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GLUTATHIONE AND THE MITOCHONDRIAL REDUCTION OF SOME DIAZENES

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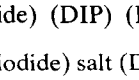
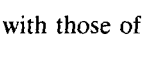
Abstract—Mitochondria reduce diazenedicarboxylic acid bis(dimethylamide) (diamide) and the corresponding bis(*N'*-methylpiperazide) (DIP) but not the bis(*N'*-methyl iodide) salt (DIP^{2+}). Without added substrates, DIP, but not diamide or DIP^{2+}, depletes mitochondrial glutathione. The depletion is less in the presence of some Krebs acids, especially succinate, and these acids proportionately increase the rate of DIP reduction. The reduction is inhibited by antimycin, thiol reagents and uncouplers. The results suggest that DIP^{2+} is impermeable, but that DIP enters mitochondria chiefly as a cation and at a more rapid rate than diamide.

Diazenes were introduced as oxidants for intracellular GSH [1].* One of them, diamide, has previously been shown to be reduced by rat liver mitochondria and evidence given that mitochondrial GSH, itself regenerated via $NADPH_2$ and glutathione reductase, is the immediate reductant [2]. The entry of diamide into mitochondria is slow and may be mediated by carriers of Krebs cycle intermediates [2]. As a result, there is very little depletion of mitochondrial GSH unless its regeneration from GSSG is also inhibited. These observations suggested that a comparison of the behaviour of diamide with that of other diazenes might yield more information about the mechanism of the reduction.

In this paper, the properties of two other diazenes (RCON:NCOR), diazenedicarboxylic acid bis(*N*-methylpiperazide) (DIP) (R, ) and its bis(*N'*-methyl iodide) salt (DIP^{2+}) (R, ) are compared with those of diamide (R, Me_2N—).

MATERIALS AND METHODS

DIP and DIP^{2+} were synthesized from diethylazodicarboxylate (Aldrich Chemical Co. Ltd., Gillingham, U.K.) as previously described [3]. The source of other reagents, the preparation of mitochondria, conditions of incubation, composition of the suspension medium and assays for GSH and protein have been previously described [2]. Mitochondrial pellets, sedimented from the suspension medium in an Eppendorf centrifuge, and the decanted supernatants were separately acidified with perchloric acid (final concentration, 2.4%) and used, respectively, for assays of GSH and diazene.

Diazene assays. DIP and DIP^{2+} and diamide lose their absorption at 310 nm when reduced with GSH. From the fall after adding a known amount of GSH, ε is $2.6 \pm 0.2 \times 10^3$ le. $mol^{-1}.cm^{-1}$ for the three diazenes. Using these values, their concentrations were determined from measurements of absorption at 310 nm of acidified supernatants before and after incubation with mitochondria. DIP was dispensed from a concentrated solution in ethanol. DIP^{2+} is insoluble in ethanol. It rapidly hydrolyses in neutral aqueous medium but is stable at low pH for several hours. A neutral solution was therefore made up immediately before use and the solution acidified within 8 min when hydrolysis did not exceed 5%.

RESULTS

The rates of reduction of three diazenes, diamide, DIP and DIP^{2+}, each added at the same initial concentration (0.35 mM) to a suspension of rat liver mitochondria have been compared (Fig. 1A). In the absence of other added substrates, diamide is lost much more rapidly than DIP, and DIP^{2+} is not significantly depleted after 5 min at 30°. Measurements of the concentration of mitochondrial GSH at intervals during the reductions (Fig. 2) show that the concentration remains unaffected by DIP^{2+}. As previously observed [2], it remains high with diamide after a small initial fall. In the presence of DIP, however, there is a substantial and sustained fall in the GSH concentration. The rate of reduction of diamide has been shown to be little affected by adding Krebs acids to supplement endogenous reductants. These acids do not induce any reduction of DIP^{2+}, but some of them substantially increase the amount of DIP reduced after a short incubation (Table 1). Pyruvate, oxaloacetate and oxoglutarate are ineffective and succinate is much more effective than the other acids used. In the presence of succinate the rate of reduction of DIP becomes much faster (about twice as fast) than the rate of reduction of diamide (cf. Figs. 1 and 2), thus reversing the order found without added substrates. When the initial concentration of DIP is lowered in the presence of succinate, no change in the rate of reduction is observed down to an initial concentration of 0.08 mM (the limits of sensitivity); this compares with the behaviour of diamide which shows a K_m of 0.075 mM [2]. Values for mitochondrial GSH in the

* GSH, reduced glutathione; GSSG, oxidised glutathione.

