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Poly(γ-glutamylcysteinyl)glycine Synthesis in *Datura innoxia* and Binding with Cadmium[1]

Role in Cadmium Tolerance

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ABSTRACT

The effects of Cd on poly(γ-glutamylcysteinyl)glycine [(γEC)$_n$G] biosynthesis and formation of (γEC)$_n$G:Cd complexes were measured in two cell lines of *Datura innoxia* with differing Cd tolerance. In addition, RNA synthesis, protein synthesis, and GSH concentrations were measured during a 48 hour exposure to Cd. Exposure to 250 micromolar $CdCl_2$ was toxic to the sensitive line, whereas the tolerant line survived and grew in its presence. Cd-sensitive cells synthesized the same amount of (γEC)$_n$G as tolerant cells during an initial 24 hour exposure to 250 micromolar $CdCl_2$. However, rates of (γEC)$_n$G:Cd complex formation differed between the two cell lines with the sensitive cells forming complexes later than tolerant cells. In addition, the complexes formed by sensitive cells were of lower molecular weight than those of tolerant cells and did not bind all of the cellular Cd. Pulse-labeling of cells with L-[^{35}S]cysteine resulted in equivalent rates of incorporation into the (γEC)$_n$G of both cell lines during the initial 24 hours after Cd. Rates of protein and RNA synthesis were similar for both cell lines during the initial 8 hours after Cd but thereafter declined rapidly in sensitive cells. This was reflected by a decline in viability of sensitive cells. The GSH content of both cell lines declined rapidly upon exposure to Cd but was higher in sensitive cells throughout the experiment. These results show that the biosynthetic pathway for (γEC)$_n$G synthesis in sensitive cells is operational and that relative overproduction of (γEC)$_n$G is not the mechanism of Cd-tolerance in a Cd-tolerant cell line of *D. innoxia*. Rapid formation of (γEC)$_n$G:Cd complexes that bind all of the cellular Cd within 24 hours appears to correlate with tolerance in these cells.

Whole plants and plant cells in culture respond to Cd stress by synthesizing (γEC)$_n$G[3] (where n = 2 or greater). These compounds are also known by the following names: cadystin, phytochelatin, γ-glutamyl metal binding peptide, Cd-binding polypeptides, and class III metallothioneins (4, 6–9, 11, 13, 18, 19, 21–23, 25). These polypeptides are structurally related to GSH in that they consist of a GSH molecule with one or more γ-glutamylcysteinyl moieties attached via α-carboxamide bonds. Additional evidence to suggest that GSH is a precursor of (γEC)$_n$G includes an observed depletion of GSH and concomitant synthesis of (γEC)$_n$G when plants or cells are exposed to Cd (8, 17, 23), replacement of the Gly by β-alanine in the (γEC)$_n$G homologues of plants that use the corresponding homologue of GSH (6), the inability of *Schizosaccharamyces pombe* mutants that lack GSH biosynthetic enzymes to synthesize these peptides (15), and an observed inhibition of (γEC)$_n$G biosynthesis by buthionine sulfoximine (8, 20, 23, 25). Buthionine sulfoximine is a specific inhibitor of γ-glutamylcysteinyl synthetase (EC 6.3,2,21), an enzyme of the GSH biosynthetic pathway (5).

In its native form, (γEC)$_n$G binds Cd to form complexes of mol wt 1.5 to 10 kD as determined by gel filtration (2, 11, 13, 18). This indicates that they consist of oligomers of the polypeptides. Murasugi *et al.* (14) have shown that, in response to Cd, *S. pombe* synthesizes two types of (γEC)$_n$G:Cd complexes, one of which contains acid-labile sulfide. Cd-binding by (γEC)$_n$G suggests its involvement in the mechanism of Cd detoxification. Inhibition of (γEC)$_n$G synthesis in Cd-tolerant cells by buthionine sulfoximine results in loss of tolerance (8, 25). Cells in suspension culture selected for tolerance to high concentrations of Cd produce large amounts of (γEC)$_n$G:Cd complexes as determined by gel filtration chromatography (11, 25). The amount of (γEC)$_n$G:Cd complex synthesized by *Datura innoxia* was found to correlate with the level of Cd-resistance in different cell lines (11). In these experiments the formation of (γEC)$_n$G:Cd complexes was measured with ^{109}Cd as a tracer and (γEC)$_n$G was not directly measured. In addition, cells were exposed to the greatest concentration of Cd that they could tolerate and were therefore not exposed to equivalent concentrations of Cd. Although these data suggest that (γEC)$_n$G overproduction is responsible for tolerance, it is possible that increased (γEC)$_n$G production in tolerant cells is a consequence of survival due to some other factor.

