The Reduction of Diamide by Rat Liver Mitochondria and the Role of Glutathione

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Diamide is reduced by mitochondria utilizing endogenous substrates with $V_{\max}$ 20 nmol/min per mg of protein and K_m 75 μ M. The reaction is inhibited by: (a) thiol-blocking reagents (*N*-ethylmaleimide, *p*-hydroxymercuribenzoate, mersalyl and 2,6-dichlorophenol-indophenol); (b) respiratory inhibitors (arsenicals, malonate and antimycin, but not cyanide or oligomycin; inhibition by antimycin is reversed by ATP); (c) uncouplers (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 2,4-dinitrophenol and valinomycin with K^+ ; inhibition by the first of these uncouplers is not reversed by cyanide); (d) reagents affecting energy conservation (Ca^{2+} , increasing pH, phosphate; phosphate inhibition is augmented by catalytic ADP or ATP and augmentation is abolished by respiratory inhibitors). Concentrations of mitochondrial glutathione are high when diamide reduction is uninhibited, but low after adding one of the above inhibitors such that the reduction rate is roughly proportional to the glutathione concentration. Endogenous ATP concentrations are lower in the presence of diamide than without, but the difference is abolished by respiratory inhibitors. With oligomycin added, however, ATP concentrations are higher in the presence of diamide and this positive increment is decreased by antimycin, *N*-ethylmaleimide and malonate. In the presence of diamide and an uncoupler, the mitochondrial glutathione content does not fall if various reducible substrates are present, although the inhibition of diamide reduction is not relieved. Some of these substrates prevent the fall in reduced glutathione concentration found with diamide and phosphate. They also relieve the inhibition of diamide reduction and the relief is sensitive to butylmalonate. The inhibition of diamide reduction by *N*-ethylmaleimide, mersalyl or *p*-hydroxymercuribenzoate is not relieved by reducible substrates, but the latter mitigate the fall in the concentration of glutathione. Inhibitors of carriers of tricarboxylic acid-cycle intermediates also inhibit reduction of diamide. The reduced glutathione concentration remains high when they are added singly, but falls when two of them are combined. It is proposed that diamide may enter the matrix as a protonated adduct formed with the thiol groups of mitochondrial carriers and then be reduced in the matrix by glutathione, which is regenerated via NADH, energy-dependent transhydrogenase and $NADP^+$ -specific glutathione reductase. Some of the high-energy equivalents required for the transhydrogenation may be generated by the substrate phosphorylation step of the tricarboxylic acid cycle.

The matrix of rat liver mitochondria contains a small amount of GSH (Jocelyn & Kamminga, 1974). This non-protein thiol does not escape from intact unswollen mitochondria, but is accessible to penetrant thiol-blocking agents (Gaudemer & Latruffe, 1975). Mitochondrial GSH is associated with glutathione reductase (EC 1.6.4.2) and glutathione peroxidase (EC 1.11.1.9), both of them present in the matrix (Flohé & Schlegel, 1971), suggesting a duplication there of the GSH redox system of the cytoplasm. This idea is further substantiated by the finding that mitochondrial GSH can be lost by

Abbreviations: GSH, reduced glutathione; GSSG, oxidised glutathione.

oxidation *in situ* (e.g. by hydroperoxides) and then recovered when tricarboxylic acid-cycle intermediates are used as substrates (Jocelyn, 1975). To determine the capacity of and constraints on such a redox system, however, it is further necessary to find, under various conditions, the rate of reduction of substances which can specifically oxidize GSH.

The diazenes ($R'-CO-N=N-R$) were introduced as GSH oxidants (Kosower & Kosower, 1969) and one of them, diamide, $(CH_3)_2N-CO-N=N-CO-N(CH_3)_2$, has since been extensively used for this purpose in various biological systems (Biaglow & Nygaard, 1973; Pillion & Leibach, 1975; Young *et al.*, 1975; Edelhauser *et al.*, 1976; Nath & Rebhun, 1976).

Diamide also rapidly oxidizes other thiols, reduced flavins, iron-sulphur proteins and, at lower rates, NAD(P)H (Brown, 1971; Kosower *et al.*, 1972; O'Brien *et al.*, 1970). Diamide also reacts with ascorbate (see the Materials and Methods section).

The present paper shows that diamide is reduced by rat liver mitochondria and presents evidence that, despite its lack of specificity, the reduction is in fact mediated chiefly through the endogenous GSH redox system.

The effect of diamide on some mitochondrial processes has previously been studied (Siliprandi *et al.*, 1974a,b, 1975).

Materials and Methods

Materials

The buffer used was 0.125M-KCl containing 0.025M-Tris/HCl, pH7.2, 0.1mM-EDTA and 5mM-NH₄Cl.

Diamide and firefly-lantern extract were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Dichloroarsenoacetic acid was a gift from Dr. H. B. F. Dixon, Department of Biochemistry, University of Cambridge. Butylmalonate was prepared from diethyl butylmalonate (Koch-Light, Colnbrook, Bucks, U.K.) as described by Vogel (1956); α -cyano-4-hydroxycinnamic acid and benzene-1,2,3-tricarboxylic acid dihydrate were from Aldrich Chemical Co., Gillingham, Dorset, U.K. These acids and the tricarboxylic acid-cycle intermediate acids used were dispensed from concentrated aqueous solutions of the sodium salts. Other chemicals were of best quality available.

Determination of diamide reduction

Diamide can be lost in solution by reduction or more slowly by hydrolysis (Kosower & Kosower, 1969). In either case the u.v. spectrum (Fig. 1a) is lost. Absorbances were determined at 310nm (Fig. 1b), whence, from the fall after the addition of a known amount of GSH, ϵ is 2.6×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹, independent of pH. Reduction can be distinguished from hydrolysis by treatment of an acidified solution with H₂O₂ at 100°C. As shown with diamide reduced by GSH, the absorbance is then recovered to the same extent as that of unreduced diamide similarly treated (Fig. 1b). The decline in the latter from its original value is attributed to partial hydrolysis under these drastic conditions.

