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DIAMIDE EXPOSURE, THERMAL RESISTANCE, AND SYNTHESIS OF STRESS (HEAT SHOCK) PROTEINS*

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Abstract—Chinese hamster ovary (CHO) cells were treated with the thiol oxidant diamide for 1 hr at 37°, incubated in diamide-free medium for 4 hr at 37°, and then exposed to hyperthermic treatment (43°) or assayed for the presence of 110, 90 and 66 kilodalton (kD) stress (heat shock) proteins. Cellular inactivation produced by the hyperthermic treatment was measured using colony formation as the end point. Low concentrations of diamide, which did not result in depletion of intracellular GSH, induced a moderate degree of protection against thermal toxicity but did not affect the pattern of protein synthesis. Exposure to 0.4 mM diamide, which reduced intracellular GSH concentrations by 50–60%, significantly reduced the rate of hyperthermic cellular inactivation. This occurred coincidentally with the synthesis of stress proteins of approximate molecular weights of 110, 90 and 66 kD. Furthermore, this concentration of diamide protected cells from thermal inhibition of protein synthesis. These results indicate that thiol oxidation by diamide can induce both the development of thermal resistance to cellular inactivation and the synthesis of stress proteins.

Therapeutic hyperthermia is currently being assessed as an adjuvant to both radiation and chemotherapy in the treatment of human neoplasia [1]. The rate of cell killing, measured after hyperthermic treatment is regulated, in part, by the intracellular concentration of glutathione (GSH‖). Depletion of intracellular GSH increases thermal sensitivity in V-79 and CHO cells [2–4]. CHO cells contain three reservoirs of GSH which, in turn, regulate thermal toxicity in a stepwise manner [5]. Removal of 50% or less of the GSH does not affect sensitivity. A small increase in sensitivity occurs when 50–80% of the GSH is removed. A significant increase in thermal toxicity occurs when greater than 80% of the GSH is depleted. The degree of GSH modification of thermal damage, however, diminishes as the oxygen tension is reduced [4].

Thermotolerance denotes a reduction in the rate of cellular inactivation produced by hyperthermic treatment [6]. It is an adaptive process which is triggered immediately after thermal stress and persists for 24–72 hr [6, 7]. The synthesis of heat shock proteins has been correlated with the development of thermotolerance, while the decay of tolerance parallels the loss of heat shock proteins from a cell [8]. Russo *et al.* [9] found that treatment with BSO reduces (a) GSH levels in V-79 cells, (b) the development of thermotolerance and (c) the synthesis of heat shock proteins. In CHO cells, depletion of GSH only inhibits the development of thermotolerance if it is triggered by a toxic heat shock, e.g. 50% of the cells are killed by the triggering dose [4]. The synthesis of heat shock proteins induced by a nontoxic heat shock is also independent of GSH concentrations (unpublished results). Once cells have developed tolerance, however, depletion of GSH does not modify thermal inactivation [3, 4].

Current research has focused only on the results produced by depletion of reduced glutathione. Equally important is the question of regulation of thermal toxicity by the redox cycling of intracellular thiols. Oxidation of cellular thiols can affect membrane integrity [10], the activity of the pentose phosphate cycle [11], calcium homeostasis [12], hydrolysis of ATP [13] and microtubules [14]. Formation of protein disulfides can protect enzymes from thermal inactivation [15]. Alteration of any one of these processes could affect thermal cell killing [16, 17]. To study the effect of thiol oxidation on hyperthermic-induced cellular inactivation, CHO cells were exposed to various concentrations of diazenedicarboxylic acid bis (*N*,*N*-dimethylamide II), i.e. diamide [18].

METHODS AND MATERIALS

Chinese hamster ovary (CHO) cells, growing exponentially in monolayer cultures, were maintained at 37° and at pH 7.4 in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2.2 g/l of sodium bicarbonate, 100 units/ml of penicillin G sodium, and 100 mg/ml of streptomycin sulfate. This will be referred to as growth medium. The cultures were found to be free of mycoplasma infection (Microbiological Associates, Bethesda, MD).

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‖ Abbreviations: GSH, reduced glutathione; CHO, Chinese hamster ovary; GSSG, oxidized glutathione; BSO, buthionine sulfoximine; HBSS, Hanks' Balanced and Salt Solution.

All experiments were performed using T-25 flasks containing 4 ml of fresh medium and 4×10^6 cells in exponential growth. The experiments commenced upon addition of freshly dissolved diamide to some of the flasks. During diamide exposure, the cells were incubated at 37° and at a pH between 7.2 and 7.4. With the exception of the experiment shown in Fig. 1, the cells were rinsed twice with Hanks' Balanced Salt Solution (HBSS) at the end of the incubation period.

The reduction of diamide by cellular thiols was measured spectrophotometrically at 296 nm according to Ref. 19.

For determination of GSH content or protein thiols, the monolayer cultures were rinsed in HBSS and then were lysed in 10% perchloric acid. The glutathione concentration in the supernatant fraction was then determined by high performance liquid chromatography [4, 20], whereas the protein SH concentration of the pellet was measured according to Ref. 21.

