Oxidation of Reduced Nicotinamide Nucleotides by Diamide (NNN'-Tetramethylazoforamide)

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Although a role for GSH in cell division was first postulated 40 years ago (Rapkine, 1931), its function in mitosis and cell division has never been elucidated. Fluctuations in the concentrations of cellular thiol groups that have been arbitrarily assigned to changes in GSH concentration may actually be due to changes in the thiol/disulphide content of a protein (Sakai & Dan, 1959; Dan, 1966). Nevertheless GSH still finds employment in theories about the control of mitosis (Szent-Györgi, 1967; Edgar, 1969). One way of testing the role of GSH in these processes would be to deprive cells of GSH and to observe the effect that this had on mitosis.

The substance diamide (NNN'-tetramethylazoforamide) has been used for the intracellular oxidation of GSH, and it has been claimed that diamide is specific in its reaction with GSH and does not affect other cellular processes (Kosower, Kosower & Wertheim, 1969). Diamide has subsequently been used to study the role of GSH in the growth of *Escherichia coli* (Wax, Rosenberg, Kosower & Kosower, 1970), in protein synthesis (Zehavi-Willner, Kosower & Kosower, 1970) and in planarian head regeneration (in this laboratory).

The present paper shows that, apart from oxidizing intracellular GSH, diamide is capable of oxidizing NADPH and NADH *in vitro* and may be a weak competitive inhibitor of glutathione reductase (EC 1.6.4.2). The value of probes that oxidize GSH in attempts to define its role is questioned, since these are likely either to be

Sigma) of yeast glutathione reductase were used to establish the K_m (GSSG) of the enzyme. Diamide (synthesized by the method of Crawford & Raap, 1963) was a gift from Professor R. H. Thomson, Department of Organic Chemistry, University of Aberdeen.

Rates of reaction were monitored by following the decrease in E_{339} and E_{338} due to oxidation of NADPH and NADH respectively in a Zeiss PMQ II spectrophotometer with the cuvette chamber maintained at 20°C. The buffer used for all reactions was 0.1 M-KH₂PO₄ adjusted with 0.5 M-NaOH to pH 7.5 for enzyme assay and to pH 7.3 for rate and equilibrium studies on the reaction between diamide and the nicotinamide nucleotides. Diamide was dissolved in 100% methanol and 0.01 ml portions were added to cuvettes.

The results are divided into two sections. Section I deals with the reaction between diamide and the nicotinamide nucleotides in the absence of any other reactant. The results presented in section I were obtained by mixing together equal molarities of the two reactants and following the decrease in extinction at 15 min intervals until equilibrium was reached (about 8 h).

Section II deals with the reduction of GSSG by glutathione reductase and NADPH in the presence and in the absence of diamide, and contains information on yeast glutathione reductase.

Results. Section I. Reactions between the nicotinamide nucleotides and diamide in the absence of GSSG and glutathione reductase are as follows:

non-specific in their oxidizing effect or to cause undesirable side effects even when specific for GSH.

Materials and methods. NADPH (tetrasodium salt) was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. GSSG (grade II) and NADH (disodium salt) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Two commercial samples (from Boehringer and from

No figure is included for the decrease in extinction against time since it is a simple exponential decline.

Both reactions are second-order, as shown by plotting the reciprocal of the concentration against time. The equilibrium constant for reaction (1) is 9.25, so that $\Delta G^0 = 1370 \text{ cal} \cdot \text{mol}^{-1}$. The equilibrium constant for reaction (2) is 0.595, so that $\Delta G^0 = 320 \text{ cal} \cdot \text{mol}^{-1}$.

Fig. 1. Progress curves for the enzymic reduction of GSSG by glutathione reductase supplied by Boehringer. Each cuvette contained $0.067 \mu\text{mol}$ of NADPH, GSSG as specified below, $1.33 \mu\text{g}$ of glutathione reductase (specific activity 90 units/mg), diamide where stated, and phosphate buffer, pH 7.5, to a volume of 3.00 ml. Δ , $0.075 \mu\text{mol}$ of GSSG/cuvette; $\circ$, $0.15 \mu\text{mol}$ of GSSG/cuvette; $\square$, $0.2 \mu\text{mol}$ of GSSG/cuvette; $\blacksquare$, $0.3 \mu\text{mol}$ of GSSG/cuvette; $\bullet$, $0.6 \mu\text{mol}$ of GSSG/cuvette; $\blacktriangle$, $0.6 \mu\text{mol}$ of GSSG + $0.57 \mu\text{mol}$ of diamide/cuvette. Reactions occurring in the cuvette containing diamide are as follows:

Reaction (1) (see under 'Results', section I)

and also:

and possibly:

The reaction constant for reaction (1) is $5.11 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$, giving $t_{\frac{1}{2}} = 187 \text{ min}$. For reaction (2) the reaction constant is $2.441 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$, giving $t_{\frac{1}{2}} = 219 \text{ min}$.

Section II. Fig. 1 shows the rate of oxidation of NADPH in the enzyme-catalysed reduction of GSSG at different starting concentrations of GSSG, and indicates the result of added diamide at one

particular concentration of GSSG. The decrease in reaction rate with time becomes less pronounced when diamide is present, indicating that reoxidation of GSH occurs rapidly under these conditions. Double-reciprocal plots of the results shown in Fig. 1 give apparent K_m (GSSG) $1.3 \times 10^{-5} \text{ M}$.

Similar experiments with the Sigma enzyme gave K_m (GSSG) $1.07 \times 10^{-5} \text{ M}$. Mize & Langdon (1962)

have shown that high concentrations of NADPH inhibit the enzyme from rat liver. The yeast enzyme is similarly inhibited. At $55\mu\text{M}$ NADPH produces almost complete inhibition in the above system, but this has not been examined in detail since intermediate rates of reaction are too rapid to be followed accurately on the equipment available.

Addition of glutathione reductase to a mixture of diamide and NADPH slightly accelerates the reaction between these two. This effect has only been observed at very high enzyme concentrations (approx. 8 units/cuvette) and may be due to catalytic traces of GSH bound to the enzyme. It is also possible that diamide can act as a substrate for the enzyme and consequently represents a competitive inhibitor of the reaction between GSSG and NADPH, but it is not possible to test this because of the rapid non-enzymic reaction between GSH and diamide.

Discussion. Diamide has been used in this laboratory in an attempt to elucidate the role of GSH in planarian regeneration. The effects observed are similar to those obtained by Wax *et al.* (1970) with *E. coli*. The progress of regeneration is significantly delayed in direct proportion to the concentration of diamide to which the animals are exposed.

This delay can be attributed to a lack of GSH provided that diamide does not affect other cellular systems. In fact it is likely that diamide causes severe depletion of reduced nicotinamide nucleotides by direct reaction, and indirectly in the case of NADPH by maintaining a consistently high intracellular concentration of GSSG. Enzymic reduction of this pool will lower the concentration of

NADPH and, even if glutathione reductase activity is extremely low in the affected cells, the direct reaction will continue to consume NADPH.

It seems unlikely that compounds that oxidize intracellular GSH can be used to determine its role in the cell because they will inevitably deplete intracellular NADPH by the continuing operation of the enzyme glutathione reductase. This problem might be avoided by use of a non-competitive inhibitor of the enzyme, but only in circumstances that also take into account the possibly rapid synthesis *de novo* of GSH (Bloch, 1949).

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