THE USE OF THE OXIDANT "DIAMIDE" FOR STUDYING THE NON-MITOCHONDRIAL REDUCING CAPACITY OF EHRLICH ASCITES TUMOR CELLS

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SUMMARY

Diazenedicarboxylic acid bis(N,N-dimethylamide), ("diamide") lowered non-mitochondrial NAD(P)H stores in Ehrlich ascites tumor cells *in vitro* by indirect reactions involving oxidation of glutathione and reduction of GSSG via glutathione reductase. The concentrations of diamide used did not alter the mitochondrial capacity to reduce NAD(P)H under anaerobic conditions. "Endogenous substrates" could be removed by multiple additions of diamide which indirectly inhibited NAD(P)H and GSH regeneration because of a lack of cellular reducing capacity. The regenerative power of the cells was restored by the addition of glucose. We conclude that diamide may prove to be a useful agent for studying the reducing capacity as well as the redox compartmentalization of cells *in vitro*.

INTRODUCTION

"Diamide" [Diazenedicarboxylic acid bis(N,N-dimethylamide), $(CH_3)_2N\text{-}CO\text{-}N{=}N\text{-}CO\text{-}N(CH_3)_2$], introduced by Kosower *et al.*, has been claimed to be a specific oxidizing agent for glutathione in anucleate erythrocytes (1). However, the agent was found to react also with the reduced nicotinamide adenine dinucleotides (NAD(P)H) in solution (2,3) although much less readily than with glutathione (3). In the intact cell diamide may cause severe depletion of NAD(P)H, either by direct reaction or indirectly by maintaining a consistently high concentration of oxidized glutathione (GSSG), since enzymatic reduction of GSSG will lower the concentration of NAD(P)H. If glutathione reductase activity in the cells is extremely low, the direct reaction of diamide will continue to consume NAD(P)H. Through the use of cellular fluorescence of NAD(P)H (3,4) we undertook the task of further quantitating the effects of diamide on the NAD(P)H and GSH content of Ehrlich ascites cells.

METHODS

Ehrlich ascites tumor (EAT) cells were collected from CF_1 mice 6-8 days

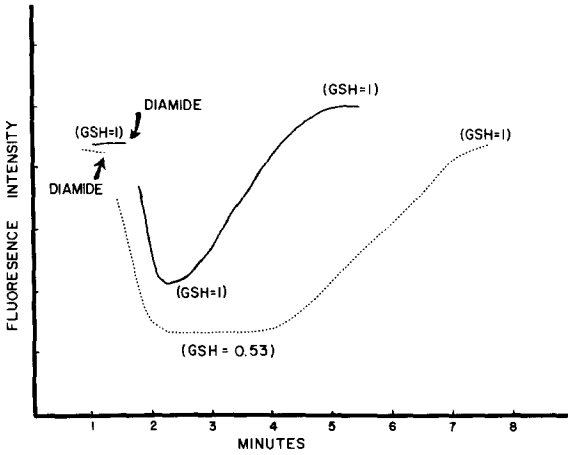

Fig. 1 Oxidation of GSH and NAD(P)H in EAT cells treated with diamide. The cells (4×10^7/3 ml) were suspended in 0.05 M phosphate buffer (pH 7.0) in physiological saline, at 37°C. The relative contents of NAD(P)H were determined by fluorescence measurements, and are indicated by the curves (solid line: 42 nmoles, dotted line: 168 nmoles of diamide added). The relative contents of GSH were determined in separate samples at times of minimal and maximal fluorescence, and are given in parentheses. The control value for GSH was 2.8×10^{-6} nmoles/cell.

after intraperitoneal inoculation with 5×10^6 EAT cells. The cells were washed twice with ice cold 25 mM phosphate buffer in physiological saline, pH 7.0, resuspended in phosphate buffer at a concentration of $1\text{-}2 \times 10^8$ cell/ml and maintained in an ice-water bath until used for measurements. Cell numbers were routinely obtained by direct counting of dilute samples with a Coulter electronic particle counter, and actual cell counts are given in the figure legends.

Acid-soluble sulfhydryl compounds were measured with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB)(5). NAD(P)H, in the intact cells, was measured by fluorometry (4). The excitation wavelength from the Eppendorf spectrophotometer was 366 nm and a 420 nm cut-off filter was interposed between the sample and the phototube to reduce stray and scattered light (3). Temperature for all experiments was 37°C. Diamide was purchased from Calbiochem.