Cell survival was determined after trypsinizing the cells and then inoculating the appropriate number of cells needed to yield 100–200 colonies in T-25 flasks after 7–10 days of growth. The error in survival was calculated according to Ref. 22.

To determine the activity of glutathione reductase, 4×10^6 cells were sonicated on ice in 1 ml of Dulbecco's phosphate-buffered saline. For this assay, 0.4 ml of cell suspension was added to a buffer containing 36.1 mM Na_2HPO_4, 1.5 mM EDTA (pH 7.4), 0.16 mM GSSG, and 0.12 mM NADPH. The decrease in absorbance at 340 nm was measured at 25°.

Protein synthesis was determined by labeling cells in 3 ml of methionine-free medium containing 25 μCi/ml of [^{35}S]methionine (sp. act. approximately 1100 Ci/mole). The cells were then incubated at 37° for 1 hr after which they were rinsed twice with 0.15 M NaCl (pH 7.4) and scraped into 1 ml of saline solution. An aliquot was removed for protein determination (Biorad protein assay), while a second aliquot was removed in order to determine the cpm incorporated into 10% trichloroacetic acid (TCA) precipitable protein. Precipitated protein was recovered on glass fiber filters and counted in 5 ml of Aqueous Counting Scintillant (Amersham) using a liquid scintillation counter. The remainder of the cellular protein was dissolved in sample buffer [62 mM Tris–HCl, pH 6.8, 2% sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0.0025% Bromophenol blue] and heated at 100° for 5 min. Proteins were electrophoretically separated using 0.75 mm thick, 7.5% polyacrylamide gel slabs with a 4% acrylamide stacking gel in the discontinuous buffer system of Laemmli [23]. Protein molecular weight was determined by comparison to molecular weight standards (myosin, 205,000; β-galactosidase, 116,000; phosphorylase *b*, 97,400; bovine albumin, 66,000; egg albumin, 45,000; and carbonic anhydrase, 29,000) [24]. After electrophoresis, the gels were fixed (50% methanol, 10% acetic acid), stained with Coomassie Brilliant Blue, destained, treated for fluorography using Enhance (NEN), dried, and exposed to Kodak SB-5 X-ray film at −70° for 24 hr. The autoradiograph was subsequently scanned and peaks were quantitated using a Zeineh scanning densitometer.

Hyperthermic treatment was administered by horizontally immersing the flasks into a water bath. Thermometry was performed with a mercury in glass thermometer traceable to NBS.

Cytofluorometric measurements of DNA content were carried out according to Ref. 25 using a cytofluorograph 50H (Ortho Diagnostic Systems) and a 2150 data processing system.

All experiments were repeated a mininum of twice.

RESULTS

Figure 1 illustrates the rate of diamide reduction when CHO cells were exposed to 0.1 mM diamide at 37°, while Table 1 shows the corresponding intracellular concentration of GSH. Under the experimental conditions shown, diamide was reduced at a rate of 2 nmoles/min/10^6 cells without a concomitant decrease in GSH concentration. This rate was similar to the activity of glutathione reductase in CHO cells (1.6 ± 0.1 nmoles NADPH/min/10^6 cells determined at 25°, data not shown). This figure indicates that exposure to 0.1 mM diamide results in steady-state reduction of diamide for the exposure interval.

Table 2 illustrates the effect of various concentrations of diamide on GSH concentration when CHO cells were incubated in full medium for 60 min at 37°. Addition of either 0.1 or 0.2 mM diamide did not affect the GSH concentration ($P > 0.05$) as determined by Student's *t*-test. Addition of 0.4 mM, however, produced a 50–60% decrease. Table 3 shows that there were no observable changes in the concentration of free protein SH groups for cells treated with 0.4 mM diamide ($P > 0.05$). A similar result was obtained for cells treated with 0.1 mM diamide (data not shown). The difference in GSH concentration seen in Tables 1 and 2 is due to the different medium used, HBSS versus McCoy's, the former containing no cysteine or cystine to support GSH synthesis.

Diamide exposure induced thermal resistance in CHO cells (Fig. 2). Figure 2 indicates that diamide-induced resistance increased as a function of diamide concentration. In these experiments, the cells were exposed to the indicated diamide concentrations for 1 hr at 37°, incubated at 37° for 4 hr, and then heated at 43° for 140 min. Survival determined in the absence of the diamide treatment was compared to that obtained in its presence using Student's *t*-test. Diamide treatment resulted in a statistically significant increase in survival ($P < 0.05$). However, survival was the same for cells exposed to 0.1 and 0.2 mM ($P > 0.05$). The half-time for the development of diamide-induced resistance was found to be 20 min (data not shown).

