GSSG or of γ-glutamyl-cysteine were found.

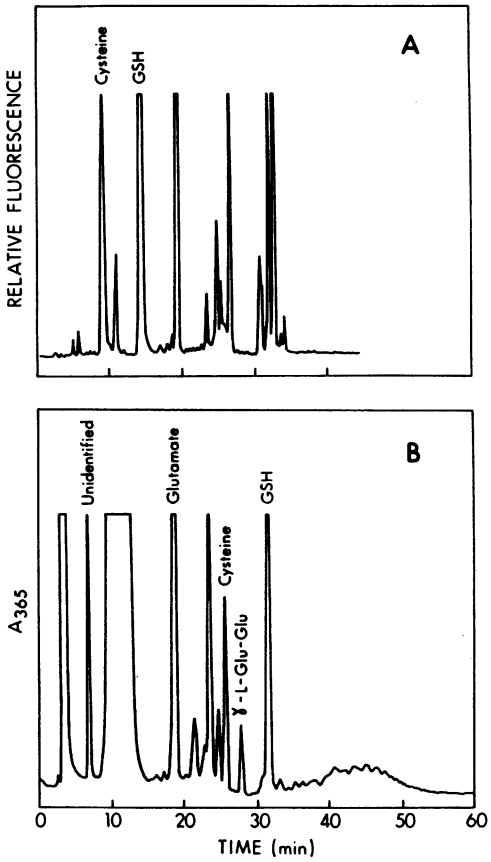

FIG. 1. HPLC of maize root extracts. Ten 1.5 cm root tips from 2-d-old maize seedlings grown at 28°C were homogenized, derivatized, and analyzed by the method of Newton *et al.* (24) (panel A) or by the method of Reed *et al.* (26) (panel B) as described in "Materials and Methods." In (B) γ-glutamyl-glutamate was used as an internal standard.

Table I. *Content of Cysteine and GSH in Maize Roots*

Ten 1.5 cm root tips from 2-d-old maize seedlings grown at 28°C were analyzed by method B (26) described in "Materials and Methods." Standard curves of cysteine and GSH were established and peak areas in HPLC profiles were used to calculate thiol concentration.

Compound	Content	
	nmol/g fresh wt	*nmol/g dry wt*
Cysteine	183	1659
GSH	743	6718

As the data in Table I indicates, one of the major thiols of maize roots is GSH, which is present at levels four times greater than the amount of cysteine on either a fresh or dry weight basis.

Levels of Cysteine and GSH during Heat Shock. Previous results in our laboratory have shown that the optimum temperature for hsp synthesis in maize roots is 40°C (5). To investigate if this temperature causes any changes in the levels of cysteine or GSH, root tips were subjected to 40°C for different lengths of time and the levels of these compounds were analyzed by HPLC using either method A or B. Figure 2 illustrates a time course of the changes of cysteine and GSH during a heat shock treatment at 40°C obtained in a typical experiment, and Table II summarizes the results of eight different experiments.

The level of free cysteine remained constant when the tissues were kept at 28°C. In contrast, a decrease in the level of cysteine