Presented here are experiments designed to determine the initial rates of (γEC)$_n$G biosynthesis in both Cd-tolerant and Cd-sensitive cells of *D. innoxia* exposed to 250 μM Cd. A modification of an assay described by Grill *et al.* (7) for the estimation of the individual peptides uses reverse-phase

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[3] Abbreviation: (γEC)$_n$G, poly(γ-glutamylcysteinyl)glycine.

HPLC of acidified extracts (22). Under these conditions $(\gamma EC)_nG$:Cd complexes are dissociated and the peptides separated according to chain length. In addition, the formation of $(\gamma EC)_nG$:Cd complexes was followed by gel filtration. The results show that sensitive cells synthesize the same amount of $(\gamma EC)_nG$ as tolerant cells during a 24 h exposure to Cd. However, the ability to form $(\gamma EC)_nG$:Cd complexes differs. Tolerant cells form the complexes earlier than sensitive cells and those formed by sensitive cells are lower mol wt and do not chelate all of the cellular Cd.

MATERIALS AND METHODS

Maintenance of Plant Suspension Cultures

Suspension cultures of Cd-sensitive and Cd-tolerant cell lines of *Datura innoxia* were maintained in the dark in 50 mL batch suspension cultures as described by Jackson *et al.* (10). When different volumes were used, the flasks that contained the cultures were selected such that the ratio of flask volume to culture volume was 5 to 1. The tolerant cells were derived from the sensitive line by a stepwise selection protocol and have been previously described (10, 11).

Long-Term Growth Experiments

The effect of Cd on growth of the cell lines was determined for 50 mL cell cultures which were diluted to equivalent densities at the start of the experiment. Culture samples (5 mL) were taken every 24 or 48 h, centrifuged at 800*g* for 1 min, and the packed cell volume was recorded.

Short-Term Effects of Cd

Cultures (250 mL) of Cd-sensitive and Cd-tolerant cells in logarithmic growth and of equal densities were transferred into fresh medium at a ratio of one part culture to three parts medium to give ten 100 mL cultures of each cell line. Two h after transfer, $CdCl_2$ (250 μM final concentration) was added to all flasks except to the 0 h time points. At specific times after the addition of the $CdCl_2$ (1, 2, 4, 8, 12, 16, 24, 36, and 48 h), cells from one flask of each cell line were analyzed. From the 100 mL culture, three 25 mL aliquots were transferred into 125 mL flasks for pulse-labeling experiments, and the remainder of the culture was analyzed for GSH, packed cell volume, and trypan blue exclusion.

Pulse-Labeling of Cells

All radioactively labeled compounds were obtained from duPont-New England Nuclear. L-[^{35}S]cysteine (62.5 μCi, >300 Ci/mmol), L-[^{3}H]leucine (40 μCi; 1.3 Ci/mmol), and [^{3}H]uridine (100 μCi; 12.5 Ci/mmol) were added separately to one of each of the 25 mL cultures as described above. After a 30 min incubation, the cultures were centrifuged (800*g* for 5 min), and the amount of label incorporated into $(\gamma EC)_nG$, RNA, or protein was determined in the cell pellets.

$(\gamma EC)_nG$ was extracted and analyzed by reverse-phase HPLC as described previously using 0.1% TFA instead of 0.05% phosphoric acid (22). Both the amount of $(\gamma EC)_nG$, determined by the thiol content, and the amount of L-[^{35}S] cysteine incorporated into $(\gamma EC)_nG$ were calculated by integrating and summing the relevant peaks from the HPLC profile. The amount of L-[^{35}S]cysteine incorporated into GSH and into the cysteine pool of the cells was estimated from the same profiles.

