GSH OXIDATION AND PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES[1]

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SUMMARY. Synthesis of protein in intact rabbit reticulocytes is inhibited by the thiol oxidizing agent, diamide, which oxidizes GSH to GSSG: 1. No incorporation of ^{14}C-leucine into soluble protein is observed during the period when GSH is absent from the cells (lag period). 2. Recovery of protein synthesis occurs following regeneration of GSH. 3. The degree to which protein synthesis recovers is correlated with the length of the lag period which depends on the amount of diamide used: five minutes absence of GSH in the rabbit reticulocyte preparation permits complete recovery of protein synthesis. Ten minutes absence of GSH results in a markedly diminished rate of protein synthesis.

The use of a new series of thiol-oxidizing agents for the intracellular oxidation of glutathione (GSH) to the disulfide (GSSG) has been reported (1). One of these compounds, $(CH_3)_2$ NCON=NCON$(CH_3)_2$, diamide, can be used to maintain a low cellular GSH content for a considerable length of time (1,2). Complete regeneration of GSH follows the lag period, the length of which depends on the amount of oxidant used. Mature red cell proteins and membrane are not affected by diamide (2). In the case of actively dividing cells, exemplified by E. coli, temporary cessation of growth followed diamide treatment (3). It seemed possible that this was due in part to inhibition of protein synthesis during the absence of GSH. We report here on the effect of diamide on rabbit reticulocytes.

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MATERIALS AND METHODS. Reticulocyte levels of white rabbits were raised to 20-30% by repeated bleeding. Suspensions of cold washed blood cells were made in medium according to Lingrel & Borsook (4), containing ^{14}C-leucine, but without added serum and with added heme (5) instead of iron salt. The cell suspensions were rapidly mixed on a vortex with diamide solution (made in buffer used in the medium), kept cold for 2 min., then incubated at 37° C. Aliquots for measurements of ^{14}C-leucine incorporation into soluble protein were lysed in H_2O. The stroma was removed by centrifugation at 25,000 x g and the concentration of hemoglobin measured by absorbance at 540 mμ. Charged tRNA was hydrolysed with 1N NaOH at 40° C for 15 min. and the protein precipitated with 10% TCA. The precipitates were collected on GF/c filters, washed with TCA, dried and counted in a gas flow counter. GSH determination was done according to Beutler et al (6).

Polysome profiles were obtained by lysing the cells in 8 volumes of reticulocytes standard buffer (RSB) (7), spinning off the stroma as described above, and layering 1.0 ml of the clarified lysate on linear sucrose gradients in RSB. The gradients ran from 15 - 40% w/v sucrose and had a volume of 36 ml. They were spun in the Spinco SW 27 head at 27,000 rpm at 4°C for 4 hours. The contents of each tube were pumped out through a flow cell in a Gilford recording spectrophotometer reading at 260 mμ.

ATP analyses were performed using the ATP-dependent light emission from a crude luciferin-luciferase system (8).

Permeability of reticulocytes to amino acids was measured by the floatation method for potassium flux in B. megatherium (9), slightly modified. Samples of cell suspension, incubated with 10^{-4}M puromycin for 5 minutes, were mixed with either ^{14}C-leucine or ^{14}C-amino acid mixture and incubated for 1 minute at 37° C The suspension was quickly layered over phthalate ester mixture precooled in an ice bath (d_4^{25} 1.062) (10) and centrifuged at 10,000 x g in a Beckman-Spinco microcentrifuge for 1 minute at 4°C. The pellet of cells was suspended in buffered saline, filtered on membranes, washed and counted.