Determination of GSH concentration

The Ellman (1959) method was used, since previous work (Jocelyn, 1975) showed that 80–90% of acid-soluble thiol from mitochondria is GSH; 0.5ml of 5,5'-dithiobis-(2-nitrobenzoic acid) solution (1mM in 0.5M-sodium phosphate, pH8) was mixed with

Fig. 1. Absorbance of diamide in buffer before and after adding GSH

(a) shows the u.v. spectrum before (above) and after (below) adding excess GSH and (b) shows the effect on the A_{310} of adding increments of GSH to 2.5 ml of diamide solution, then subsequently acidifying with 0.5 ml of 12% HClO₄ (○). The absorbance found after keeping these acid solutions at 100°C for 20 min with H₂O₂ added (final concn. 2.5%) is also shown (□). Absorbances are corrected for dilution.

0.5 ml of acid extract and the A_{412} determined. Residual diamide contaminating the pellet would deplete GSH on neutralization. However, contrary to a literature report (O'Brien *et al.*, 1970), ascorbate reduces diamide in neutral solution and its inclusion in the extracts (see under 'Incubation and assay procedure') prevents this depletion. Under these conditions, up to 50 nmol of GSH was recovered quantitatively from 0.5 ml of acid extract containing 40 nmol of diamide.

Other assays

Protein was determined by a modified biuret method (Jacobs *et al.*, 1956). ATP was assayed with luciferase (Stanley & Williams, 1969).

Preparation of mitochondria

Mitochondria were prepared from female rats as previously described (Jocelyn & Kamminga, 1974) and dispensed from a concentrated suspension in a

mannitol medium (Jocelyn, 1975) at pH 7.0 within 2 h of preparation.

Incubation and assay procedure

The standard procedure is given and any variations are noted in the individual Figures or Tables. When the reaction was to be stopped by centrifuging, incubations were carried out in Eppendorf tubes (capacity 1.5 ml).

Samples (0.1 ml) from the concentrated mitochondrial suspension containing 37 ± 3 mg of protein/ml were added to different tubes each containing the buffer (volume specified in each Table or Figure) at 0°C and other additions as specified.

Diamide ($0.5 \mu\text{mol}$, from a stock solution 20 mM in ethanol) was added to each tube just before the mitochondrial suspension. Alternatively, when it was desired first to preincubate the buffer/mitochondrial suspension mixture, diamide ($0.5 \mu\text{mol}$) was added afterwards from a prewarmed dilution in buffer (0.3 ml). This latter addition was performed on each tube simultaneously by using a multiple-pipetting device. All incubations and preincubations were at 30°C. The reaction was stopped by sedimenting the mitochondrial pellet by centrifuging for 1.5 min in a high-speed Eppendorf centrifuge. The supernatants were poured into 12% (w/v) HClO_4 (0.35 ml) and used for the assay of diamide. The drained pellets were triturated with 2.4% HClO_4 containing 0.05 M-ascorbic acid (0.5 ml) and the acid extract was used for the assay of GSH (see above).

In some experiments, when assays of GSH were not made, the reaction was stopped by adding 12% HClO_4 (0.25 ml/ml of reaction mixture) directly to the incubated mitochondrial/buffer mixture.

Diamide reduction (see above) was found from the fall in A_{310} compared with that of a diamide/buffer solution acidified after incubation, but without added mitochondrial suspension. Mitochondrial supernatants acidified after incubation without diamide show negligible absorbance at this wavelength, but appropriate corrections were made for any absorbance caused by other additions.

Expression of results

In each experiment a control tube was always included in which a mixture of diamide, buffer and mitochondrial suspension was incubated without any other additions. The amounts of diamide reduced under the same conditions but with the specified additions to the buffer are given as percentages of the amount of diamide reduced in this control ('% of control reduction') (mean $\pm$ s.d. control reduction of diamide amounted to 80 ± 52 nmol/min for eight different batches of mitochondrial suspension).

Fig. 2. Effects of varying (a) time of incubation, (b) mitochondrial concentration and (c) initial diamide concentration on the loss of diamide after incubation with a dilute mitochondrial suspension

For details, see under 'Incubation and assay procedure' (in the Materials and Methods section). The reaction was stopped with HClO_4 . Incubations were carried out in 2 ml of buffer containing: (a) mitochondrial protein (4 mg) and diamide (0.35 mM); diamide loss was assayed directly (○) and also after treatment with hot acidic H_2O_2 (□) (see Fig. 1b); (b) diamide (1 mM); the reaction was stopped after 7 min; (c) mitochondrial protein (0.3 mg); the reaction was stopped after 5 min. By using data from other similar experiments, the mean initial rate and s.d. of diamide reduction (V_{max}) is 20 ± 13 nmol/min per mg of protein (six experiments) and K_m is $75 \pm 25 \mu\text{M}$ (three experiments).

Fig. 3. Inhibition by some thiol-blocking agents of the mitochondrial reduction of diamide (○) and their effect on the GSH concentration of the sedimented pellet after incubation in the presence (□) and absence (■) of diamide

For details, see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). The same mitochondrial suspension was used throughout. The sample of this suspension was added to 1 ml of buffer containing inhibitor (see below) at the concentration shown. After preincubation for 5 min, more buffer (0.3 ml), with or without diamide, was added and the mixture centrifuged after incubating for 5 min. Broken lines show the GSH concentration when diamide was omitted. (a) Mersalyl; (b) 2,6-dichlorophenol-indophenol; (c) *N*-ethylmaleimide; (d) *p*-hydroxymercuribenzoate.

Similarly, the amounts of GSH remaining in the sedimented pellets after incubation with various additions to the buffer are taken as percentages of the

amount found in the pellet from the control tube ('% of control'). GSH concentrations found in the pellets when diamide was omitted from the incubation

medium are also given as a percentage of this control. (Mean $\pm$ s.d. control GSH concentrations after 5 min incubation amount to 19 ± 7 nmol; the amount present in 0.1 ml of the concentrated mitochondrial suspension when assayed directly is 24 ± 8.5 nmol.)

