DIAMIDE, A NEW REAGENT FOR THE INTRACELLULAR OXIDATION OF GLUTATHIONE TO THE DISULFIDE

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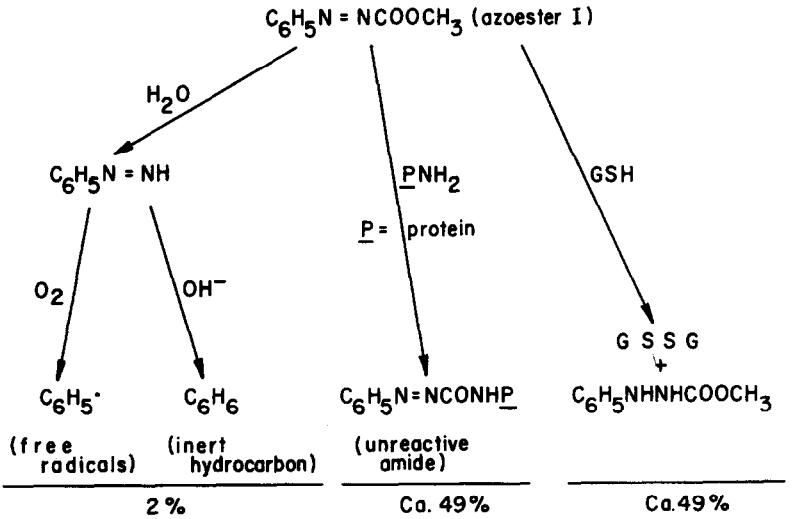
A new thiol-oxidizing agent, diamide, $(CH_3)_2NCON{=}NCON(CH_3)_2$, is shown to stoichiometrically oxidize glutathione (GSH) within the human red blood cell to the disulfide (GSSG). Conversion of GSH to GSSG is very rapid, even at 1-4°. No interference with cellular function is observed as shown by almost complete regeneration of GSH after incubation with glucose at 37°, and no alteration in hemoglobin, osmotic fragility or cell density is found.

Some time ago we introduced methyl phenyldiazenecarboxylate (azoester I) as a reagent for the oxidation of glutathione (GSH) to the disulfide (GSSG) within the human erythrocyte (1). Extensive chemical (2,3) and biological (4,5) studies have led to the following scheme for the behavior of I:



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After consumption of the GSH by reaction with azoester I, free radicals, produced via hydrolysis and reaction with oxygen, can cause damage to the cell. Although such damage is both interesting and useful (5), it was desirable to have in hand a reagent which would convert GSH to GSSG without forming free radicals in a side reaction. The design of the reagent was based on two qualities: (a) resistance to hydrolysis in neutral aqueous solution and (b) preference for the hydroxide ion reaction with the intermediate diazene (RN=NH) rather than the oxygen reaction by making R a strong electron-withdrawing group.

We here report a reagent which fits these specifications. The compound is diazenedicarboxylic acid bis(N,N-dimethylamide), II, for which we prefer the trivial name, diamide. Diamide is easily made by the synthesis of Crawford and coworkers (6). It is a yellow, non-hygroscopic crystalline solid, easily soluble in water and organic solvents, and rather stable towards hydrolysis ($t_{\frac{1}{2}}$ is estimated as 3000 hours at pH 7.4). The overall reaction of diamide with GSH is like that of the azo-ester I. (Eq. 1) (3,7)

(1) $(CH_3)_2NCON{=}NCON(CH_3)_2 + 2GSH \rightarrow (CH_3)_2NCONHNHCON(CH_3)_2 + GSSG$

Diamide II

A measured volume of diamide in aqueous buffer solution is added to a cold ($1\text{-}4^0$) suspension of washed human red blood cells in glucose-isotonic phosphate-sodium chloride buffer with rapid mixing on a Vortex. Final diamide concentrations are between $1 - 50 \times 10^{-4}$ M. After a few minutes at $1\text{-}4^0$, samples are removed and analyzed in the usual way by the method of Beutler (8). To permit regeneration of GSH within the cell, the treated suspension is incubated at 37^0 and aliquots are removed at intervals for GSH analysis (4). The results of several experiments are shown in Fig. 1, from which it may be seen that about 0.6 mole of diamide is required to oxidize 1 mole of GSH. The theoretical value for the amount of diamide required per mole of GSH is 0.5.