RESULTS. Incorporation of ^{14}C-leucine into soluble protein and GSH regeneration after diamide treatment. Protein synthesis was not affected when diamide was used in amounts insufficient for complete oxidation of intracellular GSH: no delay occurred in the onset of ^{14}C-leucine incorporation into the soluble protein and the rate of incorporation was equal to that of control untreated cells. Protein synthesis was affected when cells were treated with diamide in amounts sufficient to complete the oxidation of GSH and cause a delay in the reappearance of cellular GSH, as shown in Fig. 1: (1) No incorporation of ^{14}C-leucine occurred during the period when cellular GSH was absent. (2) The onset of incorporation lagged behind the regeneration of GSH. Incorporation of ^{14}C-leucine into protein began when about 50% of cellular GSH had been regenerated. (3) In the cases where moderate amounts of diamide were used (1-1.5 moles per 1.0 mole of cellular GSH), the inhibition of protein synthesis was completely reversible: the rate of ^{14}C-leucine incorporation became equal to the control rate after GSH regeneration reached about 80% (Fig. 1,a,b). When larger amounts of diamide were used, not only was the lag period longer, but the rate of ^{14}C-leucine incorporation into protein eventually achieved was much lower than in the control, untreated cells (Fig. 1c).

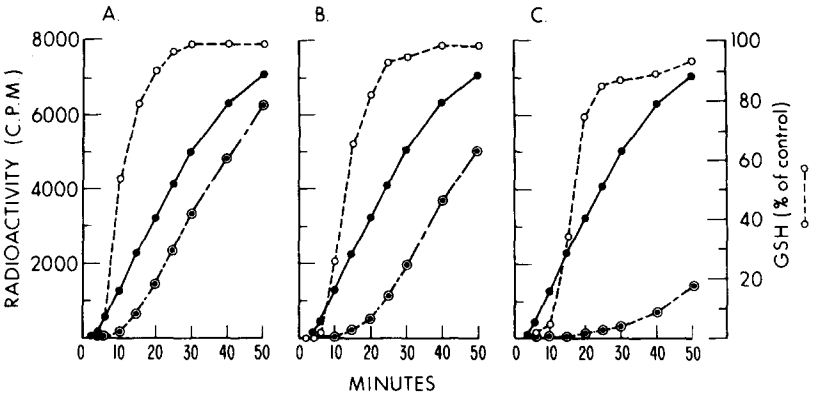

Fig. 1. Incorporation of ^{14}C-Leucine into soluble protein and GSH regeneration after treatment of cells with various amounts of diamide. Moles of diamide used per 1.0 mole of cellular GSH: a. 1.05; b. 1.34; c. 2.0. o– – –o GSH regeneration in diamide treated samples (expressed as % of control values); ●———● c.p.m. per 1.0 O.D. Hemoglobin in control non treated cells; ⊙– – –⊙ c.p.m. per 1.0 O.D. Hemoglobin in diamide treated cells.

Ribosomal patterns. When diamide was used in amounts allowing complete recovery of protein synthesis, a slight shift of polysomes to smaller aggregates and some formation of 80S ribosomes was noted during the lag period. Later on, paralleling the recovery in the rate of ^{14}C-leucine incorporation into soluble protein, a reversion to the control pattern was found (Fig. 2b). When larger amounts of diamide were used, a similar slight shift towards smaller aggregates and monosomes was noted in the period of absence of GSH. Later, a more pronounced breakdown to 80S ribosomes was noted: the ribosomal profile was composed of a large fraction of monosomes and only a small quantity of polysomes (Fig. 2c).