To determine incorporation of L-[^{3}H]leucine into protein, cells were homogenized in 1 mL of buffer (62.5 mM Tris [pH 6.8], 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 10% [w/v] glycerol) and the extract was boiled for 4 min. After centrifugation (10,000*g* for 5 min), samples (200 μL) of the extract were incubated in 2 mL of ice-cold 25% (w/v) TCA for 40 min and the resultant precipitates collected on glass-fiber filters. The filters were washed with two 8 mL volumes of 8% (w/v) TCA, dried, then placed in 4 mL of scintillant (Formula 963; duPont-New England Nuclear) and the radioactivity was measured. The amount of [^{3}H]uridine incorporated into RNA was determined for intact cells as described by Chu and Lark (3).

Assay of $(\gamma ED)_nG$:Cd Complexes

In separate experiments the formation of $(\gamma EC)_nG$:Cd complexes was followed by gel filtration. Cultures (50 mL; 1.5–2.0 mL starting packed cell volume) were exposed to 250 μM $CdCl_2$ and 15 μCi of carrier-free $^{109}CdCl_2$ for different times. A 20 mL sample was taken for the analysis of $(\gamma EC)_nG$ by a thiol assay as described above. Another 20 mL sample was used to measure $(\gamma EC)_nG$:Cd complex formation. Equivalent cultures were labeled with [^{3}H]leucine to estimate rates of protein synthesis. For the gel filtration assay, cells were collected (800*g* for 1 min) then resuspended and washed twice in ice-cold buffer that contained 10 mM Tris-Cl (pH 7.4), 10 mM KCl, and 1.5 mM $MgCl_2$. The final pellet was aspirated dry and resuspended in an equal volume of washing buffer that contained 50 mM β-mercaptoethanol. The cells were homogenized in a hand-held glass homogenizer then centrifuged at 20,000*g* for 30 min. Twenty μL of the cell extract was applied to two Spherogel-TSK 3000SW columns in series (Beckman Instruments; 300 × 7.5 mm each) that were equilibrated with 50 mM Tris-Cl (pH 7.0), 150 mM NaCl. Samples were eluted with the same buffer at 0.5 mL/min and 250 μL fractions were collected and assayed for ^{109}Cd. The elution profiles of ^{109}Cd-labeled extracts were similar to those obtained from analysis of the same samples by conventional chromatography on Sephadex G-50 (Pharmacia) (11). Recovery of ^{109}Cd was typically greater than 80% for samples which contained predominately low mol wt Cd complexes and greater than 90% for samples containing predominately $(\gamma EC)_nG$:Cd complexes.

Other Assays

Total GSH (GSH plus GSSG) concentrations were determined by a recycling enzymic assay (1). Five mL of cell culture were collected by centrifugation (800*g* for 5 min) and the cell pellet ground in 1 mL of 5% (w/v) TCA with a hand-held homogenizer. The homogenate was centrifuged at 10,000*g* for 5 min and the supernatant used for the assay. The concentrations of possible interfering substances such as TCA and Cd^{2+} were too low to affect the assay as determined

by addition of known amounts of GSH (Sigma Chemical Co.). Cells were stained with trypan blue to estimate the percentage which excluded the dye as described by Chu and Lark (3). Protein was assayed with a Coomassie blue based reagent (Bio-Rad) and BSA as a standard.

RESULTS

Effect of Cd on GSH Concentrations

GSH appears to be a precursor of $(\gamma EC)_nG$ and we hypothesized that greater production of $(\gamma EC)_nG$ by tolerant cells might be due to an initially higher endogeneous GSH concentration. It is also possible that tolerant cells are protected from initial Cd exposure by a higher GSH content since GSH is suggested to be involved in cellular protection during early Cd exposure in mammalian cells (24). However, it appears that this is not the case, since Cd-sensitive cells had higher total GSH concentrations than Cd-tolerant cells throughout the experiment (Fig. 1). An initial drop in GSH concentration in response to Cd has been observed in other plant cells and in whole plants (8, 17, 23), and in the present experiment, the concentrations fell below the detectable limits of the assay after 36 to 48 h (Fig. 1).