Results

Diamide is stable in a neutral medium for several hours without significant loss (Kosower & Kosower, 1969), but it rapidly disappears on adding to it a suspension of mitochondria at 30°C (Fig. 2). Diamide thus lost is largely recovered by subsequent reoxidation with H_2O_2 (Fig. 2a), showing that the bulk of it (and probably the whole) was convertible by mitochondria into the reduced product, $(CH_3)_2N-CO-NH-NH-CO-N(CH_3)_2$, rather than subjected to enzymic hydrolysis. This is in contrast with the mitochondrial handling of another diazene, ethyl phenylazoformate ($C_6H_5-N=N-CO_2C_2H_5$), which is catabolized by hydrolysis, decarboxylation and oxidation (Jocelyn, 1976). The amount of diamide reduced in the presence of mitochondria is roughly linear with the duration of the incubation (Fig. 2a) and with the amount of mitochondria added (Fig. 2b). Reciprocal plots of data obtained by varying the initial concentration of diamide are also linear (Fig. 2c). They yield values for the initial rate of reduction and for the K_m of respectively 20 nmol/min per mg of protein and 75 μM . Reduction of diamide is inhibited almost completely by rupture of the mitochondrial membranes either with detergents (e.g. 0.1% Lubrol) or by prior sonication.

Mitochondrial GSH concentrations during the reduction were determined by assays on the sedimented pellets. After a small initial fall (see 'Nil'

values in Fig. 8) the concentration of GSH is not much affected by incubation with diamide unless inhibitors are present. Thus, if the value for mitochondrial GSH is taken as 100% after incubation with diamide for 4–6 min (to reveal correlations with the amount of diamide reduced), the mean value obtained under the same conditions but with diamide omitted is 120%. The latter value is close to that found for the uncubated mitochondria (4–8 nmol/mg of protein).

Effect of thiol-blocking reagents

To test the possible involvement of mitochondrial GSH in the reduction of diamide, various thiol reagents were added to the suspension medium and found to inhibit the reaction. Two of these reagents were effective at a concentration below 50 μM . These thiol reagents also deplete GSH in the presence of diamide, and when their concentration was varied a close correspondence was found between the percentage decrease in the GSH concentration and the percentage fall in the rate of diamide reduction (Fig. 3). *N*-Ethylmaleimide depletes GSH to the same extent whether diamide is present in the incubation medium or not. This inhibitor is known to penetrate into the matrix and to titrate GSH directly (Gaudemer & Latruffe, 1975). The other thiol agents have, at low inhibitory amounts, little effect on the concentration of GSH when diamide is omitted, and their effect when it is included is thus indirect. *p*-Hydroxymercuribenzoate, 2,6-dichlorophenol-indophenol and mersalyl all react with GSH in free solution, but the first is a known non-penetrant (Gaudemer & Latruffe, 1975) and the other two may react preferentially with membrane thiol groups (Hadley *et al.*, 1966; Famaey & Hockel, 1973).

Table 1. *Effect of some respiratory inhibitors on the mitochondrial reduction of diamide and on the GSH concentration of the corresponding pellets sedimented after incubation with or without diamide present*

For details see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). A sample of mitochondrial suspension was added to the buffer (1.3 ml) containing the inhibitor with or without diamide present and the mixtures were centrifuged after incubation for 3–4 min. Means $\pm$ s.d. are given for at least three assays, each with a different batch of mitochondrial suspension.

Inhibitor	Diamide (% of control reduction)	GSH concentration (% of control)	
		With diamide	Without diamide
KCN (200–400 μM)	82 ± 18	100 ± 4	85
Rotenone (0.6–6 μM)	96 ± 28	95 ± 8	85
Nitrogen Antimycin (0.5–5 μM)	78 ± 8	70 ± 30	90
Oxaloacetate (2.5 mM)	60 ± 18	54 ± 30	95
Malonate (2.5 mM)	42 ± 9	45 ± 10	85
Oligomycin (3–5 $\mu g/ml$)	45 ± 12	20 ± 15	105
	93 ± 15	95 ± 10	—

Effect of respiratory inhibitors

The correlation between diamide reduction rate and GSH concentration extends to the effect of res-

piratory inhibitors (Table 1). Cyanide, rotenone and oligomycin are poor inhibitors and removal of oxygen gives only moderate inhibition. The extra oxygen consumption associated with diamide reduc-

Fig. 4. Time course of (a) mitochondrial reduction of diamide and (b) the GSH concentration of the sedimented pellet during incubation with antimycin (○) or antimycin plus ATP (□)

For details see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). Samples of the mitochondrial suspension were added directly to sets of tubes containing, in 1.3 ml of buffer, identical mixtures of diamide and antimycin ($1.5 \mu\text{M}$) with or without ATP (0.5 mM). Members of each set were centrifuged after incubation for the times shown. Results are given as percentages of the corresponding values found in a control incubated for 10 min. The intercepts at zero time represent reduction occurring during the centrifugation.

Fig. 5. Effect of arsenicals on the mitochondrial reduction of diamide (○) and on the GSH concentration of the sedimented pellets after incubation in the presence (□) and absence (■) of diamide

The results were obtained as described in Fig. 3. Broken lines show the GSH concentration when diamide was omitted. (a) Sodium arsenite; (b) phenylarsenoxide; (c) sodium dichloroarsenoacetate.

tion has a mean value of only 0.06 mol of oxygen/mol of diamide reduced. Antimycin, however, gives appreciable inhibition of diamide reduction and GSH concentration. A time course (Fig. 4) shows that there is a lag before inhibition commences. This lag may be due to consumption of endogenous ATP equivalents because the effect of antimycin is relieved by ATP and the relief is oligomycin-sensitive (results not shown). Malonate and oxaloacetate, inhibitors of succinate dehydrogenase (Webb, 1966a), strongly inhibit diamide reduction and deplete mitochondrial GSH in the presence of diamide (Table 1). Arsenicals, which do not deplete GSH in free solution (Zahler & Clelland 1967) nor when added alone, in mitochondria are also good inhibitors (Fig. 5). Their effectiveness decreases in the order phenylarsenoxide, arsenite, arsenoacetate, suggesting, since this is also the order of decreasing lipophilic character, that penetration is necessary before inhibition can occur. Arsenicals probably act, at least in part, by blocking α -oxoglutarate oxidase (EC 1.2.4.2), one of the classical sites of their inhibition (Webb, 1966b).