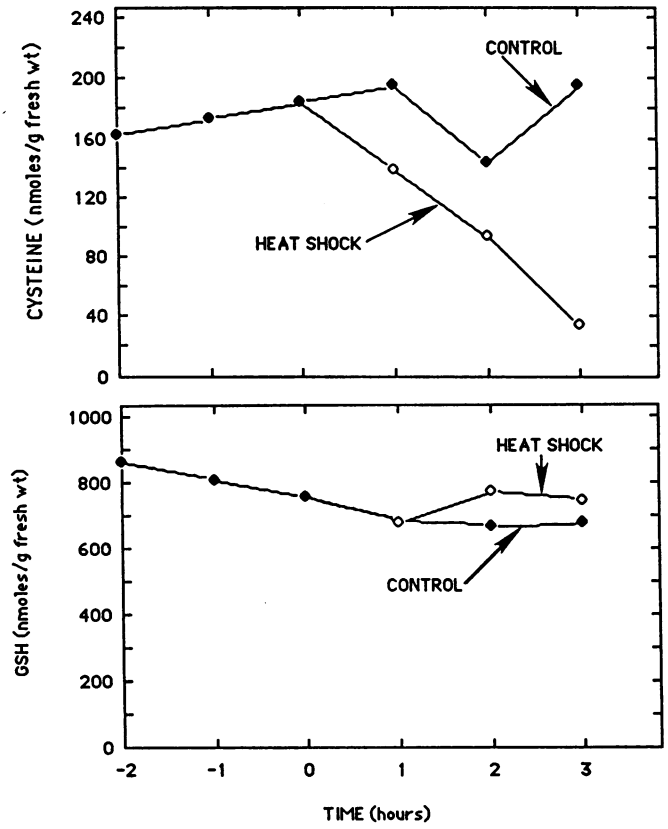

FIG. 2. Time course of cysteine and GSH content during heat shock. Twenty 2-d-old maize seedlings were preincubated in 0.1 mM $CaCl_2$ at 28°C for 2 h prior to further incubation at the indicated times at 28 or 40°C. After each treatment, 1.5 cm root tips were excised, homogenized, and derivatized for HPLC analysis by method A (24) as described in "Materials and Methods." Upper panel, cysteine content; lower panel, GSH content.

Table II. *Levels of Cysteine, GSH and the Unidentified Compound during Heat Shock as a Percentage of the Control*

Twenty 2-d-old maize seedlings were preincubated in 0.1 mM $CaCl_2$ at 28°C for 2 h prior to further incubation at the indicated times at 28 or 40°C. After each treatment, 1.5 cm root tips were excised, homogenized, and derivatized for HPLC analysis by method A (24) or B (26) as described in "Materials and Methods." Values are the averages of the ratio of heat shock/control × 100 ± SE.

Time	Heat Shock/Control		
	Cysteine	GSH	Unidentified
h		*% of control*	
1	78.0 ± 3.9	104.1 ± 4.0	412.8 ± 153.9
2	69.9 ± 5.1	117.7 ± 4.8	434.2 ± 47.9
3	17.1[a]	116.3 ± 8.7	861.5[a]

[a] Result of a single experiment.

was observed after 1 h of heat shock at 40°C and a further decrease to only 20% of the levels of control samples after 3 h of heat shock. The GSH content of seedlings grown at 28°C showed a decline during the experiment which is characteristic of many plant seeds during germination (13). However, when the tissue was heat shocked at 40°C, a 10 to 15% increase over the control levels was observed after 2 to 3 h. On a molar basis, the increase in GSH was proportional to the decrease in cysteine. No detectable levels of GSSG were found at any of the temperature treatments.

Similar results were obtained when these experiments were performed with intact seedlings or excised root tips. This ruled out the possibility that the changes we have observed were due to transport of cysteine or GSH from or to other organs of the plant. Incubation media were also analyzed for the presence of thiols during the time of incubation and no detectable levels were found, This supports the idea that the changes are not due to a differential leakage of cysteine or GSH to the incubation medium as a result of the temperature treatment but to actual changes in the metabolism of these compounds within the roots.

An Unidentified Compound Increases during Heat Shock. Table II shows that an unidentified compound increased dramatically after the 1st h of heat shock treatment. After 2 h of heat shock the levels of this compound had increased 3- to 8-fold over the control levels (see also Table III). This compound can be detected only by using the HPLC method B (26) for thiol detection. No change in the elution time of this compound was observed when reacted with NEM prior to derivatization with iodoacetate and DNFB or when the carboxymethylation step was avoided. These results suggest that this compound is probably not a thiol. However, this compound is reactive to DNFB, which suggests that it may contain at least one amino group. The elution time of this unidentified compound does not correspond with those of any of the thiol standards or other simple amines and polyamines tested so far (data not shown). We have also tested for compounds like gramine which is known to be induced by high temperature stress in barley (11). The DNB derivative of gramine has a different elution time (15.8 min) than the unidentified peak (6.5 min). Levulinic acid has been reported in acid hydrolysates of certain plants and was positive to ninhydrin reaction (32). However, this substance did not react with DNFB (data not shown). We are still in the process of identifying this compound. It appears to be present in other organs such as coleoptiles and leaves, and in other plants such as soybean and barley (Table III). The heat shock enhancement of the levels of this compound ranges from 1.2- to 8.6-fold depending upon the tissue and plant.