Diamide treatment does not interfere with normal cell function as shown by the almost complete regeneration of GSH after incubation. After the application of diamide sufficient to oxidize all of the intracellular GSH of red blood cells, incubation led to 93-95% regeneration of the GSH within 30 minutes. Larger amounts of diamide caused a lag in the beginning of regeneration and a lower rate of regeneration. However, even for a red blood cell sample to which a five-fold excess of diamide (over that

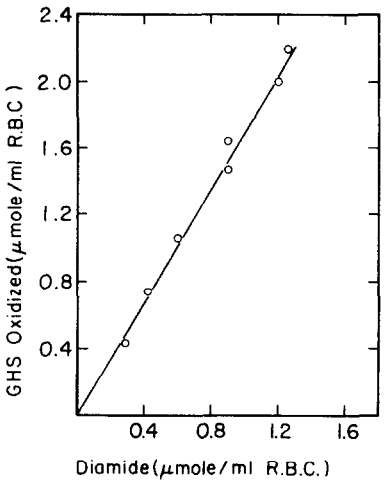

Fig. 1. Oxidation of GSH by diamide.

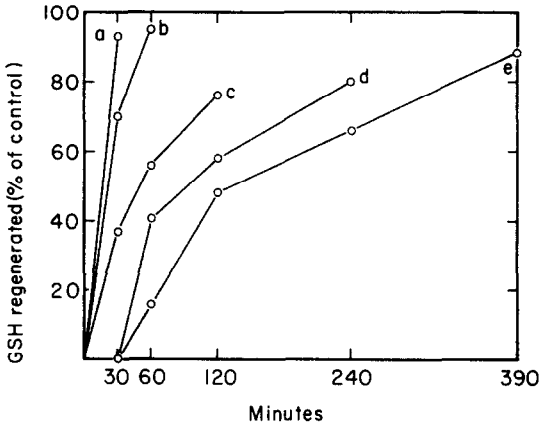

Fig. 2. Regeneration of GSH after oxidation by diamide. Moles of diamide used per mole of GSH in red blood cell sample: (a) 0.5, (b) 0.9, (c) 1.8, (d) 2.4, (e) 3.0.

required for complete oxidation of the GSH) had been applied, almost 90% of the original GSH content was eventually reformed. These results are illustrated in Fig. 2.

Diamide treatment of red blood cells has no observable effect other than the intracellular oxidation of glutathione. A large excess of diamide did not alter the hemoglobin (light absorption spectrum unchanged), the density distribution of the cells (dialkyl phthalate method) (9), the osmotic fragility (Fragiligraph) (10), or agglutinability by poly-L-lysine (11). Carbon monoxide loaded red blood cells did not hemolyze

after addition of excess diamide in contrast to the marked hemolysis found for such cells after the use of a similar quantity of azoester I (5). Heinz bodies were not seen in the light microscope nor were small electron-dense bodies seen under the electron microscope. In fact, ghosts prepared from diamide treated red blood cells appeared to be identical under the electron microscope to ghosts from untreated cells.

Diamide is a useful reagent for the intracellular oxidation of glutathione in a wide variety of biological systems. The reaction of diamide with GSH is extremely fast and close to the theoretical in stoichiometry within cells. We shall report elsewhere on some of the effects achieved with diamide in biological systems (12).

Azoester I is a useful reagent for intracellular oxidation of GSH for those cases in which it is desired to pose a serious chemical challenge (in the absence of GSH) to the cellular systems. Diamide II is preferred when it is desired to study primarily the effects of a temporary removal of GSH (as GSSG) within the cell. The two reagents constitute a pair which should continue to aid efforts to understand the biological significance of glutathione.

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