RESULTS AND DISCUSSION

The NAD(P)H measurements were made on cellular suspensions in the spectrophotofluorometer. It was assumed that the fluorescence measurements

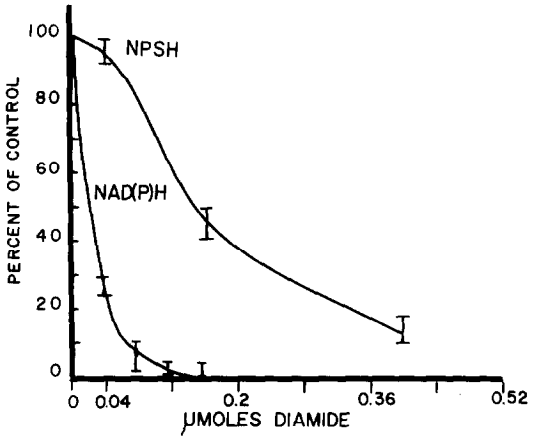

Fig. 2 Titration of GSH and NAD(P)H in EAT cells. Single doses of diamide were added to different samples of 4 x 10^7 cells/3 ml, as in Fig. 1. The recorded values for NAD(P)H represent the points of minimal fluorescence, before regeneration of reduced pyridine nucleotide occurred. GSH was measured in duplicate samples, also at the times of minimal fluorescence. The control value for GSH was 2.5 x 10^{-6} nmoles/cell.

under aerobic conditions for the most part reflect the non-mitochondrial NAD(P)H (3,4); this assumption was later confirmed directly.

As seen in Fig. 1 (solid line), the addition of 42 nmoles of diamide caused a rapid oxidation of NAD(P)H in EAT cells (4 x 10^7 in 3 ml) as indicated by a downward deflection of the recorder pen. The oxidation was complete within 0.5 min and, after a 0.2 min lag, reduction of NAD(P) to NAD(P)H occurred, as seen by an increased fluorescence signal. Regeneration of NAD(P)H to the initial control value was complete in about 2.5 minutes. Measurements of GSH, performed on aliquots of cells treated in a similar manner as those used for the fluorescence measurements, indicated that 42 nmoles of diamide caused a barely detectable (0-3%) decrease in the cellular GSH; the data for GSH are given in parentheses in Fig. 1.

The addition of 170 nmoles of diamide (dotted line) produced a decrease in the GSH to 53% of the control. The fluorescence decrease was only slightly greater than that observed with 42 nmoles of diamide. A 2 - 2.5 minute lag occurred before regeneration of NAD(P)H set in. The rate of regeneration was less than that after a 42 nmoles addition of diamide but regeneration was com-

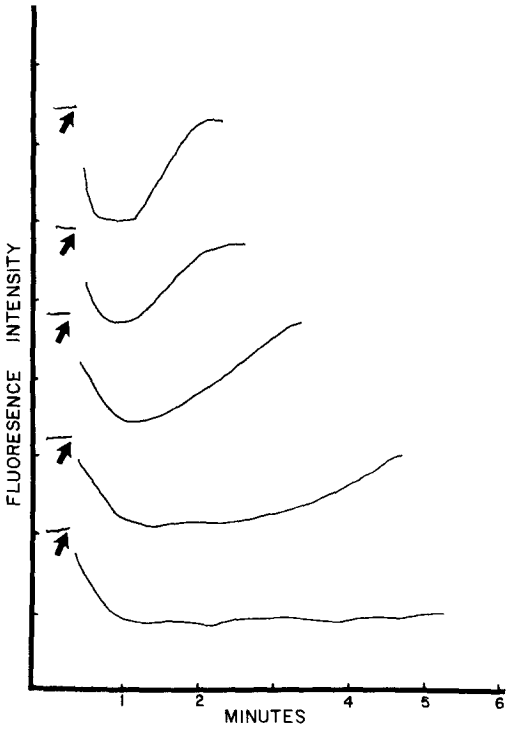

Fig. 3 The effect of repeated additions of diamide on NAD(P)H fluorescence in EAT cells. Reaction conditions were as in Fig. 1; cell concentration was 4×10^7 cells/3 ml. Each addition of 42 nmoles diamide was made after the fluorescence intensity had essentially returned to the control level.

plete in about 4 minutes. Measurements of GSH content indicated a return to the control values within the same time period.