Figure 3 illustrates the cell cycle distribution of CHO cells 4 hr after exposure to various concentrations of diamide. In the untreated control population, 59% of the cells were in G_1, which is the most resistant phase of the cell cycle, and 41% were in S/G_2. Treatment with either 0.1 or 0.2 mM diamide did not change the cell cycle distribution significantly (panel A). Treatment with 0.4 mM did not

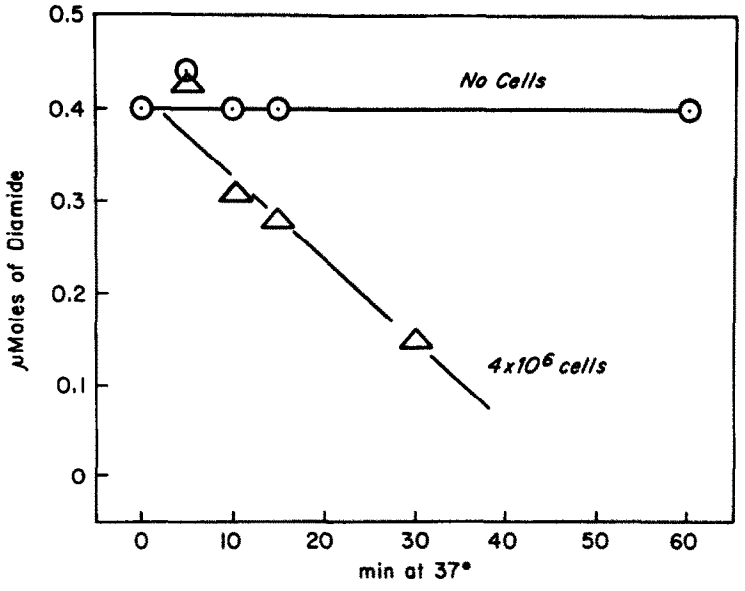

Fig. 1. Rate of diamide reduction. HBSS (4 ml) containing 0.1 mM diamide was incubated at 37° for various intervals in the absence or presence of 4×10^6 CHO cells. The diamide concentration was determined spectrophotometrically at 296 nm. Standard errors are shown in this figure, and in Figs. 2–7, when they exceed the diameter of the symbol.

result in cycle redistribution: 57% were in G_1 and 43% were in S/G_2. It should be pointed out that, below the first decade, survival of an asychronous population is determined by its most resistant cells (i.e. G_1). Figure 3 shows that, under the experimental conditions used, diamide did not alter the cell cycle distribution sufficiently to account for the changes in survival observed in Fig. 2.

Diamide exposure protected CHO cells from heat-induced inhibition of protein synthesis. CHO cells were exposed to 0, 0.1, 0.2 or 0.4 mM diamide for 1 hr at 37°, incubated in diamide-free medium for 4 hr at 37°, and then labeled for 1 hr at either 37° or 43° with 5 μCi/ml of [^{35}S]methionine. Figure 4A illustrates the incorporation obtained at either 37° or 43°. Figure 4B represents the ratio obtained when the rate of incorporation measured at 43° was divided by the rate measured at 37°. As seen in Fig. 4B, heating at 43° reduced incorporation by 82%. Exposure to either 0.1 or 0.2 mM diamide did not affect the degree of inhibition ($P > 0.05$). However, exposure to 0.4 mM diamide, 4 hr prior to heating, resulted in partial protection against thermal inhi-

Table 1. GSH concentration in 4×10^6 CHO cells exposed to 4 ml of HBSS containing 0.1 mM diamide

Minutes*	Diamide (mM)	Relative GSH content†	P‡
—	0	1.00 ± 0.03	—
5	0.1	0.87 ± 0.04	NS
10		0.85 ± 0.08	NS
15		0.95 ± 0.05	NS
30		1.00 ± 0.30	NS

* Minutes at 37°.

† Untreated cells contained 2.00 ± 0.06 nmoles GSH/10^6 cells. Values are mean ± SD.

‡ Probability of a statistically significant difference between the indicated sample and control. NS indicates $P > 0.05$, as determined by Student's t-test.

Table 2. GSH concentration of CHO cells exposed to full growth medium containing diamide

Minutes*	Diamide† (mM)	Relative GSH content	P§
—	0	1.00 ± 0.06‡	—
5	0.1	0.88 ± 0.06	NS
10		0.95 ± 0.07	NS
15		0.92 ± 0.05	NS
30		0.99 ± 0.07	NS
60		0.92 ± 0.09	NS
5	0.20	0.86 ± 0.05	NS
10		0.87 ± 0.07	NS
15		0.83 ± 0.05	NS
30		0.98 ± 0.06	NS
60		0.76 ± 0.08	NS
5	0.40	0.44 ± 0.03	**
10		0.34 ± 0.08	**
15		0.51 ± 0.05	**
30		0.58 ± 0.06	**
60		0.75 ± 0.06	NS

* Minutes at 37°.

† Cells (4×10^6) were incubated in 4 ml of full medium for 60 min with the indicated concentrations of diamide.

‡ Untreated cells contain 3.9 ± 0.2 nmoles GSH/10^6 cells. Values are mean ± SD.

§ Same as in Table 1: NS indicates $P > 0.05$; the double asterisk (**) indicates $P < 0.05$.

