GLUTATHIONE IX. NEW THIOL-OXIDIZING AGENTS: DIP, DIP+1, DIP+2

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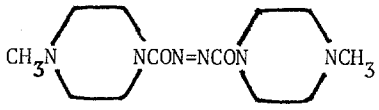
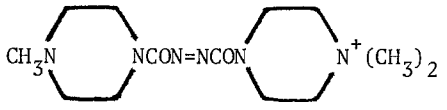
New thiol-oxidizing agents, DIP (diazene dicarboxylic acid bis(N'-methylpiperazide), DIP+1(N'-methyl iodide salt of DIP), and DIP+2 (bis-N'-methyl iodide salt of DIP) have been applied to human red blood cell suspensions. At 20°C, DIP leads to fairly rapid oxidation of intracellular glutathione, GSH. Although both DIP+1 and DIP+2 are slightly more reactive towards GSH than DIP, neither agent causes any change in intracellular GSH. Possible applications of the new series are outlined.

The thiol oxidizing agent, diamide (1), causes the release of neurotransmitter from frog neuromuscular junctions (2,3). In order to probe the possibility that thiol groups accessible to extracellular reagent might be involved in the neural response, we needed a series of compounds which varied in their ability to enter cells. The molecular design problem was solved through the addition of a basic nitrogen to the diamide structure together with enough carbon atoms to make the compounds stable, compact, synthesizable and readily accessible from usual starting materials. The basic nitrogen made the preparation of quaternary salts possible, and it was anticipated that the cations thus produced would enter cells much less readily than the potentially neutral compounds. The structures of DIP, DIP+1 and DIP+2 are given below. The syntheses will be described elsewhere. (4)

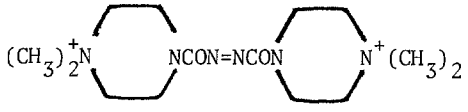
The three compounds, DIP, DIP+1 and DIP+2, are easily soluble yellow solids, which are much more reactive towards glutathione (GSH) than is diamide.



DIP



DIP+1



DIP+2

The overall reaction occurs in two stages with rates which are similar within an order of magnitude, according to Eqs. 1 and 2. Kinetic and mechanistic studies will be reported elsewhere (4). The rate of reaction of GSH with DIP+2 is ca. 3000 times faster than with diamide.

(1) $GSH = H^+ + GS^-$; $GS^- + -N{=}N- \xrightarrow{H^+} GS-\underset{|}{N}-NH-$

(2) $GS^- + GS\underset{|}{N}-NH \xrightarrow{H^+} GSSG + -NHNH-$

The higher reactivity of the DIP series as compared with diamide is also expressed in the reaction with water, with half-lives for hydrolysis for the compounds at pH 7.2 given as follows: DIP (5.5 hours), DIP+1 (1.5 hours) and DIP+2 (1.2 hours). Solutions must therefore be made up immediately before use and long-term experiments must be planned with due regard for the loss of agent through hydrolysis.

A measured volume of freshly prepared DIP solution in isotonic buffer is added to a suspension of washed human red blood cells in isotonic phosphate-sodium chloride buffer maintained at the desired temperature, mixing rapidly with a Vortex. After an appropriate time, aliquots are quickly mixed with ten volumes of ice-cold buffer, centrifuged, and resuspended in buffer. [In some experiments, aliquots were mixed with GSH solution, centrifuged, washed and

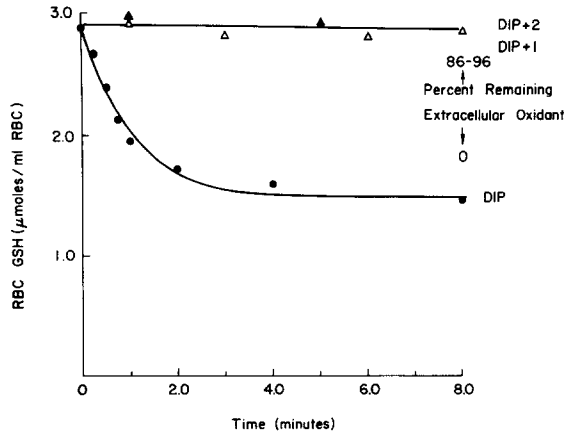


Fig. 1 Oxidation of intracellular GSH by DIP, DIP+1 and DIP+2. The amounts of oxidant remaining outside of the cells after 8 minutes are shown.

resuspended in buffer. GSH determinations on the supernatant were made in these cases]. Analyses of the intracellular GSH content are then made according to the previously described method (5) on deproteinized samples.