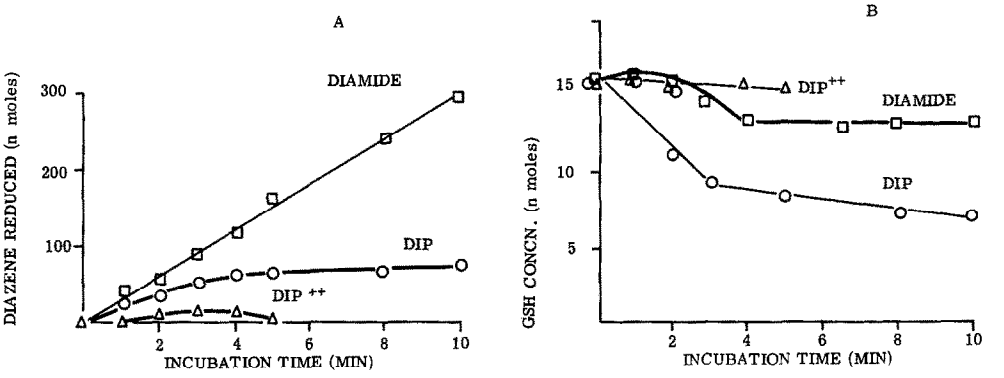


Fig. 1. Time course of reduction of diamide (□), DIP (○) and DIP^{2+} (△) by mitochondria (A) and corresponding GSH concentration (B). Mitochondria (0.1 ml; ~4 mg protein) were added to the diazene (0.35 mM) in buffer medium (1.3 ml), the solutions incubated at 30° and centrifuged at the times indicated.

Table 1. Mitochondrial reduction of DIP and the corresponding GSH concentration after incubation with Krebs acids (2.5 mM)

Krebs acid	Fall in DIP	GSH
Nil	100	31 ± 5
Pyruvate	127 ± 10	29 ± 5
Oxaloacetate	35 ± 35	42 ± 4
Citrate	200 ± 25	48 ± 16
Isocitrate	170 ± 40	58 ± 20
α-Oxoglutarate	100 ± 25	32 ± 8
Succinate	400 ± 30	82 ± 6
Malate	185 ± 50	83 ± 2
3-Hydroxybutyrate	170 ± 30	42 ± 15

Conditions are as for Fig. 2. Incubation was for 5 min. GSH is given as a percentage of the value before incubation and DIP as a percentage of the fall obtained without added Krebs acid. Values are the means of two assays.

presence of DIP and Krebs acids show that acids increasing the rate of reduction of DIP also raise the concentration of GSH above the value found with the control. Under these conditions, however, there is no clear correlation between the two parameters. Thus, malate, which is much less effective than succinate in increasing the reduction of DIP, raises the GSH level by the same amount. The reason for these discrepancies is not clear. However, a close correlation is found in the time courses of DIP reduction and GSH concentration in the presence of succinate or isocitrate (Fig. 2). Thus the rate with isocitrate falls off as the GSH concentration falls, while the rate with succinate remains linear and the GSH concentration is largely maintained.

The effect of some inhibitors on DIP reduction in the presence of succinate has been studied (Table 2). As with diamide reduction, the amount of reduc-

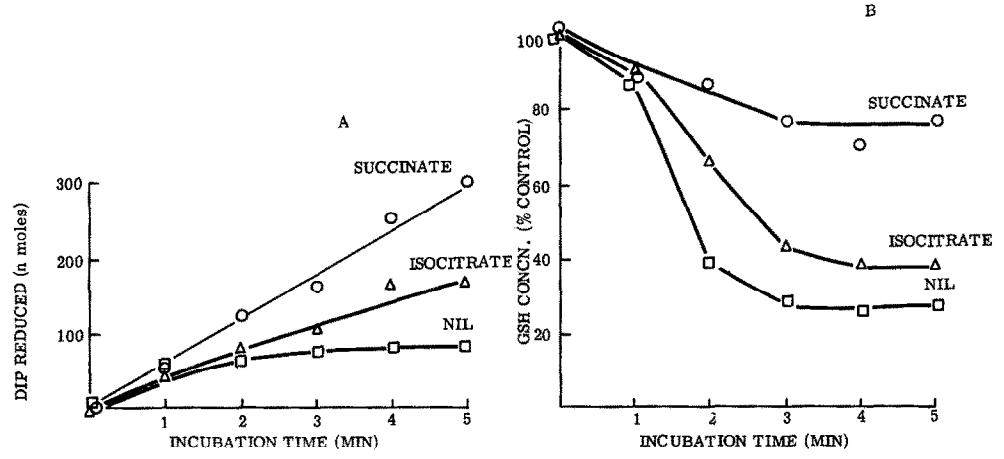


Fig. 2. Reduction of DIP (A) and the corresponding GSH concentration (B) in the presence of nil (□), isocitrate (△) or succinate (○). Conditions were as for Fig. 1. Mitochondria were preincubated with the Krebs acid (2.5 mM) for 2 min before adding DIP. The control value (17.5 nmole) was obtained by sedimenting at once and without adding DIP.