Table 3. Effect of 0.4 mM diamide on protein sulfhydryl groups

Minutes*	Diamide (mM)	SH (nmoles/10^6 cells)	P†
—	0	13.5 ± 1.3‡	NS
10	0.4	12.6 ± 0.9	NS
30	0.4	14.5 ± 1.3	NS
60	0.4	11.8 ± 2.6	NS

* Minutes at 37°.
† Same as in Table 1: NS indicates $P > 0.05$.
‡ Mean ± SD.

bition of protein synthesis (Fig. 4B, 82% vs 53%, $P < 0.05$). As seen in Fig. 4A, the diamide treatment by itself reduced incorporation. The protein that was being synthesized 4 hr after exposure to 0.4 mM diamide was apparently resistant to thermal inhibition.

Figure 5 presents a fluorograph of cell extracts prepared 4 hr after being heated for 15, 30 or 45 min at 43°. Heat shock increased the synthesis of three specific proteins having molecular weights of approximately 110, 90 and 68–66 kD.

Figure 6 illustrates the pattern of proteins synthesized 4 hr after exposure to diamide. Cells were exposed to 0, 0.1, 0.2 or 0.4 mM diamide for 1 hr, incubated at 37° in diamide-free medium for 4 hr, and then labeled for 1 hr at 37° with [^{35}S]methionine. The pattern of protein synthesis after exposure to either 0.1 or 0.2 mM diamide was similar to control. Because there was no inhibition of protein synthesis at either 0.1 or 0.2 mM (see Fig. 4), loading the gel on an equal protein basis resulted in equal cpm being placed on each gel. This is important because, if protein synthesis were inhibited, then loading on an equal protein basis would minimize detection of stress proteins. This is illustrated for protein synthesis which occurs after exposure to 0.4 mM diamide (Fig. 6). Electrophoresis of proteins synthesized immediately, or 2 hr, after exposure to 0.1 mM diamide also yielded the same pattern of protein synthesis as control (data not shown). In this case, the relative rate of proteins synthesized was estimated using a method based on that described by Tomasovic *et al.* [26]. Fluorographs loaded on an equal protein basis

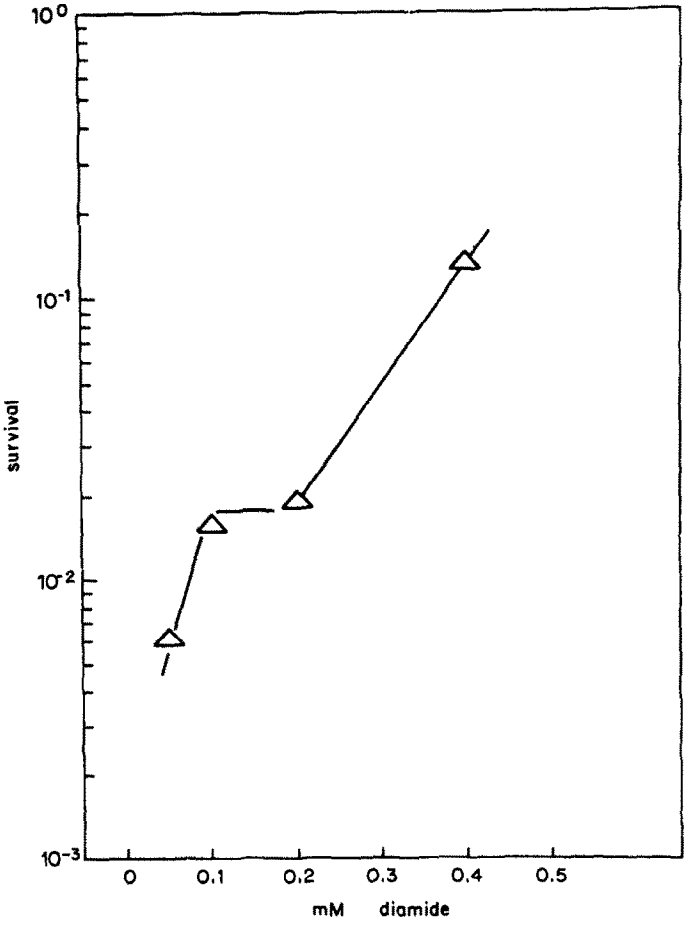

Fig. 2. Survival after heating as a function of diamide concentration. Cells were treated for 1 hr at 37° in the absence or presence of the indicated diamide concentrations, incubated for 4 hr at 37° in diamide-free medium, and then heated at 43° for 140 min. The plating efficiencies were 0.98 ± 0.03, 1.07 ± 0.03, 0.98 ± 0.03 and 0.65 ± 0.03 for cells exposed to 0, 0.1, 0.2, or 0.4 mM diamide at 37° respectively.