Involvement of high-energy intermediates

Although the mitochondrial reduction of diamide is unaffected by oligomycin it is inhibited by un-

couplers (Table 2). The rate falls to about one-third of the uninhibited value and there is also a similar fall in the concentration of mitochondrial GSH. GSH is not depleted by uncouplers in the absence of diamide. Uncouplers do not inhibit simply by stimulating the oxidation of reduced coenzymes via the respiratory chain and so depleting a pool that might be required for diamide reduction. This is shown by the fact that cyanide does not appreciably relieve the inhibition of diamide reduction by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Diamide reduction is also inhibited and GSH concentration depleted by Ca^{2+} (Fig. 6). Mg^{2+} is not an inhibitor at similar concentrations. This effect of Ca^{2+} may be due to its depleting ATP equivalents required for its own transport (Brand & Lehninger, 1975). This explanation would account for the observation

Fig. 6. Inhibition by CaCl_2 of mitochondrial diamide reduction (○) and its effect on the GSH concentration of the corresponding sedimented pellet after incubation in the presence (□) and absence (■) of diamide

For details see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). A sample of mitochondrial suspension was added to 1.3 ml of buffer containing CaCl_2 at the concentration shown. The reaction was stopped after incubation for 5 min. Broken lines show the GSH concentration when diamide was omitted.

Fig. 7. Mitochondrial reduction of diamide (○) and GSH concentration of the sedimented pellet (□) after incubation at different pH values

For details, see under 'Incubation and assay procedures' and 'Expression of results' (in the Materials and Methods section). The buffers contained *N*-ethylmorpholine (0.025 M) to replace the Tris present in the usual buffer (see under 'Materials') and were brought to the stated pH by titration with HCl. A sample of the mitochondrial suspension was added to 1.3 ml of the buffer containing diamide and the mixture centrifuged after incubation for 5 min. The results are given as percentages of the control results obtained with the usual buffer.

Table 2. Action of uncouplers on the mitochondrial reduction of diamide and on the GSH concentration of the corresponding sedimented pellet

Conditions were as for Table 1, except that the incubations were for 4–5 min.

Inhibitor	Diamide (% of control reduction)	GSH concentration (% of control)	
		With diamide	Without diamide
Dinitrophenol (3–7 μM)	29 $\pm$ 12	54 $\pm$ 15	
Valinomycin (0.5 μM)	26 $\pm$ 18	28 $\pm$ 15	105 $\pm$ 11
Triethyl tin (1–10 μM)	38 $\pm$ 17	28 $\pm$ 15	85 $\pm$ 40
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone (0.3–0.6 μM)	35 $\pm$ 9	42 $\pm$ 13	95
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone + cyanide (0.2–0.6 mM)	38 $\pm$ 25	58 $\pm$ 26	

that diamide also decreases the uptake of Ca^{2+} ions by rat liver mitochondria (Siliprandi *et al.*, 1974b). Such a loss of ATP equivalents may also explain the effect of pH on diamide reduction and mitochondrial GSH concentration. When the pH is increased above 7, the former is progressively inhibited and the latter correspondingly depleted (Fig. 7). This observation contrasts with the fact that, like most reactions in-

volving thiols, the rate of diamide reduction directly by GSH increases with increasing pH (Kosower & Kanety-Londner, 1971). Diamide reduction is inhibited by phosphate at concentrations below 5 mM. A time course (Fig. 8) shows that GSH concentrations, unaffected by phosphate alone, fall proportionately. Similar results have been obtained with pyrophosphate and sulphate, but not with other anions (e.g.

Fig. 8. Influence of different phosphate concentrations on (a) the time course of the mitochondrial reduction of diamide and (b) on the GSH concentration of pellets sedimented after incubation with (○) or without (●) diamide

For details, see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). Samples of mitochondrial suspension were added to identical sets of tubes, each set containing the stated amount of potassium phosphate with or without diamide in 1.3 ml of buffer. A member of each set was centrifuged after incubation for the time shown. Zero-time values represent reaction occurring during the centrifugation. Results are given as percentages of the corresponding values of a control incubated for 10 min. The broken line shows the amount of GSH found in pellets sedimented after incubation with 2.5 mM-phosphate in the absence of diamide.

nitrate, acetate, bromide, oxalate). Assays of GSSG present in the pellets after incubation with phosphate show that most of the GSH loss (approx. 80 %) is accounted for as the disulphide. This is in contrast with the escape of GSH into the surrounding medium, which has been observed at higher phosphate concentrations (10mM) in the absence of diamide (Jocelyn, 1975). The inhibitory effect of phosphate at low concentrations (1mM) on diamide reduction and GSH concentration is considerably augmented by small amounts of ADP or ATP (i.e. 0.1–0.2mM), which have no consistent effect by themselves (Fig.

9). This inhibition by phosphate and ADP or ATP is only slightly relieved by oligomycin, but much more so by respiratory inhibitors (Table 3). These inhibitors do not affect the inhibition by phosphate alone.

Endogenous ATP concentrations have been compared in mitochondria incubated with or without diamide (Fig. 10). There is a greater decrease in ATP when diamide is present, but this difference is abolished by cyanide (which itself rapidly depletes mitochondrial ATP). However, these findings are reversed in the presence of oligomycin; in that case, ATP decreases less when diamide is present and the

Fig. 9. Effect of phosphate (1 mM) (□), ADP (0.15 mM) (○), ATP (0.15 mM) (●), ADP (0.15 mM) with phosphate (1 mM) (△) and ATP (0.15 mM) with phosphate (1 mM) (▲) on the time course of (a) mitochondrial diamide reduction and (b) the GSH concentration of the sedimented pellet

The data plotted were obtained in the same way as for Fig. 8, except that the results are given as percentages of the control values found after incubation for 8 min.

Table 3. Effect of respiratory inhibitors on mitochondrial reduction of diamide and the corresponding GSH concentration of the sedimented pellet after incubation with ADP and phosphate

For details see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). A sample of mitochondrial suspension was added to 1.3 ml of buffer containing diamide, ADP (0.15 mM) and potassium phosphate (1 mM). The mixtures were centrifuged after incubation for 4 min. Means $\pm$ s.d. are given for three assays, each with a different batch of mitochondria.