Effect of Cd on $(\gamma EC)_nG$ Biosynthesis

Cd stimulated the synthesis of $(\gamma EC)_nG$ in both Cd-tolerant and Cd-sensitive cells. The amount of $(\gamma EC)_nG$ accumulated was the same for sensitive and tolerant cells during the initial 24 h of exposure to Cd (Fig. 2). After 48 h exposure to Cd, sensitive cells had accumulated less $(\gamma EC)_nG$ but this coincided with cell death occurring between 24 and 48 h. The data from four independent experiments showed that, after 24 h exposure to Cd, sensitive cells accumulated 99, 112, 96, and 78% of the $(\gamma EC)_nG$ accumulated by tolerant cells. Pulse-labeling experiments showed that exogenously supplied L-[^{35}S]cysteine incorporation into $(\gamma EC)_nG$ increased with time after Cd and that the rates were also the same for both cell lines during the first 24 h of Cd (Fig. 3). Although the total amount of $(\gamma EC)_nG$ synthesized as determined by a thiol assay was the same, synthesis of the various species differed. Tolerant cells synthesized more of the longer chain species (n = 3, 4, and 5) than sensitive cells by 24 h (Fig. 4). Polypeptides of n = 4 and 5 were detectable in tolerant cells but not in sensitive cells from 4 to 8 h after addition of Cd.

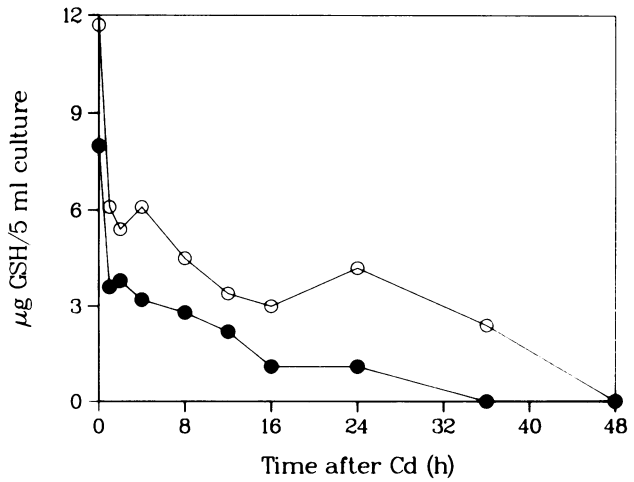

Figure 1. Effect of 250 μM $CdCl_2$ on GSH concentrations in sensitive (○) and tolerant (●) cells. Total GSH (GSH plus GSSG) was estimated by an enzymic assay (1) in the cells obtained from 5 mL of culture.

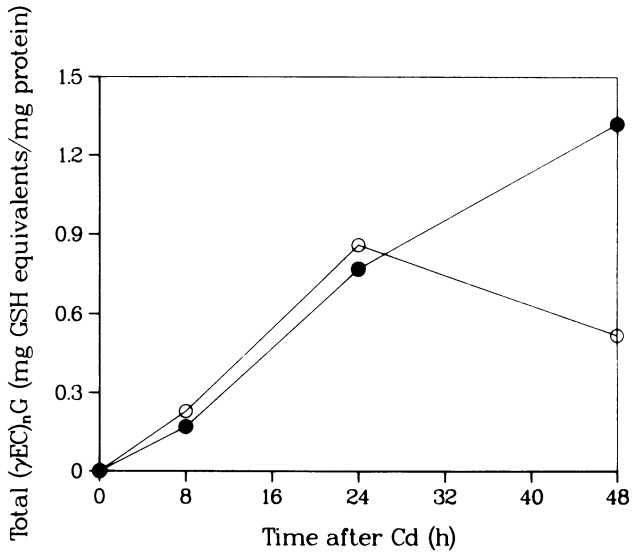

Figure 2. Effect of Cd on $(\gamma EC)_nG$ accumulation in sensitive (○) and tolerant (●) cells of *D. innoxia*. Cells were exposed to 250 μM $CdCl_2$ and extracted at the times shown. $(\gamma EC)_nG$ was separated by reverse-phase HPLC and total thiols were estimated by Ellman's reagent with GSH to calibrate the assay (22). Under the conditions of the assay $(\gamma EC)_nG$:Cd complexes are dissociated and the individual $(\gamma EC)_nGs$ separated according to length. The data are expressed on the basis of mg of starting (time 0) protein in the cultures since, at the later time points, protein synthesis in sensitive cells was greatly reduced.