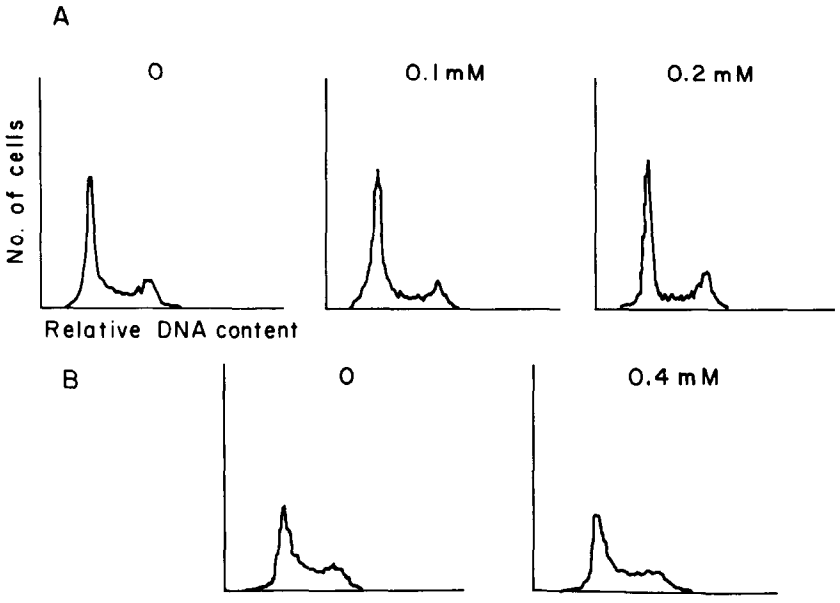

Fig. 3. Effect of diamide on cell cycle redistribution. DNA histograms were obtained 4 hr after exposure to (A) 0, 0.1 or 0.2 mM diamide or (B) 0 or 0.4 mM diamide.

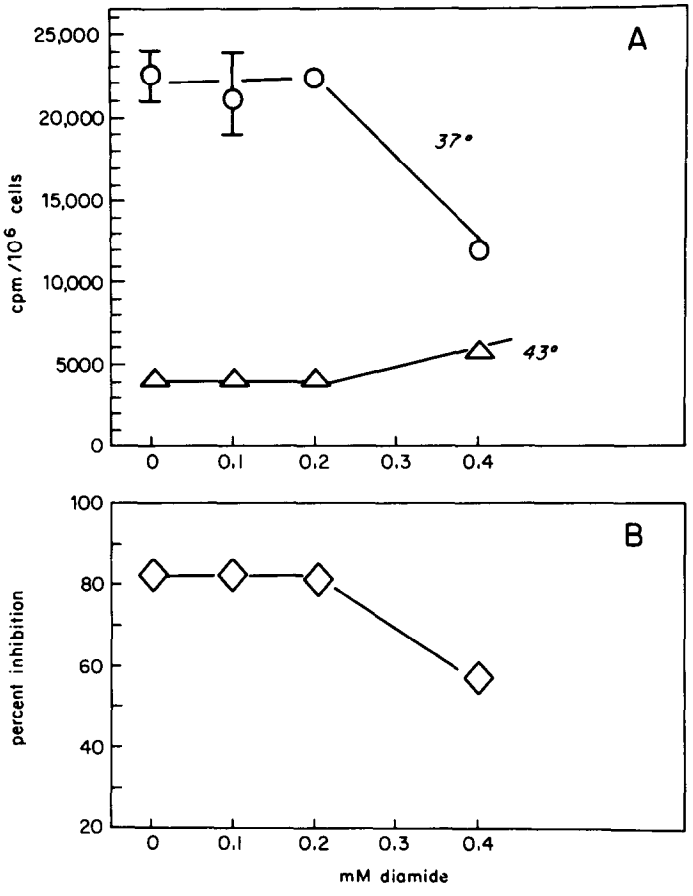

Fig. 4. Inhibition of protein synthesis by hyperthermic treatment. (A) Cells were treated for 1 hr at 37° at the indicated diamide concentrations, incubated for 4 hr in diamide-free medium, and then labeled for 1 hr at 37° (○) or 43° (△) with 5 μCi/ml of [^{35}S]methionine. The incorporation of label into TCA-precipitable protein was determined immediately after the labeling period. (B) The rate of incorporation measured at 43° was divided by the rate measured at 37°; the ratio is shown as a function of diamide concentration.

