OXIDATION OF A VARIETY OF NATURAL ELECTRON DONORS BY THE THIOL-OXIDISING AGENT, DIAMIDE

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1. Introduction

Kosower and Kosower [1] have reported that members of a new series of thiol-oxidising agents can rapidly oxidise intracellular glutathione. The most promising of these reagents is *N,N,N', N'*-tetramethylazoformamide, to which they have given the trivial name, diamide [2]. This compound displays comparatively great hydrolytic stability and shows little or no tendency to produce free radicals in aqueous solution, yet it rapidly effects the stoichiometric and O_2-independent oxidation of reduced glutathione, both *in vitro* and intracellularly e.g. in human erythrocytes [2]. Subsequently reported biological effects of diamide, which include inhibition of (a) protein synthesis in rabbit reticulocytes [3], (b) growth of *Escherichia coli* [4] and (c) RNA synthesis in *E. coli* [5], have all been deemed the consequence of its intracellular oxidation of glutathione, and possibly also of cysteine and homocysteine.

We recently used diamide in an attempt to establish the extent to which the toxicity of oxygen to a Clostridium could be attributed to oxidation of intracellular thiol compounds. It was in the course of this study that we discovered that diamide was capable of oxidising a variety of important, intracellular electron carriers. In this communication we draw attention to the fast oxidation of reduced lipoate and slower oxidation of reduced coenzyme A by diamide; we further report that diamide very rapidly oxidises reduced flavin nucleotides and reduced ferredoxin, and more slowly oxidises NADH and NADPH. Both spectrophotometric and polarographic procedures have been developed to follow the reduction of diamide in these reactions.

2. Experimental procedures

Diamide and dihydrodiamide were synthesised by the method of Crawford and Raap [6]. Aqueous solutions of diamide (pH 7) displayed an unsatisfactorily broad and minor peak of absorbance around 450 nm; the major peaks of absorbance were at 296 nm and 205 nm. Spectrophotometric assay of the rates of diamide reduction by thiol compounds and by NADH and NADPH was performed at 290 nm; ϵ_{290} of diamide equals 2.35×10^3 $M^{-1} cm^{-1}$. Polarographic measurements were made with a Radiometer PO4 Polariter using a dropping mercury electrode and a saturated calomel reference electrode. Solutions were deoxygenated with nitrogen and current-voltage curves were recorded automatically.

3. Results

Table 1 shows that in aqueous solution at pH 7 diamide very rapidly oxidised a number of thiol compounds (including reduced lipoate), and more slowly oxidised others such as reduced coenzyme A. More surprisingly, diamide also rapidly oxidised reduced flavin nucleotides ($FMNH_2$ and $FADH_2$), and reduced ferredoxin; NADH and NADPH were also oxidised by diamide though at a slower yet still significant rate.

These spectrophotometric findings were confirmed by polarographic measurements. Diamide itself produced a well-defined polarographic reduction wave (fig. 1), whose height varied linearly with concentration; no wave was produced by dihydrodiamide. It was

Table 1
Rates of reduction of diamide by thiol compounds and natural electron donors.

Compound	Rate of reduction of diamide (μmoles/min)
A. *Thiols*	
Glutathione (reduced)	Very rapid[a]
Cysteine	
N-Acetylcysteamine	
Dimercaptopropanol (BAL)	
Dithiothreitol	
Lipoic acid (reduced)	
Mercaptoethanol	0.20
Coa	0.17
Thioglycollic acid	0.03
B. *Non-thiol, natural electron donors*	
$FMNH_2$	Very rapid[a]
$FADH_2$	
Ferredoxin (reduced)	
NADH	0.04
NADPH	0.03
Ascorbic acid (reduced)	Nil

Equimolar concentrations (4×10^{-4} M) of test compound and diamide in 0.1 M tris-HCl buffer pH 7 were incubated at 30°. Reactions were followed spectrophotometrically at 290 nm, or by the polarographic procedures described in the text.

[a] These reactions were so fast that their initial rates were not measurable by the spectrophotometric and polarographic procedures employed.

to be expected that the polarographic reduction of diamide would be a 2-electron process. Consistent with this was our finding that the half-wave potential of diamide was linearly related to pH with a slope of 59 mV/pH unit. Incidentally, this relationship enabled us to assign a value of +215mV (versus the hydrogen electrode) to the E_0' for diamide at 25°, pH 7. In the polarographic investigation of the reaction of diamide with NADH, with $FMNH_2$ and with reduced lipoate, use was also made of the distinctive polarographic behaviour of these electron donors.