Table 2. Effect of inhibitors on the reduction of DIP and GSH concentration with or without succinate (2.5 mM)

Inhibitor	Fall in DIP	GSH concentration
Nil	100	82 ± 6
N-ethylmaleimide (0.125 mM)	20 ± 11	38 ± 6
p-hydroxymercuribenzoate (0.15 mM)	5 ± 2	38 ± 10
Potassium cyanide (0.25 mM)	97 ± 5	90 ± 5
Antimycin (1.3 μM)	20 ± 10	49 ± 10
Rotenone (2.0 μM)	77 ± 8	91 ± 5
FCCP (0.35 μM)	17 ± 17	65 ± 13
FCCP less succinate	15 ± 5	30 ± 10

Mitochondria are preincubated for 2 min with inhibitor before adding DIP, then reincubated for 5 min. DIP reduction rate with succinate only is taken as 100%. GSH is given as a percentage of the value found before incubation.

tion of DIP in the presence of succinate is little affected by cyanide or rotenone, but is inhibited by antimycin. Thiol reagents (*N*-ethylmaleimide and *p*-hydroxymercuribenzoate) are also effective inhibitors. In all these cases, inhibition is accompanied by a corresponding decrease in the mitochondrial GSH concentration. DIP reduction is also inhibited by uncouplers. The rate falls to about the same basal value with or without Krebs acids present and the inhibition is thus most marked with succinate. However, as previously found with diamide, the concentration of GSH is not much affected by the uncoupler, carbonylcyanide-*p*-trifluormethoxyphenyl hydrazone (FCCP) in the presence of succinate.

DISCUSSION

The finding that DIP^{2+}, which react readily *in vitro* with GSH, is not reduced by mitochondria nor able to deplete mitochondrial GSH shows that this substance, despite the favourable membrane potential of coupled mitochondria, cannot penetrate into the matrix. Presumably this is because DIP^{2+}, an ethanol-insoluble dication, is insoluble in the membrane lipids. This substance has previously been found to be unable to penetrate into erythrocytes [4] and cultured chinese hamster cells [5]. In contrast, DIP readily does so and it also probably penetrates into mitochondria more rapidly than diamide in conformity with the presence of lipophilic groups in its structure. Thus, unlike diamide, its rate of entry is fast enough to overwhelm the GSH regeneration system and so deplete mitochondrial GSH unless an exogenous reductant is also added. The low rate of reduction of DIP, observed after an initial spurt, is attributed to toxic effects (e.g. on the GSH regeneration system) due to the accumulation of DIP within the matrix. This explanation is supported by considering the big increase in the reduction rate of DIP, but not of diamide, obtained when the reducing capacity of the mitochondria are supplemented with some exogenous Krebs acids, especially succinate. The rate then considerably exceeds that obtained with diamide. The corresponding elevation by these Krebs acids of the concentration of GSH is evidence that the reduction of DIP, like that of diamide, is mediated through GSH and that in uninhibited mitochondria the rate of reduction is roughly proportional to its concentration. The inability of pyruvate and α-oxoglutarate to increase the rate of DIP reduction or to elevate the GSH concentration may be due in part to preferential oxidation by DIP of the SH group of coenzyme A required for their dehydrogenation. These acids and oxaloacetate were previously found to be unable to reduce GSSG in lysed mitochondria or in intact mitochondria when formed by incubation with tert-butylhydroperoxide [6]. An inhibition of oxygen uptake from pyruvate and α-oxoglutarate in the presence of diamide or butylhydroperoxide has also been reported recently [7].

Although isocitrate can form NADPH required for the reduction of GSSG directly, it is less effective than succinate in supporting DIP reduction. NADPH formation from succinate involves reversed electron transport, which is known to be a rapid process, and this may explain the sensitivity of the reaction to antimycin, though the poor inhibition by rotenone is an anomaly. The effect of other inhibitors on DIP reduction in the presence of succinate is similar to their effect on diamide reduction. In particular, the severe inhibition obtained with FCCP can be attributed to a requirement for ATP equivalents for energy-dependent transhydrogenation. In the presence of succinate, FCCP does not much deplete mitochondrial GSH, showing that penetration of DIP is restricted in the uncoupled state. It has been proposed that diamide has to be protonated before it can traverse the mitochondrial membrane and a similar requirement may also apply to DIP.

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