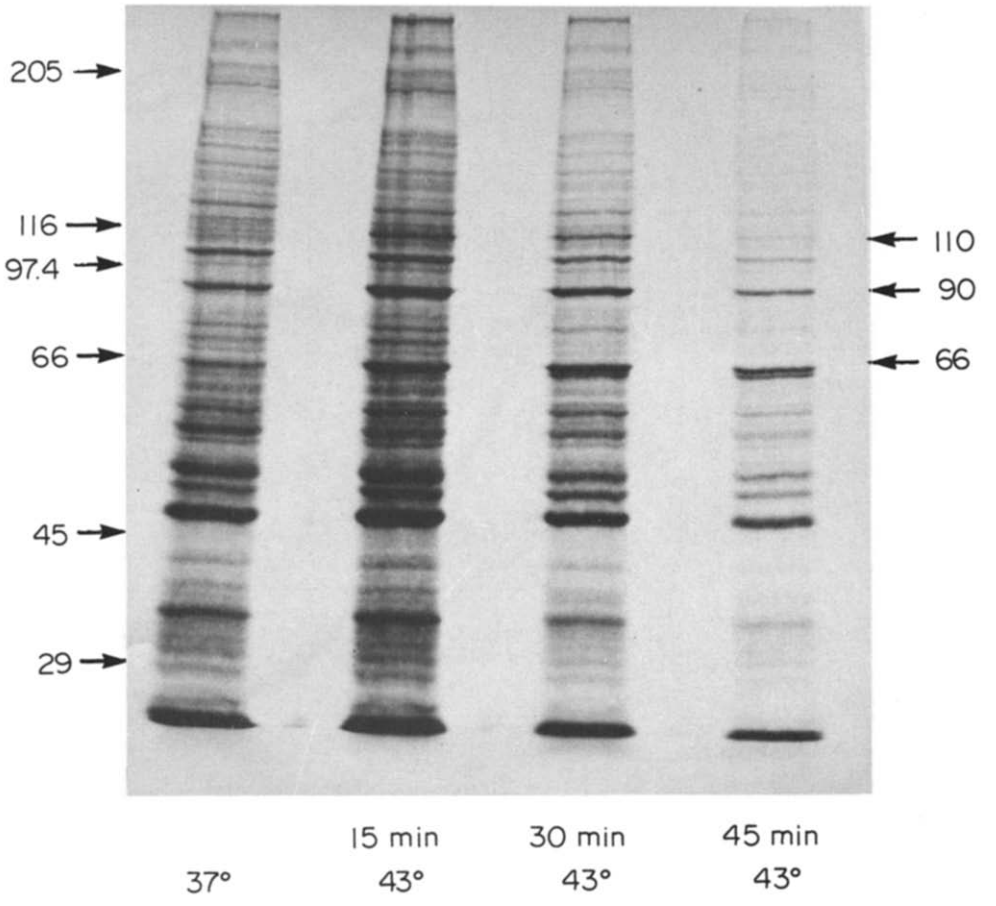

Fig. 5. Fluorograph showing the synthesis of heat shock proteins. Cells were heated for 15, 30, or 45 min at 43°, and then incubated for 4 hr at 37°. After the incubation period, the cells were labeled at 37° with [^{35}S]methionine for 1 hr, and then the protein was electrophoresed and a fluorograph was prepared. A constant amount of protein, approximately 10 mg, was loaded on each lane. The arrows represent the molecular weight standards; 110, 90, and 66 indicate the stress proteins.

were scanned by a densitometer and the area under each peak was integrated. The relative rate of synthesis for a particular protein was calculated from the ratio obtained upon dividing the area of the peak in question by the sum of the areas from peaks in that same lane representing proteins of 30, 42, 50 and 105 kD. The relative rate of 110, 90 or 66 kD proteins synthesized after diamide treatment was the same as control.

At a diamide concentration of 0.4 mM, however, the synthesis of proteins having molecular weights of approximately 110, 90 and 66 kD was enhanced relative to the total protein synthesized (Fig. 6). This is seen very clearly in Fig. 7 which shows a second experiment in which the gel was loaded on the basis of equal cpm. Untreated controls were separated in lanes a, b and c, while diamide-treated cells were in lanes a′, b′ and c′. A similar pattern of protein synthesis was observed when cells treated with 0.4 mM diamide were assayed 4, 6 or 8 hr later and electrophoretically separated in 10% polyacrylamide gels (data not shown). Comparison of Figs. 7 and 5 indicates that both thermal stress and diamide exposure enhanced the synthesis of proteins that have been termed heat shock or stress proteins.

DISCUSSION

This present investigation has shown that exposure to diamide prior to hyperthermic treatment reduced the rate of cellular inactivation as measured by colony formation. The rationale for using diamide was to produce various degrees of thiol oxidation. As with any drug, however, the specificity can be questioned [19, 27]. Although nonspecific interactions cannot be ruled out, it is most probable that the results observed were due to thiol oxidation. This is based on the observations of Harris and Biaglow [27] who have shown that diamide oxidation of pyridine nucleotides occurs only after total GSH oxidation. In an authoritative paper on the subject, Kosower *et al.* [28] re-examined the specificity of diamide by carefully measuring the rate constants for reaction with diamide and the intracellular concentration of oxidizable constituents. They concluded that oxidation of GSH is the more important reaction by many orders of magnitude. For example, diamide reacts approximately 86 times faster with GSH compared to NADH and there is approximately 30 times more intracellular GSH than NADH [28]. Under the conditions used in this study, only 50–60% of the