GSH Synthesis *in Vivo* during Heat Shock. Since cysteine is one of the precursors in the pathway leading to GSH biosynthesis (21, 22), the question arose as to whether or not the decrease in cysteine levels was due to an increase in the rate of GSH synthesis or to its utilization. The decrease in cysteine is even more remarkable considering that during a heat shock at 40°C the incorporation of [^{35}S]cysteine into protein can be 40 to 60% less

Table III. *Content of the Unidentified Compound upon Heat Shock in Different Plant Tissues*

Equal amounts of fresh excised tissue were preincubated in 1 ml of 20 mM Na succinate (pH 5.0), 0.1 mM $CaCl_2$ at 25°C for 3 h prior to further incubation for 2 h at either 25°C or 40°C. At the end of the incubation, tissues were homogenized and derivatized for HPLC analysis by method B (26) as described in "Materials and Methods." Values shown are the ratios of heat shock: control peak areas of the unidentified compound after treatment.

Plant	Tissue	Heat Shock/Control
		peak area ratio
Maize	Excised root tips	8.6
Maize	Excised proximal root segments	2.7
Maize	Root tips from intact seedlings	6.2
Maize	Excised coleoptiles	2.1
Maize	Excised leaf discs	1.9
Soybean	Excised roots	1.7
Soybean	Excised hypocotyls	1.6
Soybean	Excised leaf discs	1.2
Barley	Excised root tips	1.2
Barley	Excised coleoptiles	4.6

than at the control temperature. ^{35}S was rapidly incorporated into GSH or GSSG at both 28 and 40°C when labeling with [^{35}S] cysteine. Typically, 70 to 80% of the ^{35}S radioactivity was incorporated into GSH and GSSG; 8 to 12% remained as cysteine, and 8 to 22% was in a fraction containing methionine and noncarboxymethylated material. No radioactivity was found in fractions corresponding to the unidentified compound.

Figure 3 shows a time course of the rates of synthesis *in vivo* of GSH and GSSG which was established by giving tissues 8 min pulses of [^{35}S]cysteine. When root tips were pulse labeled with [^{35}S]cysteine during heat shock, the rate of incorporation of ^{35}S into GSH or GSSG was higher in the heat shocked samples than in the controls. Heat shock:control ratios for the rates of incorporation of ^{35}S into GSH and GSSG were: 0.8 after 1 h of heat shock, 1.9 after 2 h, 1.6 after 3 h, and 1.9 after 4 h.