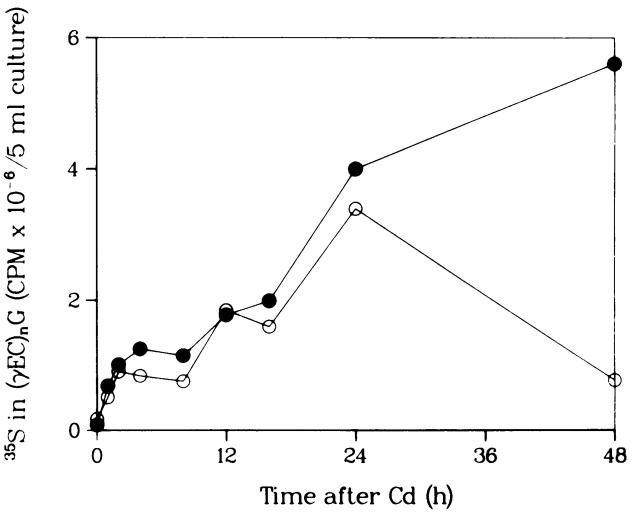

Figure 3. Effect of 250 μM $CdCl_2$ on the rate of L-[^{35}S]cysteine incorporation into $(\gamma EC)_nG$ in Cd-tolerant (●) and Cd-sensitive cells (○). At different times after Cd exposure, 25 mL of cells were labeled with L-[^{35}S]cysteine (62.5 μCi) for 30 min then samples were prepared and analyzed by reverse-phase HPLC as described previously (22).

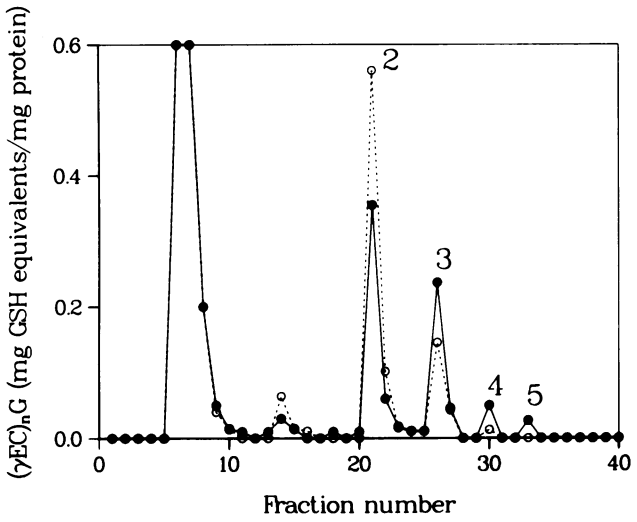

Figure 4. Synthesis of $(\gamma EC)_nG$ of different chain lengths in sensitive (---) and tolerant (——) cells 24 h after 250 μM $CdCl_2$. $(\gamma EC)_nG$ was extracted and separated by reverse-phase HPLC, and fractions were analyzed for thiols by previously described methods (22). The *n* value is shown above the corresponding peaks. The major peak at fractions 5 to 9 is a result of β-mercaptoethanol in the extraction buffer.

Formation of $(\gamma EC)_nG$:Cd Complexes

Tolerant cells formed $(\gamma EC)_nG$:Cd complexes earlier than sensitive cells after exposure to Cd. Figure 5 shows the data obtained from a typical experiment. In all of the experiments, Cd-tolerant cells showed a similar pattern of $(\gamma EC)_nG$:Cd formation with 95 to 100% of the Cd accounted for by $(\gamma EC)_nG$:Cd complexes at 24 h. By contrast, sensitive cells showed greater variability in the ability to form $(\gamma EC)_nG$:Cd complexes in four independent experiments (Table I). In the three experiments where sensitive cells did synthesize a $(\gamma EC)_nG$:Cd complex by 24 h, it eluted as a peak with a greater retention time than the $(\gamma EC)_nG$:Cd complexes of tolerant cells, indicating it is smaller or has a different structure. In addition, a large proportion of the Cd was complexed by smaller compounds of unknown composition (Table I).

At 4 h, tolerant cells had formed $(\gamma EC)_nG$:Cd complexes which accounted for 29% of the total Cd, whereas in sensitive cells all of the Cd was associated with low mol wt complexes which eluted either coincident with or slightly before a Cd-β-mercaptoethanol complex. Some of these low mol wt complexes, which were also apparent in the tolerant cells at 4 h, might have resulted from free Cd extracted from the cells which was subsequently complexed by β-mercaptoethanol present in the extraction buffer. A proportion may have been precursor forms for the larger $(\gamma EC)_nG$:Cd complexes and could consist of single $(\gamma EC)_nG$ peptides complexed to Cd prior to forming the larger oligomers. The total amount of Cd accumulated was the same for both cell lines indicating that exclusion of Cd is not the tolerance mechanism. The soluble fraction accounted for 95 to 100% of the Cd accumulated in both cell lines.