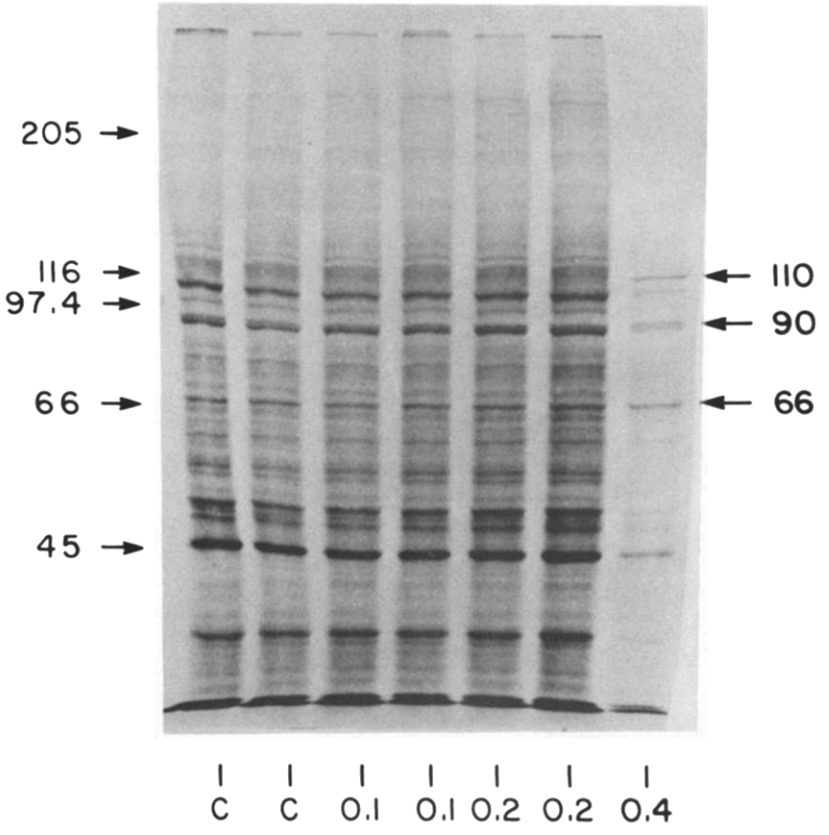

Fig. 6. Fluorograph showing protein synthesis after diamide exposure. Cells were exposed to 0, 0.1, 0.2 or 0.4 mM diamide for 1 hr at 37°, incubated in diamide-free medium for 4 hr at 37°, and then labeled at 37° for 1 hr with [^{35}S]methionine. The protein was then electrophoresed and an autoradiograph was prepared. A constant amount of protein was loaded on each gel. C equals untreated control; 0.1, 0.2, and 0.4 indicate the diamide concentration (mM). The arrows indicate M_r; 110, 90, and 66 indicate the stress proteins.

GSH was depleted upon exposure to diamide and 13% or less of the protein thiols were oxidized (Tables 2 and 3). Such conditions should result in minimal nonspecific oxidation.

Grimm *et al.* [29] have shown that addition of 0.5 mM diamide to *in vitro* cultures of rat heart cells results in formation of protein mixed disulfides. This occurs within 15 min of the addition of the diamide. Using gel electrophoresis, they found that the thiol groups in 21 protein bands having molecular weights ranging from 20,000 to 120,000 were affected. The measurement of protein mixed disulfides was performed under nondenaturing conditions, in contrast to the present study. It is quite conceivable that addition of 0.4 mM diamide increased the concentration of mixed disulfides in CHO cells but that the DTNB assay performed in the presence of SDS was insufficiently sensitive to measure it.

A correlation was observed between GSH concentration and subsequent metabolic functions. Low concentrations of diamide (e.g. 0.1 mM) did not result in depletion of intracellular GSH (Table 2). Although 0.1 mM diamide induced a moderate degree of protection against thermal toxicity (Fig. 2), it did not protect against thermal inhibition of protein synthesis (Fig. 4). Only after a diamide challenge, sufficient to deplete 50–60% of the GSH, were stress proteins of 110, 90 and 66 kD synthesized. Their synthesis occurred coincident with a significant reduction in thermal cell killing (Fig. 2) and resistance to thermal inhibition of protein synthesis (Fig. 4). The synthesis of low molecular weight stress proteins (e.g. 27 kD) was not studied. Diamide has also been shown to induce stress proteins in sea urchin blastulae [30] and to modify thermal inactivation in CHO cells [31].

Hahn and Shiu [32] have shown that the degree of thermal inhibition of protein synthesis is diminished as mammalian cells develop thermotolerance. The experiment shown in Fig. 4 indicates that a similar occurrence was observed when CHO cells were exposed to diamide. Protein synthesis, resistant to diamide inhibition, is apparently also resistant to thermal inhibition. This could reflect the synthesis of stress proteins.

Zehavi-Willner *et al.* [33] have shown that addition of diamide to rabbit reticulocytes inhibits protein synthesis. This effect is dependent upon the levels of GSH and GSSG present. Oxidation of GSH to GSSG, upon addition of diamide, reduces protein synthesis, whereas recovery of synthesis occurs after reduction of GSSG. Addition of GSSG to rabbit

