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Diamide for Dummies

by

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Abbreviations

$GS^{\cdot}$, glutathiyl radical
GSH, glutathione
GSSG, glutathione disulfide
$PS^{\cdot}$, protein thiyl radical
PSH, protein thiol
PSSG, protein-glutathione mixed disulfide

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Abstract

Diamide is a small thiol oxidant that is used in many laboratories. It reacts rapidly with glutathione, oxidizing it to glutathione disulfide. Its main use in the laboratory is as an oxidant probe that specifically oxidizes glutathione. Diamide also interferes with translation and initiation of protein synthesis. Additionally, hexose uptake and metabolism is affected by diamide treatment. Diamide may be detected by UV/Vis spectroscopy. This review summarizes the reaction of diamide with GSH as well as other effects of diamide on cells.

Introduction

Diamide is a small oxidant molecule that has been commonly used in laboratories for about thirty years to oxidize glutathione (GSH) to the disulfide (GSSG). Diamide is useful for studying GSH because it is a highly specific, rapidly-acting oxidant. Although it is a very specific oxidant, there are many potential consequences of diamide treatment that should be considered when using this reagent. However, armed with this information, diamide becomes a powerful oxidant probe that perturbs the redox balance of cells by oxidation of GSH.

In 1969, Kosower *et al*. published a paper describing the properties of a new reagent called diamide to preferentially oxidize glutathione in cells [1]. Prior to this, methyl phenyldiazenecarboxylate (azoester I) was used for this purpose. However, the reaction of azoester I with GSH produces free radicals in a side reaction, which could then go on and damage cellular components (see Figure 1). Another disadvantage to using azoester I to oxidize GSH was that it would lyse the cell [2].

Diamide was therefore investigated as a reagent and was found to be more useful for the oxidation of glutathione to the disulfide. Diamide and azoester I undergo the same reaction scheme, as shown in Figure 1. However, three criteria make diamide a preferable tool for glutathione oxidation. First, diamide does not hydrolyze as easily as azoester I when in neutral aqueous solution [1]. In fact, diamide has a $t_{½}$ at pH 7.4, 25°C of approximately 3000 h [1]. Second, when diamide does hydrolyze, the intermediate diazene prefers to react with OH^- rather than dioxygen (see Figure 1) [1]. Thus, diamide oxidizes GSH without producing free radicals to any great extent. Finally, unlike azoester I, diamide does not kill the cell [2].

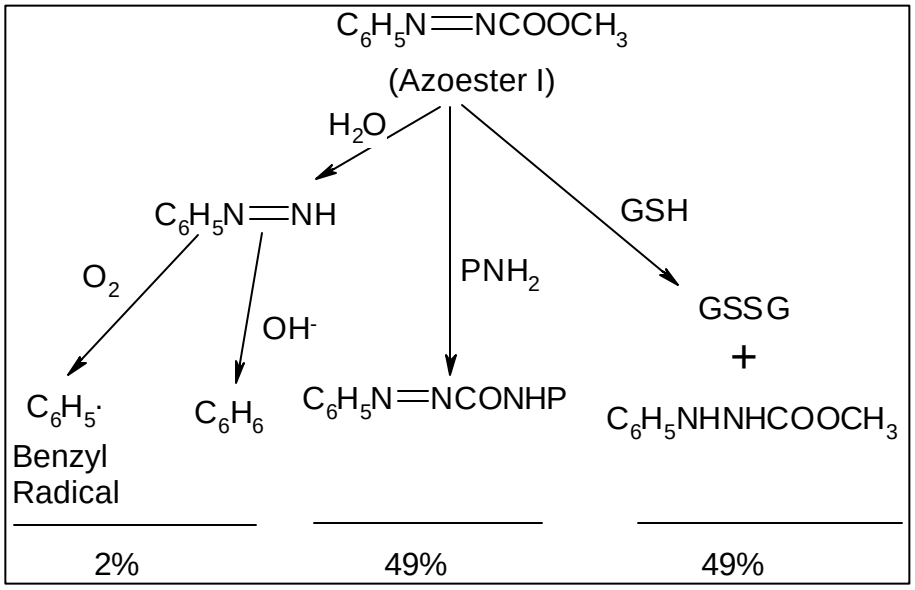


Figure 1: Azoester I as a reagent for the oxidation of glutathione. Although less than two percent of azoester I goes through the pathway involving dioxygen, this side reaction creates free radicals that can confound an experimental system. Kosower *et al.* does not speculate as to what the other products in this reaction scheme might be. Adapted from [1].