Addition	Diamide (% of control reduction)	GSH concentration (% of control)
Nil	42 $\pm$ 8	37 $\pm$ 17
Cyanide (0.3 mM)	73 $\pm$ 2	75 $\pm$ 3
Rotenone (0.5 μ M)	75 $\pm$ 8	75 $\pm$ 18
Antimycin (0.5 μ M)	73 $\pm$ 2	62 $\pm$ 10
Oligomycin (30 μ g/ml)	57 $\pm$ 2	50 $\pm$ 6

difference is not abolished by cyanide. This difference in the ATP concentration caused by diamide in the presence of oligomycin responds to the addition of other substances in the same way that these substances affect diamide reduction. Thus antimycin, thiol reagents and malonate largely abolish the difference, whereas rotenone does not. In the presence

of malonate, reducible substrates, which relieve the inhibition of diamide reduction by malonate (see Table 7), also affect this oligomycin-sensitive ATP difference caused by diamide. However, succinate and 3-hydroxybutyrate abolish it, whereas with oxoglutarate and isocitrate it is considerably increased (Table 4).

Fig. 10. *Effect of diamide on ATP concentrations during incubation with or without cyanide and oligomycin present* A sample of mitochondrial suspension (0.1 ml; approx. 4 mg of protein) was added to 2 ml of buffer in the presence (○, ●) or absence (□, ■) of diamide (0.35 mM). KCN (0.3 mM) was either included (■, ●) or not included (□, ○). (a) No oligomycin; (b) oligomycin present at 2.5 µg/ml. Incubations were at 30°C and the reaction was stopped with HClO₄ (0.2 ml; 12%). ATP concentrations (see the Methods section) are given as the total amounts found.

Table 4. *Oligomycin-dependent increments of ATP associated with diamide reduction in the presence of some inhibitors and substrates*

After incubation of mitochondria (approx. 2 mg of protein) in 1 ml of medium containing oligomycin (2.5 µg), cyanide (0.3 mM) and the stated addition (reducible substrate, 2.5 mM) for 2–4 min at 30°C, proteins were precipitated with HClO₄ (0.1 ml) and ATP was determined. Increments of ATP associated with diamide were obtained in each case by subtracting values found with the inhibitor when diamide (0.35 mM) was present from those obtained when it was not. Means ± S.D. are given for three assays.

Addition	ATP increment (nmol)	
	No cyanide	With cyanide
None	4.2 ± 2.2	2.2 ± 1.0
Antimycin (1.3 µM)	0.25 ± 0.35	
Rotenone (2.0 µM)	1.7 ± 1.4	
<i>N</i> -Ethylmaleimide (200 µM)		0.65 ± 0.25
2,6-Dichlorophenol-indophenol (50 µM)		0.8 ± 0.2
Oxaloacetate (2.5 mM)		0.3 ± 0.1
Malonate (2.5 mM)		1.0 ± 0.1
Malonate (2.5 mM) + isocitrate (2.5 mM)		7.5 ± 3.0
Malonate (2.5 mM) + oxoglutarate (2.5 mM)		4.8 ± 2.0
Malonate (2.5 mM) + succinate (2.5 mM)		-0.9 ± 0.9
Malonate (2.5 mM) + 3-hydroxybutyrate (2.5 mM)		-0.3 ± 0.3

Effect of mitochondrial carriers on diamide reduction

The substances α -cyano-4-hydroxycinnamate, *n*-butyl malonate and benzene-1,2,3-tricarboxylate are known inhibitors of the carriers for respectively monocarboxylates (Halestrap & Denton, 1974), dicarboxylates (Robinson & Chappell, 1967) and tricarboxylates (Robinson *et al.*, 1971). The reduction of diamide without reducible substrates present is moderately inhibited by each of these substances when added singly at concentrations commonly used to inhibit the carriers and more so when two of them are present together. The concentration of GSH is not appreciably affected by them singly, but is synergistically depleted by combinations, e.g. when the dicarboxylate and tricarboxylate inhibitors are both present (Table 5). Since these combinations do not affect the concentration of GSH in the absence of diamide, these results suggest that the carriers are required for both the transfer of reducing equivalents between diamide and GSH and also for the regeneration of

GSH from GSSG. Isocitrate largely relieves the inhibition of diamide reduction and the loss of GSH by the carrier inhibitors. Hydroxybutyrate is less effective and does not relieve the inhibitions by pairs of inhibitors.

Effect of reducible substrates on diamide reduction

The reduction of diamide by mitochondria and the corresponding GSH concentration of the sedimented pellet are each only slightly affected by reducible substrates (i.e. tricarboxylic acid-cycle intermediates) (Table 7) unless an inhibitor is also present, in which case they may influence the action of the inhibitor (Tables 6–8). The most notable finding is that these substrates (and also rotenone) prevent the fall in the GSH concentration of the pellet obtained with an uncoupler alone (see Table 6), but without significantly relieving the inhibition of diamide reduction.

Table 5. Action of inhibitors of the mitochondrial carriers for tricarboxylic acid-cycle intermediates on the mitochondrial reduction of diamide and the GSH concentration of the corresponding sedimented pellet with or without diamide or reducible substrate present

For details, see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). A sample of mitochondrial suspension added to 1 ml of buffer containing the inhibitors was preincubated for 2 min, then diluted diamide or buffer (0.3 ml) was added and the mixture centrifuged after reincubation for 4–5½ min. When isocitrate or 3-hydroxybutyrate was used, 1 vol. of 0.5 M-acid or 1 vol. of buffer was first added at 0°C to 9 vol. of the concentrated suspension and the dilution compensated for by taking 0.11 ml of sample within 1 h. Inhibitors were present at the following concentrations: *n*-butylmalonate (10 mM); 1,2,3-benzenetricarboxylate (25 mM); α -cyano-4-hydroxycinnamate (1 mM). When the latter inhibitor was present its high absorption at 310 nm necessitated a different method for assaying the loss of diamide: 0.1 ml of the acid extract was added to a solution of GSH (0.075 μ mol) in 0.4 ml of 0.5 M-phosphate buffer, pH 8, and the fall in GSH assayed after 10 min. Means $\pm$ s.d. are given for at least four assays (no reducible substrates present) or two assays (hydroxybutyrate or isocitrate present). Each assay utilized a different batch of mitochondrial suspension. GSH concentrations (in parentheses) are given immediately below the corresponding values for diamide reduction (not in parentheses).