Effect of Cd on RNA Synthesis, Protein Synthesis, and Call Viability

The effect of Cd on these parameters was consistent with the ability of the cells to tolerate Cd. Relative rates of RNA and protein synthesis in sensitive cells declined rapidly 8 h after addition of Cd (Fig. 6A) and were reflected in the ability of cells to exclude a vital stain (Fig. 6B). At 48 h, the sensitive cells were dead and did not grow when transferred to media that contained no Cd. Tolerant cells showed an initial drop in cell viability but continued to grow and synthesize RNA and protein at increasing rates as the experiment progressed. These data show that vital processes in sensitive cells are

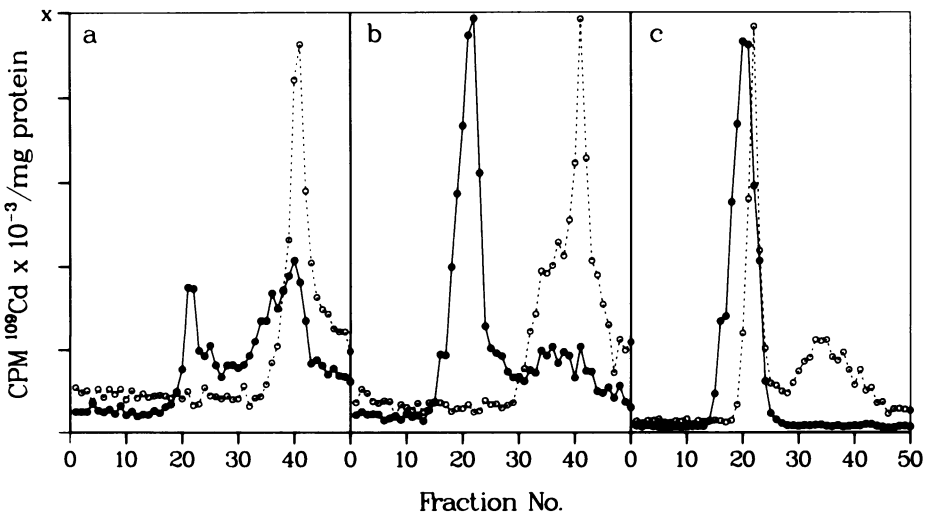

Figure 5. Formation of $(\gamma EC)_nG$:Cd complexes in sensitive (○) and tolerant (●) cells 4 h (a), 8 h (b), and 24 h (c) after exposure to 250 μM $CdCl_2$ and 15 μCi of ^{109}Cd (panels a, b, and c; x = 50, 70, and 325 respectively). Extracts of ^{109}Cd-labeled cells were separated by gel filtration HPLC as described in "Materials and Methods." Collection of fractions was started 30 min after injection of sample onto the column. $(\gamma EC)_nG$:Cd complexes were identified from previous experiments (11; fractions 25 and greater) and the elution position of a Cd:β-mercaptoethanol complex is 49.5 min (fraction 39). The void volume eluted at 21 min (not shown) and did not have any Cd associated with it, while the total column volume eluted at 53 min (fraction 46). Complexes eluting before fraction 26 are designated as $(\gamma EC)_nG$:Cd complexes; those eluting between fractions 26 and 36 (inclusive) are designated as intermediate complexes; and those eluting after fraction 36 are designated as coincident with β-mercaptoethanol:Cd complexes.

Table I. *Time Course of Formation of Cd Complexes in Cd-Sensitive Cells Showing the Variation Obtained from Four Independent Experiments*

Cells were labeled with ^{109}Cd, extracted, and analyzed by gel filtration HPLC as described in "Materials and Methods." Complexes eluting before fraction 26 are designated as $(\gamma EC)_nG$:Cd complexes, those eluting between fractions 26 and 36 (inclusive) are designated as intermediate complexes and those eluting after fraction 36 are designated as coincident with β-mercaptoethanol:Cd complexes. The retention times of the various complexes are shown in Figure 5.