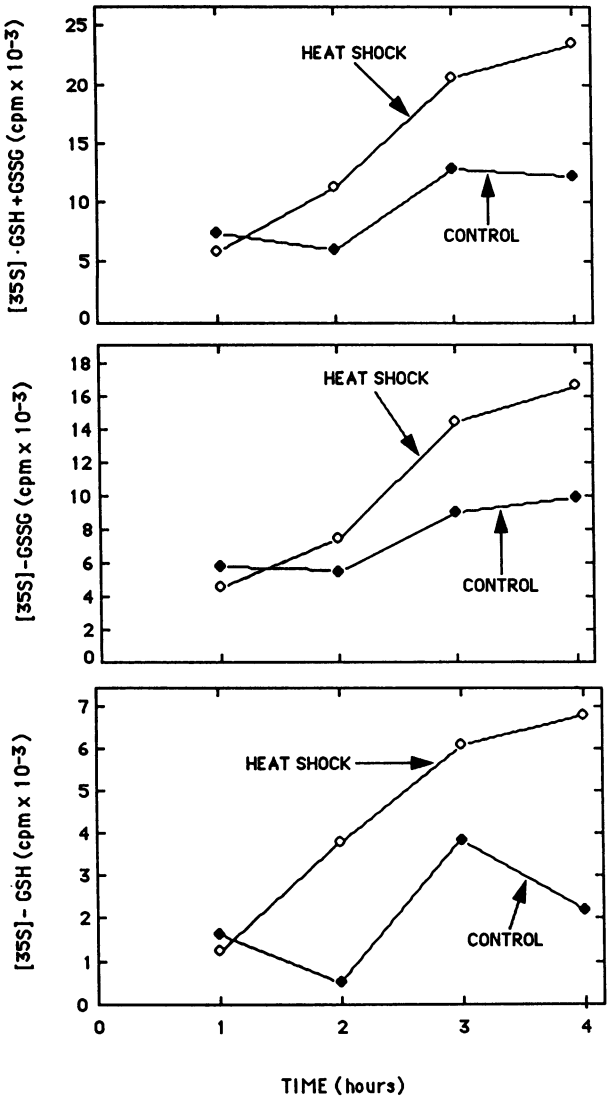

FIG. 3. Rates of glutathione synthesis during heat shock. Ten 1.5 cm root tips were excised from 2-d-old maize seedlings and preincubated for 3 h in 2 ml of 20 mM Na succinate (pH 5.0), 0.1 mM $CaCl_2$ at 28°C prior to further incubation for the indicated times at 28 or 40°C. Labeling with 40 μCi/ml of [^{35}S]cysteine took place in the final 8 min of incubation. After labeling, the medium was removed and the roots rinsed with 1 mM nonradioactive cysteine. Roots were homogenized and derivatized for HPLC analysis by method B (26). Column eluant was collected in 1 ml fractions, counted in the liquid scintillation counter, and the radioactivity corresponding to GSH or GSSG plotted. Upper panel, ^{35}S incorporated in total glutathione; middle panel, ^{35}S incorporated in GSSG; lower panel, ^{35}S incorporated in GSH.

To study the utilization of GSH, pulse-chase experiments were conducted. Root tips were pulsed for 30 min with [^{35}S]cysteine at 28°C and chased with nonradioactive cysteine either at 28°C or 40°C for 1, 2, 3 and 4 h. No net loss in radioactivity was observed after 4 h at any temperature and in some cases the radioactivity in GSH and GSSG was higher than at the start of the chase. This could indicate that during the chase period ^{35}S already incorporated into protein or in other metabolic compartments was turned over; its reutilization causing the accumulation of more radioactivity in GSH and GSSG during this time period.

To overcome the complications of the pulse-chase experiments and get an answer about the extent of utilization of GSH or GSSG, pulse experiments were performed in which the pulse lengths were varied. It became evident that the shorter the pulse of [^{35}S]cysteine the greater the heat shock enhancement on incorporation of ^{35}S into GSH and GSSG. For instance, the heat shock:control ratios of the rates of incorporation of ^{35}S into GSH and GSSG after a 2 h heat shock were: 1.1 for a 32 min pulse, 1.5 for a 24 min pulse, and 1.7 for an 8 min pulse.

This set of experiments indicate that the rates of GSH synthesis obtained during very short pulses of [^{35}S]cysteine (*i.e.* 8 min) are a better approximation to the actual rates of GSH synthesis. The decreased ratios of heat shock:control rates of GSH and GSSG synthesis during long pulses may indicate a higher rate of GSH and GSSG utilization during heat shock.

To test whether the increased rate of incorporation was a result of an increase in the specific activity of [^{35}S]cysteine during heat shock or an increase in the activity of the GSH biosynthesis enzymes (*i.e.* γ-glutamyl-cysteine synthetase and glutathione synthetase), we incubated 2-d-old maize seedlings in medium containing 10 mM nonradioactive cysteine for 2 h at either 28 or 40°C. After incubation the medium was removed, the tissue washed three times extensively with cysteine-free medium and the cysteine and GSH content of the root tips was analyzed by method B. The level of free cysteine in the heat shocked sample was 20% less than the control at 28°C, however, the level of GSH in the heat shocked sample was 22% more than the control level (Nieto-Sotelo and Ho, 1986; unpublished data). Therefore, under saturating conditions of cysteine, the only explanation for GSH elevation is an increase in the activity of the biosynthetic enzymes. Additionally, the increased synthesis of GSH may explain the concomitant elevation of GSH levels and the decrease of cysteine levels during heat shock.