Inhibitor added	Diamide omitted	Diamide present with:		
		Nil	Hydroxybutyrate	Isocitrate
None	— (112 $\pm$ 7)	100 (100)	106 $\pm$ 30 (96 $\pm$ 10)	94 $\pm$ 16 (96 $\pm$ 1)
<i>n</i> -Butylmalonate	— (110 $\pm$ 5)	65 $\pm$ 10 (96 $\pm$ 10)	81 $\pm$ 12 (99 $\pm$ 17)	93 $\pm$ 7 (94 $\pm$ 3)
α -Cyano-4-hydroxycinnamate	— (125 $\pm$ 24)	75 $\pm$ 17 (97 $\pm$ 6)	88 $\pm$ 41 (103 $\pm$ 21)	86 $\pm$ 1 (88 $\pm$ 2)
Benzene-1,2,3-tricarboxylate	— (110 $\pm$ 10)	67 $\pm$ 6 (88 $\pm$ 6)	77 $\pm$ 17 (78 $\pm$ 20)	91 $\pm$ 4 (95 $\pm$ 5)
<i>n</i> -Butylmalonate +	—	47 $\pm$ 20	62 $\pm$ 11	85 $\pm$ 21
α -Cyano-4-hydroxycinnamate	(110 $\pm$ 15)	(55 $\pm$ 12)	(—)	(90 $\pm$ 11)
<i>n</i> -Butylmalonate +	—	42 $\pm$ 14	47 $\pm$ 9	77 $\pm$ 5
1,2,3-Benzenetricarboxylate	(110 $\pm$ 5)	(45 $\pm$ 2)	(42 $\pm$ 13)	(89 $\pm$ 7)
1,2,3-Benzenetricarboxylate +	—	60 $\pm$ 20	49 $\pm$ 6	87 $\pm$ 18
α -Cyano-4-hydroxycinnamate	(100 $\pm$ 5)	(63 $\pm$ 13)	(61 $\pm$ 16)	(80 $\pm$ 2)
<i>n</i> -Butylmalonate +	—	38 $\pm$ 17	40 $\pm$ 16	77 $\pm$ 17
1,2,3-Benzenetricarboxylate +	(98 $\pm$ 3)	(42 $\pm$ 12)	(40 $\pm$ 10)	(75 $\pm$ 14)
α -Cyano-4-hydroxycinnamate				

Table 6. *Effect of reducible substrates or rotenone on the mitochondrial reduction of diamide and the corresponding GSH concentration of the sedimented pellet after incubation in the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine*

For details see under 'Incubation and assay procedures' and 'Expression of results' (in the Materials and Methods section). A sample of mitochondrial suspension was added to 1.3 ml of buffer containing diamide, the uncoupler (0.3–0.6 μ M) and reducible substrate (2.5 mM) or rotenone (0.6 μ M). The mixtures were centrifuged after incubation for 4–5 min. Means $\pm$ s.d. are given for at least three assays, each with a different batch on mitochondrial suspension.

Reducible substrate	Diamide (% of control reduction)	GSH concentration (% of control)
Nil	35 $\pm$ 9	42 $\pm$ 13
Pyruvate	20 $\pm$ 2	84 $\pm$ 3
Citrate	15 $\pm$ 1	109 $\pm$ 2
Isocitrate	29 $\pm$ 9	92 $\pm$ 10
Oxoglutarate	10 $\pm$ 2	85 $\pm$ 4
Succinate	46 $\pm$ 16	90 $\pm$ 9
Malate	23 $\pm$ 5	97 $\pm$ 3
3-Hydroxybutyrate	36 $\pm$ 16	74 $\pm$ 8
Rotenone	41 $\pm$ 15	85 $\pm$ 19

Reducible substrates also affect the lowered mitochondrial GSH concentration and inhibition of diamide reduction obtained with thiol reagents alone (see Fig. 3) and with malonate (Table 7). Inhibition of diamide reduction by 2,6-dichlorophenol-indophenol is significantly relieved only by isocitrate and succinate, which also largely prevent the fall in mitochondrial GSH concentration, but none of the reducible substrates significantly relieves the inhibition of diamide reduction by *N*-ethylmaleimide, mersalyl or *p*-hydroxymercuribenzoate. With the last-mentioned inhibitor, however, mitochondrial GSH concentrations in the presence of the substrates

are significantly higher than the corresponding values for diamide reduction. The inhibition by malonate is relieved to some extent by all the substrates, but hydroxybutyrate is the least effective. Phosphate inhibition of diamide reduction (see Fig. 8) is overcome by succinate and isocitrate, but not by pyruvate, oxoglutarate or citrate, and only slightly by malate. Involvement of the phosphate/dicarboxylate carrier (Klingenberg, 1970) is suggested by the effect of adding *n*-butylmalonate together with the substrates. The action of succinate and isocitrate is largely prevented by butylmalonate. Oxaloacetate also utilizes this carrier (Gimpel *et al.*, 1973) and its

Table 7. *Effect of reducible substrates on mitochondrial diamide reduction and the corresponding GSH concentration of the sedimented pellet in the presence of thiol-blocking reagents*

For details see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). A sample of mitochondrial suspension was added to 1 ml of buffer containing the substrate (2.5 mM) and the inhibitor (concentration shown). After preincubation for 2 min diamide was added and the mixture centrifuged after 5 min. Values expressed as percentages of the control values, are means $\pm$ s.d. of at least three assays, each using a different batch of mitochondrial suspension. GSH concentrations (in parentheses) are given immediately below the corresponding values for diamide reduction (not in parentheses).