Experiment	Time after Cd	Percent of Total Cd Bound		
		$(\gamma EC)_n$:G:Cd	Intermediate complexes	Coincident with β-mercaptoethanol:Cd
1	24	0	12	88
	48	0	3	97
2	8	2	69	29
	24	71	17	12
	48	79	13	8
3	4	0	1	99
	8	0	23	77
	24	62	30	8
4	4	0	34	66
	8	52	14	34
	24	61	22	17

affected by Cd before accumulation of $(\gamma EC)_nG$ is affected between 24 to 48 h after exposure to Cd.

DISCUSSION

Previous studies on $(\gamma EC)_nG$ synthesis by whole plants or plant cells in culture have implicated its involvement in metal tolerance (6–11, 21, 22, 25). If $(\gamma EC)_nG$ synthesis is inhibited, for example by buthionine sulfoximine, then tolerant cells show increased sensitivity to Cd (8, 25). These and other data suggested that overproduction of $(\gamma EC)_nG$ in tolerant cell lines relative to sensitive cells is the basis of resistance. The results in this paper show that this appears not the case for Cd-tolerant cells of *D. innoxia*. Cd-sensitive *D. innoxia* cells synthesize equivalent amounts of $(\gamma EC)_nG$ to tolerant cells during the initial 24 h after exposure to a toxic concentration of Cd. After 48 h, the sensitive cells synthesized about half the amount synthesized by tolerant cells, but this is likely to be a consequence of cell death. Rates of protein and RNA synthesis begin to decline in the sensitive cells 8 h after Cd exposure (Fig. 6) and by 48 h the cells have died. These data taken together indicate that, while $(\gamma EC)_nG$ synthesis is an essential component of the tolerance mechanism, it is not sufficient in itself to produce the tolerant phenotype. The tolerance mechanism in *D. innoxia* must therefore include some other factor.

The equivalent synthesis of $(\gamma EC)_nG$ in the two cell lines of *D. innoxia* is not reflected by an equivalent formation of $(\gamma EC)_nG$:Cd complexes. Sensitive cells form $(\gamma EC)_nG$:Cd complexes later than tolerant cells, and a proportion of the Cd remains associated with smaller, uncharacterized complexes (Fig. 5). Similar results have been observed in a Cu-tolerant ecotype of *Agrostis gigantea*. In this species, tolerance appears to be associated with the ability to rapidly form complexes with a Cu-binding protein when plants are stressed with excess Cu (16). In *Schizosaccharomyces pombe*, two forms of the complexes have been identified (14, 18). One form contains acid-labile sulfide (complex II) and has a higher capacity for Cd, while the other is devoid of sulfide and contains only Cd and $(\gamma EC)_nG$ (complex I). It appears that complex II is derived from complex I but this has not been proven. On gel filtration, complex II elutes earlier than complex I, and they can be separated as two close peaks or as a combined broad peak with complex II being predominately at the front and complex I predominately at the trailing end of the peak (14, 18). Mutoh and Hayashi (15) have identified Cd-sensitive mutants of *S. pombe* which are able to synthesize complex I but not complex II, indicating the importance of complex II for Cd tolerance in this species.

Figure 5 shows that, in the current study, tolerant cells form complexes which elute as a broad peak consistent with a mixture of complex I and II (Fig. 5, fractions 15–26). By contrast, sensitive cells form a complex after 24 h exposure to Cd which elutes at a position consistent with only complex I (Fig. 5, fractions 20–26). It is possible that the larger complexes synthesized by the tolerant cells have a higher binding capacity and/or affinity for Cd than the complexes formed by sensitive cells. This could be due to the presence of sulfide in the complex as observed for complex II from *S. pombe*. However, when the complexes from tolerant cells were analyzed for acid-labile sulfide in preliminary studies (data not shown) by the method of King and Morris (12), very little could be detected. Indeed, a concentrated sample of complex partially purified from Cd tolerant cells yielded sulfide in the ratio of 1 to 38 mol Cd, much lower than that reported for *S. pombe* (14, 18) even if it is considered that a mixture of complex I and complex II were present. These data indicate that there is either a difference in the sulfide content of complexes between *S. pombe* and *D. innoxia* or that something is interfering with the sulfide assay in our samples.