Addition of DIP to r.b.c. leads to the oxidation of GSH, with a half-time for the reaction of 30-60 seconds at 18-23°C. The half-life for GSH oxidation at 0-4°C is about 20 minutes. Thus, the rates of intracellular GSH oxidation produced by diamide are higher than those observed for DIP even though the latter has a much higher intrinsic reactivity towards GSH. The difference can be explained readily and its usefulness will be the subject of a separate report (6).

Neither DIP+1 nor DIP+2 caused the oxidation of intracellular GSH as shown in Fig. 1, but could be shown to effect a stoichiometric oxidation of GSH added later. DIP, in contrast, disappeared completely from the medium. Approximately 0.65-0.7 moles of DIP are required to oxidize 1 mole of GSH, somewhat above the theoretical value of 0.5. (Fig. 2).

Like diamide (1, 7), DIP does not interfere with normal red cell function as exhibited through the regeneration of GSH. After addition of DIP sufficient to oxidize essentially all of the intracellular GSH, incubation at

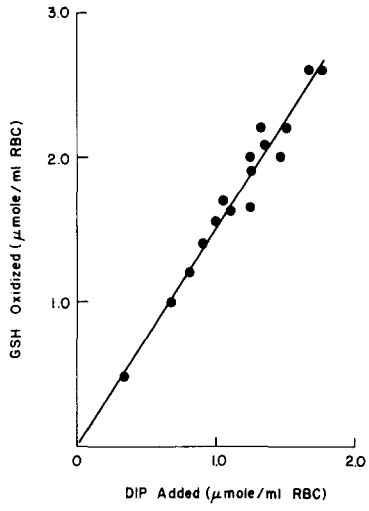


Fig. 2 Stoichiometry of oxidation of intracellular GSH by DIP. (Data pooled from six different experiments).

37°C led to complete regeneration of GSH within 30 minutes. Larger amounts of DIP caused a delay in regeneration of GSH. No oxidation of hemoglobin SH groups and no change in hemoglobin spectrum occurred in erythrocytes treated with DIP in excess of that sufficient for GSH oxidation (3-6 μmoles/ml. rbc).

The DIP series fulfilled the anticipated need in experiments on frog neuromuscular junctions. DIP promoted neurotransmitter release in a fashion parallel to that stimulated by diamide, whereas DIP+1 and DIP+2 had no effect on the m.e.p.p. rate (8). In another study, lymphocyte transformation by phytoagglutinins was shown to be inhibited by the intracellular oxidation of GSH but was unaffected by DIP+1 (9).

The DIP series offers a useful new way of testing whether or not intracellular or extracellular thiol oxidation is involved in a biological process. The ability to discriminate between sites of thiol oxidation might also be useful in the case of isolated organelles like mitochondria. We have found the DIP series a useful addition to our other thiol-oxidizing agents, azoester and diamide (10-17) in our efforts to elucidate the effects of perturbation in the GSH-GSSG status of biological systems (18).

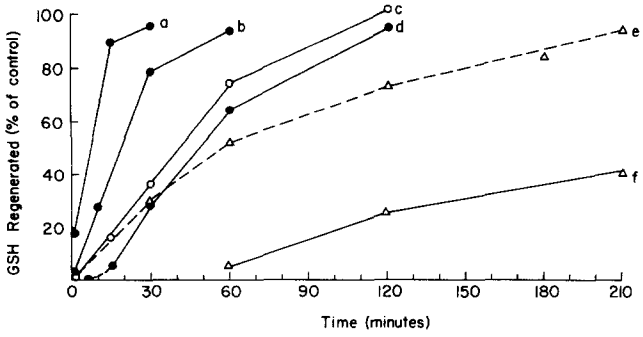


Fig. 3 Regeneration of GSH after oxidation by DIP within red blood cells. DIP was added to washed r.b.c. suspension at room temperature. After 5 minutes, glucose was added (final concn. 10 mM) and samples were incubated at 37°C. DIP added (as moles/mole of GSH in r.b.c. sample): (a) 0.5; (b) 0.76; (c) 1.1 (r.b.c. washed free of DIP before addition of glucose); (d) 1.1; (e) 2.5 (r.b.c. washed free of DIP before addition of glucose); (f) 2.5 [zero time was taken as the start of incubation at 37°C].

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