Inhibitor	Diamide (% of control reduction) and GSH concentration (% of control) with inhibitor and:					
	Nil	Isocitrate	Oxoglutarate	Succinate	Malate	Hydroxybutyrate
None	100 (100)	114 $\pm$ 30 (108 $\pm$ 5)	92 $\pm$ 14 (109 $\pm$ 3)	117 $\pm$ 30 (112 $\pm$ 3)	91 $\pm$ 13 (106 $\pm$ 5)	99 $\pm$ 6 (109 $\pm$ 3)
2,6-Dichlorophenol-indophenol (35–50 μ M)	15 $\pm$ 8 (33 $\pm$ 16)	52 $\pm$ 25 (76 $\pm$ 6)	37 $\pm$ 21 (—)	76 $\pm$ 25 (96 $\pm$ 10)	20 $\pm$ 8 (—)	33 $\pm$ 13 (57 $\pm$ 12)
<i>N</i> -Ethylmaleimide (35–70 μ M)	25 $\pm$ 16 (42 $\pm$ 8)	30 $\pm$ 8 (39 $\pm$ 8)	35 $\pm$ 20 (57 $\pm$ 19)	34 $\pm$ 8 (45 $\pm$ 6)	24 $\pm$ 5 (42 $\pm$ 3)	27 $\pm$ 3 (38 $\pm$ 10)
Mersalyl (150–500 μ M)	28 $\pm$ 13 (40 $\pm$ 5)	33 $\pm$ 16 (48 $\pm$ 3)	32 $\pm$ 17 (54 $\pm$ 7)	29 $\pm$ 7 (—)	17 $\pm$ 15 (—)	32 $\pm$ 2 (—)
<i>p</i> -Hydroxymercuribenzoate (100–200 μ M)	13 $\pm$ 13 (29 $\pm$ 23)	22 $\pm$ 6 (52 $\pm$ 10)	15 $\pm$ 15 (48 $\pm$ 15)	15 $\pm$ 2 (51 $\pm$ 17)	8 $\pm$ 14 (45 $\pm$ 22)	28 $\pm$ 14 (41 $\pm$ 2)
Malonate (2.5 mM)	42 $\pm$ 15 (39)	102 $\pm$ 22 (108)	81 $\pm$ 8 (104)	92 $\pm$ 1 (108)	82 $\pm$ 5 (108)	52 $\pm$ 16 (72)

Table 8. *Effect of reducible substrates on the inhibition of mitochondrial diamide reduction by phosphate with or without n-butyldmalonate present*

For details see under 'Incubation and assay procedure' and 'Expression of results' (in the Materials and Methods section). A sample of mitochondrial suspension was added to 2 ml of buffer containing 2.5 mM-potassium phosphate, with or without butyldmalonate (10 mM) and the reducible substrate. The reaction was stopped with acid after incubation for 7 min. Values are means $\pm$ S.D. for three assays, each with a different batch of mitochondrial suspension.

Reducible substrate	Diamide (% of control reduction)	
	With phosphate	With phosphate and butyldmalonate
Nil	45 $\pm$ 11	40 $\pm$ 4
Pyruvate	42 $\pm$ 10	49 $\pm$ 14
Citrate	37 $\pm$ 14	37 $\pm$ 2
Isocitrate	124 $\pm$ 8	67 $\pm$ 5
α -Oxoglutarate	38 $\pm$ 4	50 $\pm$ 5
Succinate	106 $\pm$ 10	74 $\pm$ 5
Malate	61 $\pm$ 6	63 $\pm$ 4
Oxaloacetate	25 $\pm$ 17	63 $\pm$ 15

inhibitory action on diamide reduction is largely prevented by butyldmalonate (Table 8).

It is noteworthy that the inhibition by phosphate in the absence of the substrates is reversed as well as prevented when succinate is present. Thus preincubation of mitochondria (3 mg/ml of medium) with 2.5 mM-phosphate and 0.35 mM-diamide at 30°C gave a fall in the mitochondrial GSH concentration to 25% of the value found without phosphate. Addition of succinate and reincubation restored the GSH value to 75–80% and the diamide reduction rate to 85–90% of the control values. These recoveries were unaffected by storage at 0°C for up to 50 min after the preincubation but before reincubation with succinate, indicating that GSSG is stable and does not appreciably escape from mitochondria as it does from hepatocytes (Oshino & Chance, 1977).

Discussion

Diamide is reduced by mitochondria in State 4 (Chance & Williams, 1956) at rates of 20–30% of the rate of electron transfer to succinate in State 3. Reducing equivalents are ultimately provided by endogenous substrates via the tricarboxylic acid cycle, as shown by inhibiting the reaction with the classical inhibitors, malonate and arsenicals. The inhibitory potency of uncouplers (which lower rates to about 35%) is evidence that ATP equivalents are also necessary for the reaction. Their role (in part) could be to drive the formation of NADPH from NADP⁺ and NADH via energy-dependent transhydrogenase (EC 1.6.1.1) (Rydström, 1977). The involvement of this enzyme would account for the inhibition by non-penetrant thiol reagents, since the rat liver mitochondrial transhydrogenase is known to be inhibited by them (Sweetman *et al.*, 1974).

A requirement for NADPH is easily explained if

the immediate reductant for diamide is mitochondrial GSH, because the glutathione reductase of the matrix, like the cytoplasmic enzyme, specifically utilizes NADPH as reducing agent for GSSG (P. C. Jocelyn, unpublished work). GSH reduces diamide rapidly *in vitro* (see the introduction), but its involvement in the mitochondrial reduction could only be catalytic, since the GSH present is only approx. 6 nmol/mg of protein, whereas about 8 times the equivalent amount of diamide is reduced per min. However, the determined activities of transhydrogenase (Pedersen, 1976) and mitochondrial glutathione reductase (Flohé & Schlegel, 1971) seem adequate to be able to regenerate GSH from GSSG at the required rate. Direct evidence that GSH is in fact required is that various substances which inhibit diamide reduction also lower the GSH concentration by the same percentage. This is most strikingly shown for the thiol reagents, where the parameters vary in a very similar way with various concentrations of inhibitor, but the correlation is also evident in the inhibitions by antimycin (and relief by ATP), by pH, by phosphate, with or without ADP or ATP present, and by uncouplers. The involvement of ATP equivalents in diamide reduction, required by the outlined route, also accounts for other observed inhibitions. Thus Ca²⁺ ions, which (like K⁺ and valinomycin) deplete ATP equivalents by active transport, and increasing pH, which depletes them owing to the decreased Δ pH (Mitchell, 1976), both progressively inhibit diamide reduction.