Figure 2 shows the effect of increasing amounts of diamide on NAD(P)H and GSH. Diamide was introduced by closely spaced additions of 42 nmoles. Measurements of GSH were made in separate samples at the times of minimal fluorescence. NAD(P)H decreased more rapidly than GSH and complete oxidation of NAD(P)H was obtained with 130-170 nmoles of diamide while it required 420 nmoles or more to obtain 80-90% oxidation of cellular GSH in the present experiment (4×10^7 cells). The oxidizing effect of diamide is influenced by the presence of "endogenous substrates" that may donate reducing equivalents for the reduction of $NAD(P)^+$ and GSSG. As demonstrated in Fig. 3, it is possible to reduce this endogenous pool by repeated additions of diamide. The fifth addition of 42 nmoles resulted in a prolonged period of depressed

Table 1. The cells (4.2×10^7/3 ml) were suspended in 0.05 M phosphate buffer (pH 7.0) in physological saline at 37° C. NAD(P)H was determined at the time of maximal fluorescence change after glucose addition (30 sec); GSH was determined at the same time. GSH content in the control cells was 2.5×10^{-6} nmoles/cell, or 0.105 umoles in the total sample.

Additions									
Diamide (umoles)	0			0.17			0.42		
Glucose (umoles)	0	0.1	10	0	0.1	10	0	0.1	10
Contents:									
NAD(P)H (rel. units)	1.0	1.0	1.7	0.3	0.8	1.4	0.0	0.6	1.0
GSH (rel. units)	1.0	1.0	1.0	0.5	0.5	1.0	0.1	0.3	0.7

fluorescence, suggesting depletion of "endogenous substrates" from reduction of diamide. However, longer time studies, after even larger amounts of diamide (420 nmoles), indicated that the cell has additional stores of substrates that may eventually be mobilized for reduction of diamide and regeneration of GSH as was indicated in Fig. 2. Further work is required to better quantitate the "endogenous substrate" pools. However, the present results do demonstrate an endogenous potential for reduction in substantial excess of the cellular GSH.

The addition of glucose stimulated the regeneration of both NAD(P)H and GSH in cells treated with diamide by providing reducing equivalents in excess of the capacity of the "endogenous substrates". As seen in Table 1, the amounts of NAD(P)H produced may even exceed the control level, as in the case of 10 μmoles of glucose added to control cells or to the cell suspension treated with 0.17 μmoles of diamide. GSH was never observed to exceed the levels found in the control cells.

Within the concentration ranges used, diamide does not appear to influence mitochondrial reduction of $NAD(P)^+$. This was demonstrated in the following way (Fig. 4). As indicated earlier, the endogenous substrates and the ability to regenerate non-mitochondrial NAD(P)H were exhausted by repeated additions of diamide. A "steady state" of low NAD(P)H was reached after addition of 420

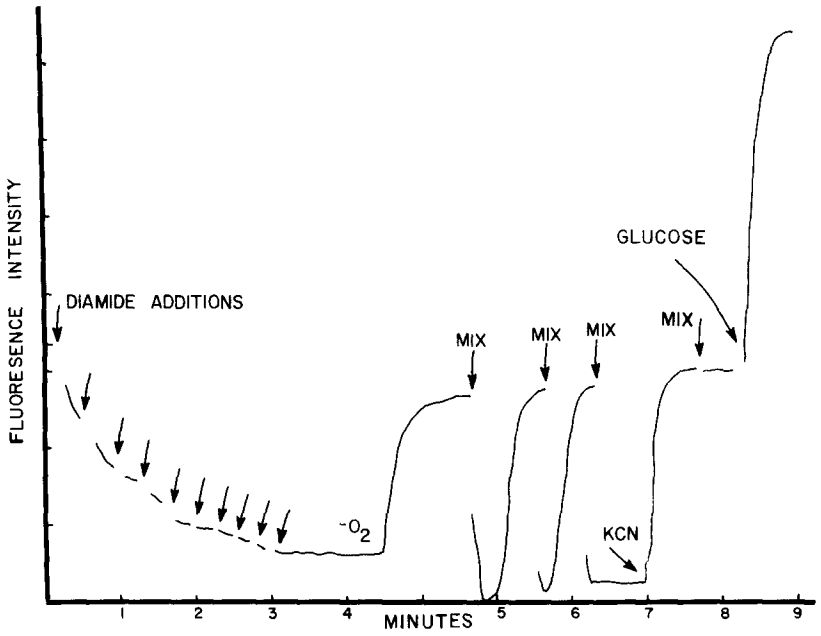

Fig. 4. The effects of oxygen depletion, cyanide, and glucose on NAD(P)H fluorescence in EAT cells previously depleted of non-mitochondrial NAD(P)H by repeated diamide additions. Oxygen depletion was caused by cellular respiration. Oxygen was re-introduced into the medium by stirring, and KCN (4 mmoles) and glucose (10 mmoles) were added at the times indicated by the arrows. Cell density was 7.8 x 10^7 cells/3 ml; all other reaction conditions were as in Fig.1.