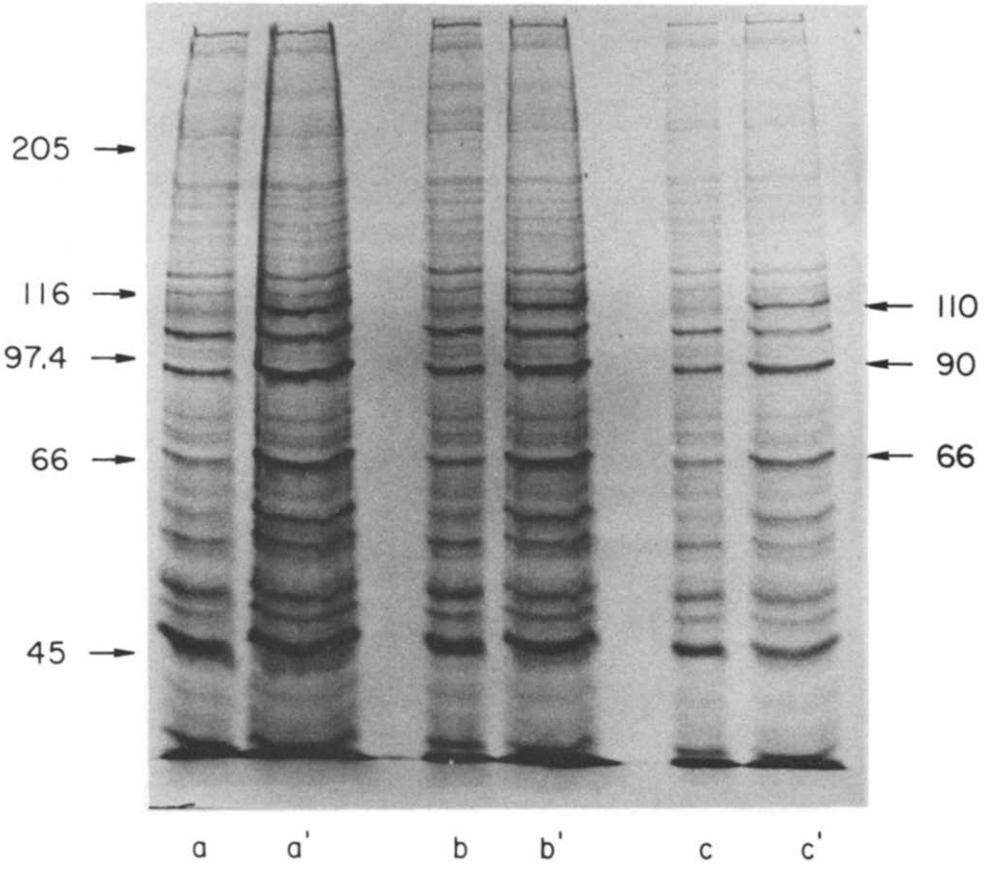

Fig. 7. Fluorograph from cells labeled for 1 hr at 37° 4 hr after exposure to 0 or 0.4 mM diamide. A constant level of cpm was loaded on each lane. Key: (a, b, and c) untreated controls; (a', b', and c') cells treated for 1 hr at 37° with 0.4 mM diamide; (lanes a and a') 46,000 cpm (b and b') 32,000 cpm, and (c and c') 23,000 cpm; 110, 90, and 66 indicate the stress proteins.

reticulocyte lysates containing GSH also inhibits protein synthesis [34]. The effect is abolished if the GSSG is converted to GSH, thus showing that the inhibition of protein synthesis by addition of diamide is due to the formation of GSSG. There is evidence to suggest that this inhibition is due to activation of a cAMP-independent protein kinase which phosphorylates the initiation factor eIF-2 [35]. Interestingly, this factor is also phosphorylated after heat shock [36]. The initiation factor, eIF-2, is composed of three subunits, α, β, and γ. Phosphorylation of the α subunit (M_r approximately 37K) inhibits initiation of protein synthesis [37].

There is evidence that the synthesis of stress proteins can modify thermal toxicity. Finkelstein and Strausberg [38] have provided evidence for a direct relationship between the synthesis of a stress protein and the rate of thermal inactivation. They isolated the gene which codes for a stress protein having a molecular weight of approximately 90 kD in yeast and then introduced the gene into *Saccharomyces cerevisiae*. The result was an increase in the synthesis of the protein coded for by this gene and a concomitant decrease in thermal sensitivity. For example, survival after 5 min at 54° was 0.007 for control cultures compared to 0.37 in cells expressing an abundance of heat shock protein M_r 90K. The thermal sensitivity of thermotolerant cells was unaffected by the presence of this gene, however. Landry *et al.* [39] found a temporal correlation between the synthesis and degradation of the 107, 89, 70 and 68 kD heat stress proteins and the induction and decay of thermotolerance. No correlation was noted for the 27 kD heat stress protein. Similar results were obtained by Li [40] using Chinese hamster HA-1 cells and their heat-resistant sublines tested either in plateau phase or in exponential growth. Again, no correlation was noted for the 22–27 kD heat stress proteins. If, however, stress proteins do modify the rate of thermal inactivation, the mechanism remains obscure.

The current study presents several novel observations. Oxidation of cellular thiols by diamide induced thermal resistance in Chinese hamster ovary cells. Although we cannot rule out oxidation of some other component as the cause, kinetically, thiols seem the most likely target. The induction of thermal resistance occurred as a step function rather than a linear one. Low concentration of diamide produced a moderate level of resistance. Higher concentrations of diamide, which deplete GSH, increased the synthesis of three proteins having a molecular weight of

approximately 110, 90 and 66 kD. This occurred concomitantly with a dramatic increase in thermal resistance and protection against thermal inhibition of protein synthesis.

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