Physical Properties and Structure

Diamide is a yellow [3], non-hygroscopic [1], crystalline solid at room temperature [3]. Its melting point is between 113 and 115°C [3] and it is soluble in water and organic solvents [G]. Diamide is a diazene dicarbonyl compound [4], as depicted in Figure 2.

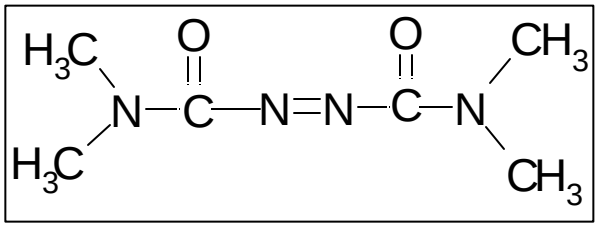
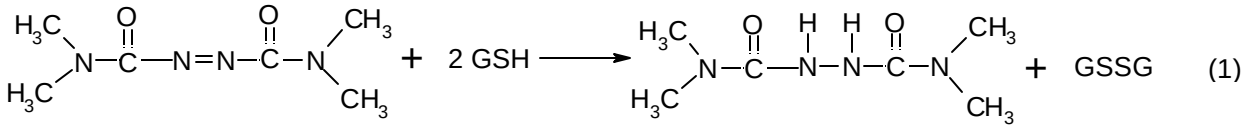


Figure 2: Structure of diamide, a diazene dicarbonyl compound. Adapted from [4].

Reaction of Diamide with Glutathione

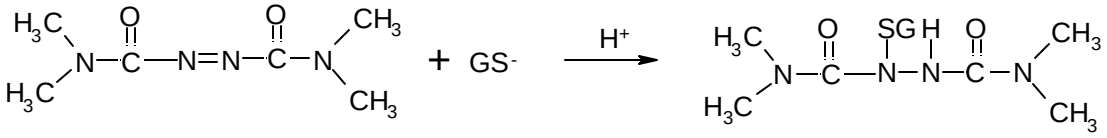
Overall, the reaction of glutathione with diamide is similar to its reaction with azoester I (Figure 1). See reaction 1 below [1]. Theoretically, 0.5 moles of diamide should be required



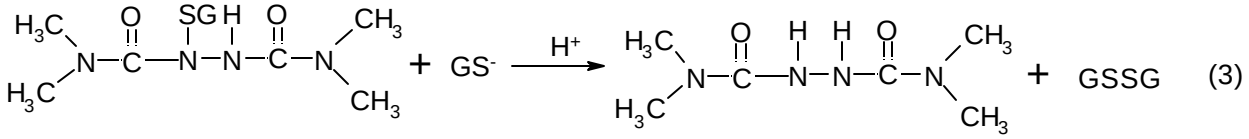
(1)

to react with each mole of GSH. Experimentally, it takes about 0.6 moles of diamide to oxidize each mole of GSH to GSSG [1].

The reaction of diamide with thiols like GSH can be broken into two steps. First, GS- adds to the double bond of the diazene, forming a sulfenylhydrazine (equation 2). Then, this sulfenylhydrazine reacts with another GS^- anion, giving a disulfide and a hydrazine (equation 3). It is also possible for GSH-protein mixed disulfides (PSSG) to form. This happens when PS^- replaces GS^- in reaction 3 [4].



(2)



(3)

Overall, diamide reacts with glutathione more rapidly than other thiol substrates that have been measured. As can be seen in Table 1, the reaction of diamide with glutathione has the largest rate constant of all the reagents examined [5]. Since glutathione is also the most abundant non-protein thiol in cells, it is easy to understand why diamide oxidizes glutathione so specifically. Thus, diamide reacts very slowly with non-GSH thiols, and even more slowly with non-thiols. Additionally, diamide is more likely to react with a small thiol like GSH than with a protein thiol because GSH has less steric hindrance [4].