nmoles. The cells, through their own metabolism, then depleted the oxygen from the medium and reduction of mitochondrial $NAD(P)^+$ occurred. It was further demonstrated that the increased fluorescence was due to mitochondrial reduction by stirring in a small amount of oxygen with the addition of another aliquot of diamide and allowing the aerobic/anaerobic transition to occur again. An alternative means to inhibit electron flow is the use of specific electron transfer inhibitors such as amytal or cyanide. Both of these chemicals will specifically inhibit mitochondrial electron transfer reactions, the net result being an increase in NAD(P)H with a corresponding increase in fluorescence. Increased fluorescence was observed when either of these chemicals was added to the cell suspension in the presence of O_2. The aerobic addition of glucose to this sample (previously titrated with diamide) caused the nonmitochondrial nucleotides to become reduced to a greater extent than in the control.

Much greater additions of diamide (2.5 μmoles) to similar samples caused inhibition of cellular respiration (3). Because of the absorbancy of unreacted diamide at the excitation wave length of NAD(P)H, however, any concomitant fluorescence change was obscured under these conditions.

DISCUSSION

The addition of diamide to EAT cells caused an immediate decrease in the fluorescence of the cells. This may best be explained by considering the following series of equations in which we assume that the amounts of NAD(P)H oxidized directly by diamide are negligible (3) compared with the amounts consumed in the enzymatic reduction of oxidized glutathione (reaction 2).

1) $2GSH + (CH_3)_2N\text{-}CO\text{-}N{=}N\text{-}CO\text{-}N(CH_3)_2 \longrightarrow GSSG + (CH_3)_2N\text{-}CO\text{-}NH\text{-}NH\text{-}CO\text{-}N(CH_3)_2$

The oxidized GSSG may then be reduced via glutathione reductase:

2) $GSSG + NAD(P)H + H^{+} \rightleftarrows 2\ GSH + NAD(P)^{+}$

The sum of equations 1 and 2 explains the net effect of diamide on the fluorescence decrease:

3) $NAD(P)H + H^{+} + (CH_3)_2N\text{-}CO\text{-}N{=}N\text{-}CO\text{-}N(CH_3)_2 \longrightarrow$

$$NAD(P)^{+} + (CH_3)_2N\text{-}CO\text{-}NH\text{-}NH\text{-}CO\text{-}N(CH_3)_2$$

The endogenous regeneration may be described by the following reaction:

4) Reduced Substrate + $NAD(P)^{+} \rightleftarrows NAD(P)H + H^{+}$ + Oxidized Substrate.

The reaction of diamide with cytoplasmic glutathione, and indirectly with NAD(P)H and "endogenous substrates", provides an innocuous means for studying redox compartmentalization as well as hydrogen transfer mechanisms. Diamide thus provides an alternative to the use of hydrogen peroxide for such studies (6).

Our interest in diamide has been further enhanced by a recent report that it sensitizes anoxic cells to ionizing radiation (7). The concentration of diamide needed for this radiosensitization was 20-100 fold in excess of that required for stoichiometric oxidation of GSH and NAD(P)H, both of which may act as scavengers of free radicals produced by irradiation (7). Concentrations as high as these will also alter macromolecular syntheses, inhibit

cellular oxidation, oxidize protein sulfhydryl groups (3) as well as deplete the "endogenous substrates". Which of these effects are the more important in radiation sensitization currently remains an open question.

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REFERENCES

1. Kosower, N.S., Kosower, E.M. and Wertheim, B., Biochem. Biophys. Res. Commun. 37 (1969) 593.
2. Brown, J.S., Biochem. J. 124 (1971) 665.
3. Harris, J.W. and Biaglow, J.E., Biochem. Biophys. Res. Commun. 46 (1972) 1743.
4. Estabrook, R.W. and Maitra, P.K., Biochem. 3, 369-382 (1962).
5. Harris, J.W. and Patt, H.M., Exptl. Cell Res. 56 (1969) 131.
6. Galeotti, T., Cittadini, A., and Chance B., Biochem. Biophys. Acta 267 (1972) 106.
7. Harris, J.W., and Power, J.A., Radiat. Res. 1973 (in press).