3.1. *Oxidation of NAD(P)H by diamide.*

When this reaction was followed spectrophotometrically at 290 nm, a value of 7.1×10^2 $M^{-1} cm^{-1}$ was assumed for ϵ_{290} of NAD(P)H. To determine the stoichiometry of the reaction the following reaction mixture was employed: tris-HCl buffer, pH 7, 100 μmoles, diamide, 1 μmole, NADH or NADPH, 2 μmoles in a final volume of 1 ml. After incubating at room temperature for 75 min, the diamide was completely reduced and the residual NAD(P)H and newly formed NAD(P) were assayed using standard enzymic procedures [7]. It was found that reduction of 1 μmole of diamide was coupled with the disappearance of 1.09 μmoles of NADH and the production of 1.02 μmoles of NAD. Similar results were obtained with NADPH.

In the polarographic investigation of this reaction, use was made of the fact that NAD but not NADH is active at the mercury electrode, producing a reduction wave whose height is proportional to concentration. Anaerobic mixtures of NADH and diamide were prepared as described above, and current-voltage curves were periodically recorded. From the measured heights of the waves due to diamide and NAD, it was possible to follow the disappearance of diamide and the concomitant production of an equimolar amount of NAD, thereby confirming the 1:1 stoichiometry of the reaction which can therefore be written as in eq. 1:

$$NADH + H^+ + (CH_3)_2NCON{=}NCON(CH_3)_2 \rightarrow NAD + (CH_3)_2NCONHNHCON(CH_3)_2 \quad (1)$$

3.2. *Oxidation of $FMNH_2$ by diamide*

The stoichiometry of this fast reaction was determined polarographically. Polarographic waves are produced both by FMN (cathodic wave) and by $FMNH_2$ (anodic wave). The method we employed was as follows. A solution containing a known concentration of FMN on 0.1 M tris-HCl buffer pH 7, was deoxygenated, and a polarogram was recorded. Less than one equivalent of sodium dithionite was then added to reduce a substantial proportion of the FMN and yet to leave no excess dithionite; a second polarogram was recorded. A quantity of diamide was then added and a further polarogram was immediately taken. Oxidation of the $FMNH_2$ proceeded extremely rapidly and was reflected in the decrease in the magnitude of the anodic $FMNH_2$ wave and the corresponding increase in the cathodic FMN wave. Using various amounts of diamide it was possible to determine the "end point" quantity required to oxidise all of the $FMNH_2$. At this end point, no polarographic waves

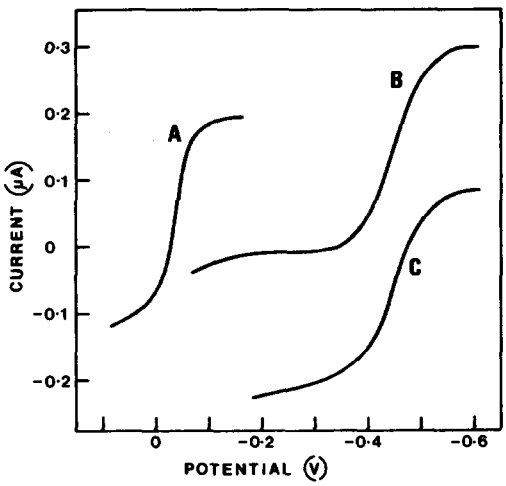

Fig. 1. Polarographic current-voltage curves. A) 0.05 mM diamide; B) 0.1 mM FMN; C) composite anodic/cathodic wave of FMN plus $FMNH_2$ produced by the addition of less than one equivalent of dithionite to the solution in B. The further addition of diamide to this mixture resulted in the conversion of this polarogram to one identical with B. All solutions were in 0.1 M tris-HCl buffer, pH 7 at 25°. Potentials were measured versus a saturated calomel electrode.

due to $FMNH_2$ or diamide were discernible. Only beyond this end point when excess diamide had been added, did a diamide wave appear. Since these experiments were performed under strictly anaerobic conditions, it was clear that the $FMNH_2$ was indeed being oxidised by the diamide. Fig. 1 shows the polarographic waves obtained in such an experiment. It was found that 1 μmole of diamide oxidised some 0.95 μmole of $FMNH_2$, demonstrating the 1:1 stoichiometry of the reaction.

3.3. *Oxidation of thiols by diamide*

Though the interaction of thiols with diamide could, in general, be followed spectrophotometrically, polarographic measurements enabled the stoichiometry of these oxidation-reduction processes to be more easily determined. For example, during its titration with diamide we followed consumption of reduced lipoate by observing the decrease in its anodic wave height. The titration was continued until this anodic wave was just eliminated, when it was found that the reduced lipoate had been oxidised by an equimolar quantity of diamide.

4. Conclusions

The finding that diamide oxidises (a) thiol coenzymes other than glutathione, and (b) several natural, non-thiol, electron donors (including reduced flavin and pyridine nucleotides), complicates interpretation of the consequences of diamide addition to biological systems. Certainly, the effects of diamide on microbial cultures and cell suspensions cannot be held to be due solely to the intracellular oxidation of glutathione.

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