The data in Figure 3 show that the rate of L-[^{35}S]cysteine incorporation into $(\gamma EC)_nG$ increases with time after exposure to Cd. The increased incorporation may reflect a greater

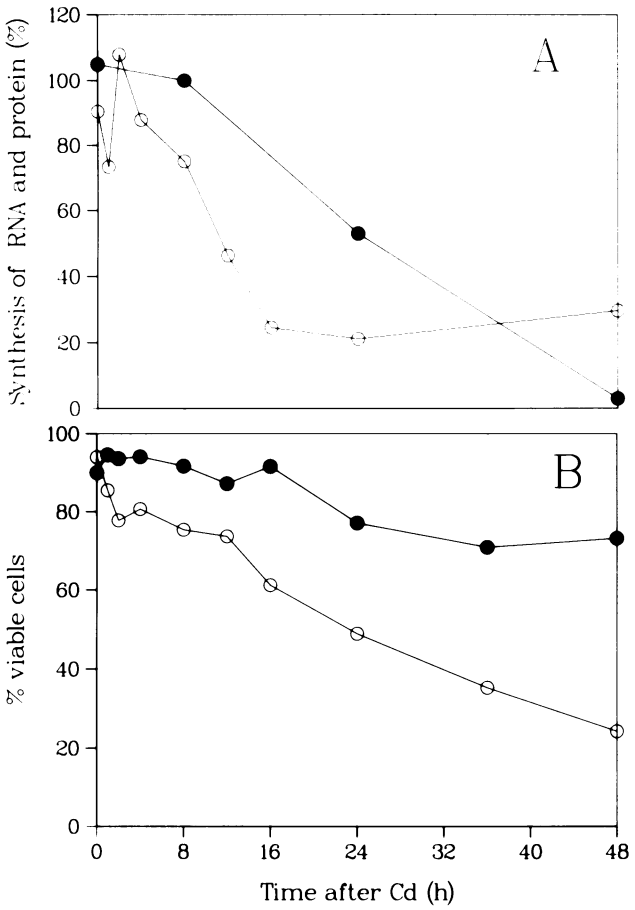

Figure 6. A, effect of 250 μM $CdCl_2$ on rates of RNA and protein synthesis of Cd-sensitive cells. Cells were pulse-labeled with 40 μCi of [^{3}H]uridine or 40 μCi of L-[^{3}H]leucine for 30 min prior to estimation of the amount of radiolabel incorporated into RNA or protein. The amount of radioactivity incorporated into RNA (○) and protein (●) by the sensitive cells is expressed as a percentage of the amount incorporated by tolerant cells. B, cell viability in sensitive (○) and tolerant (●) cells after exposure to 250 μM $CdCl_2$. Cell viability was estimated by the ability of cells to exclude trypan blue (4).

specific radioactivity of L-[^{35}S]cysteine as time progresses due to depletion of endogenous and exogenous cold cysteine and does not necessarily indicate an increased rate of $(\gamma EC)_nG$ biosynthesis. This is supported by the data on the amount of $(\gamma EC)_nG$ accumulated which show a linear increase in $(\gamma EC)_nG$ with time, as expected for a constant rate of synthesis (Fig. 2). However, degradation of $(\gamma EC)_nG$ or conversion into forms not detected by the reverse-phase HPLC assay might contribute to an underestimation of total $(\gamma EC)_nG$ synthesized over a given time. Both of these factors may also be important in the tolerance mechanism. In addition, Bernhard and Kägi (2) have shown that $(\gamma EC)_nG$:Cd complexes isolated from corn under conditions to prevent oxidation of sulfhydryl groups possess a large number of disulfide bonds. It is possible that the larger complexes observed in tolerant cells contain disulfide linkages which stabilize the complex. The synthesis of longer chain species of $(\gamma EC)_nG$ (n = 4, 5) by tolerant cells might be important in the formation of such stabilized complexes even though they represent only a small proportion of the total $(\gamma EC)_nG$.

Further experiments are being directed toward characterizing the $(\gamma EC)_nG$:Cd complexes from Cd-tolerant and Cd-sensitive cells. These include comparisons of the amounts of acid-labile sulfide, disulfide bonds, and longer chain $(\gamma EC)_nG$ species in fully purified preparations. The biochemical basis of the early formation of $(\gamma EC)_nG$:Cd complexes by tolerant cells is also being investigated.

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