DISCUSSION

Our results showed that in maize roots two of the main low mol wt thiol compounds are GSH and cysteine, the GSH content being four times greater than that of cysteine. This observation is consistent with recent surveys showing that GSH occurs uniformly in eukaryotes as the major thiol, while cysteine is a minor thiol in most of the cases (7). The analysis of thiols in heat shocked samples indicated that whereas the cysteine content diminished with time, the GSH pools built up (Fig. 2; Table II). The decrease in cysteine levels might reflect a cysteine scavenging mechanism during the stress. Toxic effects of cysteine have been well documented. Cysteine enhanced the hyperthermia-induced cell killing when applied to Chinese hamster cells (17). This toxic effect was seen to be mediated through H_2O_2 production since the addition of catalase blocked the effect (15). However, the GSH increase induced by cysteine application was not prevented by catalase.

Our labeling experiments showed an increase in the rate of GSH synthesis during heat shock which could explain the cysteine decline and GSH increase. A decrease in the utilization of

GSH during heat shock was ruled out on the basis of a comparison of the rates of ^{35}S incorporation in GSH and GSSG with changing pulse time lengths. When the lengths of pulse were shortened the results showed a further heat shock enhancement of the incorporation of ^{35}S into GSH and GSSG.

Elevation in GSH levels under heat shock in mammalian cells have been reported previously by Mitchel *et al.* (23). They also showed that agents that bind GSH or inhibitors of GSH synthesis reduced the thermotolerance of Chinese hamster V79 cells, indicating that an increased synthesis of GSH is responsible for thermotolerance. Our results confirm these findings and we provide direct evidence for increase in GSH synthesis *in vivo* during heat shock. We do not know if these changes are due to an increase in the activity of γ-glutamyl-cysteine synthetase and glutathione synthetase as a result of thermal activation of these enzymes or an increase in the synthesis *de novo* of these enzymes. γ-Glutamyl-cysteine synthetase is considered to be the key enzyme in GSH synthesis (21, 22). Studies are under way in our laboratory to determine whether or not this enzyme is an hsp.

GSH participates in a great variety of enzymic and nonenzymic reactions such as the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection against the toxic effects of drugs, foreign compounds and O_2 (21, 22, 27). It is possible that temperatures above the optimum for cell growth potentiate damage in some of these pathways and GSH participation in the prevention or repair of damage is important under stress conditions.

The possibility exists that GSH elevation during heat shock reflects an accumulation of H_2O_2 or related oxygen radicals. Heat shock has been proposed to be an oxidative stress (18). AppppA and related dinucleotides accumulate after conditions of oxidative stress as well as heat shock. The involvement of GSH in protection against oxidative damage has been reported. Smith *et al.* (31) have found increased GSH levels in barley shoots exposed to the catalase inhibitor aminotriazole or shoots of a catalase deficient barley mutant exposed to air after 8 h in the light. In a normal atmosphere, this mutant suffers oxidative damage due to H_2O_2 accumulation. Recently, a dependence on O_2 tension and increased thermal sensitivity to GSH depletors was found (8). A reduction in O_2 tension decreased the sensitivity to heat shock in cells treated with the GSH depleter diethylmaleate. The authors suggested that the possible role of GSH under heat shock may be the protection against O_2 damage.

An alternative possibility to explain GSH elevation during heat stress could be its function as precursor of phytochelatins (9, 16). These compounds are small peptides whose structure is (γ-glutamyl-cysteinyl)*n*-glycine (n = 3–7). Phytochelatins are induced in higher plants by heavy metals such as cadmium, copper, mercury, lead, and zinc. It is known that cadmium and other heavy metals induce the synthesis of the hsp (1, 19, 28). The possibilities that GSH serves as a precursor of phytochelatin synthesis and that phytochelatins may accumulate and play a role in the heat stress response, are worth future study.

We also found an unidentified compound that increases upon heat shock. We have been able to further purify the fraction corresponding to the unidentified peak by subsequent separation with a different HPLC system (data not shown). Preliminary data on the MS of this fraction indicate that the mol wt of this compound is below 200 (data not shown). Further characterization of the compound is necessary in order to study its presumptive relevance during heat shock.

Acknowledgments—We gratefully acknowledge the help from people in Dr. D. J. Reed's laboratory, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR, on the use of HPLC technique to analyze thiol compounds. We also thank Dr. J. E. Varner for critically reading the manuscript.

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