Table 1: Rate Constants for Diamide Oxidation Reactions. Adapted from [5]

Compound	Rate Constants (M^{-1} s^{-1})	
	Kosower *et al*. [5]	O'Brien *et al.* [6]
Glutathione	300	>25*
Dithiothreitol	100	>25*
Cysteine		>25*
N-Acetylcysteamine		>25*
Dimercaptopropanol		>25*
Lipoic acid (reduced)	30-60	>25*
Flavin mononucleotide (reduced)		>25*
Flavin adenine dinucleotide (reduced)		>25*
1-Benzyl-3-carbamido-1,4-dihydropyridine	44	---
1-Benzyl-3-carbamido-1,4-dihydro[4,4-$^2H_2$]pyridine**	13	---
Mercaptoethanol		21
Coenzyme A		18
NADH	3.5	4.2
NADPH		3.2
Ferredoxin (reduced)		>25*
Ascorbic acid		0***
Mercaptoacetic acid		3.2

* Rate reported as "too fast to measure"
** Less that 4% 2H in 4 position
*** No observed reaction
--- Not tested

Other Effects of Diamide on Cells

First, it is important to recognize that while diamide treatment will not kill most cells, it will slow cell growth. *E. coli* are able to tolerate diamide concentrations of up to at least 3 mM [2]. The cells experienced a dose-dependent decrease in growth rate with increasing diamide [2]. This effect was reversible with additon of excess GSH to the media [2]. The decrease in growth rate does not diminish diamide's utility, however. Azoester I effectively oxidizes GSH and inhibits growth, but also causes extensive lysis of the cells--50% in 1.8 hours [2].

Diamide is also able to temporarily stop protein synthesis in cells that are actively synthesizing protein [7]. Using GSH-rich rabbit reticulocytes, Zehavi-Willner *et al.* [7] demonstrated that diamide affected both translation and initiation of protein synthesis, although

initiation is more sensitive to a diamide-induced drop in GSH concentration. When 40 to 60 % of the original GSH concentration has recovered, translation and release resume in a short time. By contrast, recovery of initiation requires that 70 to 80% of the GSH be regenerated. If the cell has endured a long period with no GSH, initiation will never recover completely [7].

Diamide also affects transport of hexoses in human platelets [8, 9]. At low diamide concentrations (1 mM), glucose uptake increases over untreated platelets [8]. When 2 to 3 mM diamide is used, however, glucose uptake decreases dramatically [8]. In red cell ghosts, diamide inhibits glucose transport [9]. Fructose uptake in platelets, by contrast, steadily decreases with increasing diamide concentrations in the same range [8].

The activity of glycolytic enzymes may be affected by high diamide concentrations [8]. Of some thirteen glycolytic enzymes tested in human platelets, the activity of phosphoglycerate kinase, phosphofructokinase, pyruvate kinase, enolase, and glucose 6-phosphate dehydrogenase were all inhibited by 2 mM diamide to varying degrees [8]. By far the most pronounced inhibition is of pyruvate kinase, which showed a 13-fold reduction in activity.

Detection of Diamide

Diamide may be quantified by its UV/Vis spectrum. When the solvent is CH_2Cl_2, the extinction coefficient is 1800 $M^{-1}cm^{-1}$ at λ_{max} 290 nm [4]. When dissolved in water (135 mM NaCl/10 mM phosphate buffer, pH 7.4), the extinction coefficient is 3000 $M^{-1}cm^{-1}$ at 296 nm [4]. It is also possible to follow the reaction of diamide with thiols with a UV/Vis spectrophotometer between 300 and 325 nm [4]. When diamide becomes reduced, the diazene dicarboxylic acid bis(N, N-dimethylamide) is formed. This species does not absorb in the 300 to 325 nm range.

Summary

Thus, diamide is an important laboratory oxidant because it is able to react with glutathione so rapidly and specifically. Diamide is more useful than azoester I for oxidation of glutathione, because it does not cause cellular damage through free radical production. Although there is some effect on protein synthesis and hexose transport and metabolism, diamide has been and will continue to be useful to researchers.

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