Effect of diamide on ATP and on the permeability of cells to amino

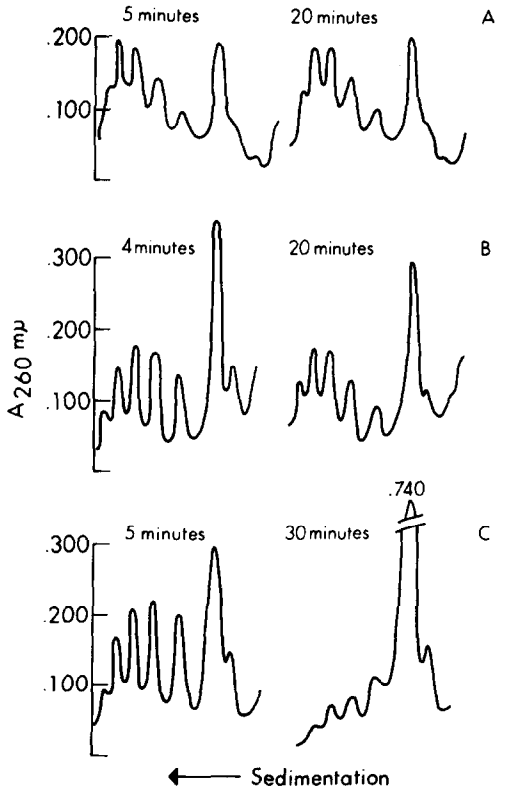

Fig. 2. Ribosomal patterns of incubated cells after treatment with Diamide. a. control cells; b. cells treated with 1.3 moles Diamide per 1.0 mole of cellular GSH; c. cells treated with 2.2 moles Diamide per 1.0 mole of cellular GSH. Aliquots for ribosomal profiles taken at times indicated on the figures.

Table I. The Effect of Diamide on ATP levels and the Permeability of Amino Acids into Reticulocytes

Diamide moles/1.0 mole cellular GSH	Incubation time (min.)	ATP μmoles/ml cells	Amino acid uptake (c.p.m./ml. cells) ^{14}C-leucine	^{14}C-amino acid mixture
0	0	1.56	29,000	90,000
0	10	1.80	25,500	82,000
0	20	1.71	26,000	95,000
1.3	0	1.65	28,000	95,000
1.3	10	1.72	26,500	98,000
1.3	20	1.73	25,000	90,000
2.6	0	1.77	29,500	88,000
2.6	10	1.58	28,700	96,000
2.6	20	1.70	27,200	90,000

For experimental details see "Materials and Methods."

acids. Regardless of the amount of diamide used, including quantities which resulted in largely irreversible inhibition of protein synthesis, no change could be detected in the ATP levels of the cells (Table 1). Diamide had no effect on the permeability of the cells to amino acids, either as a mixture or the single compound, leucine (Table 1).

Effect of dihydrodiamide $(CH_3)_2NCONHNHCON(CH_3)_2$. The product of the reaction of diamide with GSH, dihydrodiamide, had no effect on protein synthesis in this system when added either to control or diamide treated cells.

Effect of added cysteine. Addition of cysteine in amounts of 20% of the cellular GSH content to cells after washing off excess diamide did not shorten the observed lag period.

CONCLUSION. In this paper we show that lowering the intracellular GSH concentration to zero has a direct, close link to protein synthesis. The lack of incorporation of amino acids into protein that results from adding diamide to a suspension of reticulocytes is not the result of impaired ATP levels or failure to allow the entry of amino acids or any effect of the reduced form of diamide. The completeness and immediacy of the inhibition suggest that protein synthesis

is being directly affected because one or all of the processes of initiation, translation or release are halted. The fact that a moderate dose of diamide, one that allows complete recovery, causes very little disaggregation of polysomes during the period of absence of GSH implies that either translation and/or release is inhibited. The polysome profiles from cells treated with large amounts of diamide do not differ initially from those of cells treated with low dose. However, later on, when GSH has been regenerated, a considerable breakdown of polysomes to single ribosomes occurs. This suggests that initiation has been permanently impaired in these cells, leading to a low rate of protein synthesis. These inferences from the incorporation of ^{14}C-leucine and the polysome profiles are consistent with the knowledge that some of the enzymes involved in protein synthesis require sulfhydryl groups for activity. Certain activating enzymes (11), transfer factor II (12), and the I factor of Miller and Schweet (13) all have such a requirement. The present data do not allow us to draw any detailed conclusions as to which stages in the overall process are primarily affected. Further studies aimed at finding the affected sites, their relative sensitivities and their potentials for reversion to normal activity are in progress.

We believe that the results obtained thus far reflect a heretofore unsuspected importance for GSH in maintaining normal physiological activity in cells.

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