The inhibitory effect of phosphate may also be due, in part, to its competing with diamide reduction for high-energy intermediates. Its mode of action is clarified by the synergistic effect of catalytic amounts of ADP or ATP, which suggests the operation of a 'futile' cycle. Phosphate utilizes endogenous ADP and high-energy intermediates in forming ATP, which then hydrolyses as a result of the appearance of

adenosine triphosphatase activity known to be induced by diamide (Siliprandi *et al.*, 1974a). Deactivation of this enzyme by respiratory inhibitors perhaps accounts for their counteracting the effect of ADP or ATP on diamide reduction with phosphate, although it is not clear why oligomycin is such a poor inhibitor. This interpretation is, however, supported by direct assays of endogenous ATP during diamide reduction, which show that steady-state concentrations are lower in the presence of diamide than without, and this difference is abolished by cyanide or antimycin.

ATP equivalents required for diamide reduction do not need to be formed via the electron-transport chain, since the reaction is little affected by cyanide or rotenone. In coupled mitochondria the oxidation of NADH accompanying diamide reduction would allow flux through succinic thiokinase (EC 6.2.1.4), the substrate-phosphorylation site of the tricarboxylic acid cycle (Lowenstein, 1971a). Some of the diamide-dependent ATP formed in this way via GTP and nucleoside diphosphate kinase (EC 2.7.4.6) would be expected to be trapped by oligomycin and its concentration decreased by inhibitors of diamide reduction. These predictions have been confirmed and are supported by the increases found with oxoglutarate and isocitrate (Table 4), but, since diamide reduction is oligomycin-insensitive, it is uncertain at present whether this can be the primary high-energy source for the reaction.

Carrier dependence for diamide transport

The saturation kinetics obtained with various initial diamide concentrations (Fig. 2c), the high steady-state concentration of mitochondrial GSH throughout uninhibited diamide reduction (Fig. 8) (which contrasts with the effect of diamide on the GSH of erythrocytes; Kosower *et al.*, 1969) and the fact that the rate of reduction is unaffected by adding reducible substrates to uninhibited mitochondria (Table 7) when taken together suggest that a carrier mechanism is required for the transport of diamide into the matrix. Specific inhibition of this transport would prevent diamide reduction, but not affect the mitochondrial GSH concentration, which would then be protected from the oxidant. This situation has been observed most strikingly with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in the presence of added reducible substrates. Without the latter, GSH values, despite the smaller oxidative stress, are low, because regeneration of GSH from GSSG utilizing energy-dependent transhydrogenation is inhibited even more than diamide transport. The effect of the substrates (and of rotenone) is to increase the pool of reduced coenzymes and thus to shift the equilibrium to favour GSH. This evidence that diamide transport is inhibited by uncouplers may indi-

cate that it is proton-driven. Azo groups are weakly basic (Jolles, 1956) and a possible explanation is that the species $(\text{CH}_3)_2\text{N}-\text{CO}-\text{NH}^+=\text{N}-\text{CO}(\text{CH}_3)_2$ is preferred for transport by coupled mitochondria. Known inhibitors of the carriers for tricarboxylic acid-cycle intermediates also inhibit diamide reduction while maintaining a normal concentration of GSH, suggesting that each of them plays a part in the transport of diamide. Involvement of the phosphate/dicarboxylate carrier is further suggested by the inhibition of diamide reduction by phosphate and the effects of tricarboxylic acid-cycle intermediates and butylmalonate on it (Lowenstein, 1971b).

These carriers probably all possess thiol groups, since they are sensitive to mercurials (Papa & Paradies, 1974; Palmieri *et al.*, 1974; Passarella & Quagliariello, 1976), and those of the phosphate/dicarboxylate carrier are also protected by phosphate (Klingenberg *et al.*, 1974). Diamide can form adducts with protein thiol groups (Harris & Biaglow, 1972) and hence might be carried into the matrix in this form. Such adducts are cleaved by thiols (Kosower & Kanety-Londner, 1971) so that on the matrix side the carrier would be regenerated and diamide reduced in two stages by cleavage with GSH. Carriers do have an affinity for glutathione, as has been demonstrated in a different connection (Kun *et al.*, 1977). A proposed mechanism is shown in Scheme 1. By this mechanism the overall rate is determined by the rate of transition from stage (c) to stage (d) and thus to the concentration of the two substrates for stage (c). This is because stages (d)–(e) would be extremely rapid since a rate constant for the reduction of diamide by GSH of $300\text{ M}^{-1}\cdot\text{s}^{-1}$ has been found at pH 7.3 (Kosower *et al.*, 1972). During the reaction the steady-state concentrations of these two substances would remain constant because of rapid replenishment of the carrier–diamide complex by stages (a)–(b) and of GSH by stages (e)–(g). The overall rate would thus fall if either of these steady-state concentrations were depleted. The concentration of inward-facing diamide–carrier complex would be depleted by direct titration of carriers with carrier inhibitors or by inhibition of stage (a). The GSH concentration would be depleted by direct titration (e.g. with *N*-ethylmaleimide) or by inhibition of stages (e)–(h), and in this case the overall rate would then be decreased to the same extent as the GSH concentration was lowered, as found for various inhibitors.

If membrane thiol groups are involved in diamide entry, their blockage with thiol reagents in the presence of reducible substrates should inhibit diamide reduction while maintaining the GSH concentration. However, thiol reagents affect various other mitochondrial processes (Gautheron, 1973), and this test applied to various thiol reagents has so far revealed (with *p*-hydroxymercuribenzoate)

Scheme 1. Possible mechanism for the reduction of diamide

Abbreviations: ~, high-energy expenditure (not stoichiometric); D, diamide; CSH, thiol-dependent carrier; TH, transhydrogenase; GR, glutathione reductase. Inhibitors may prevent reactions at the various stages as follows: (a)–(b), uncouplers, carrier inhibitors, phosphate, *p*-hydroxymercuribenzoate; (c)–(e), *N*-ethylmaleimide; (f) uncouplers, thiol-blocking reagents; (g) thiol-blocking reagents?; (h) malonate, arsenicals.

only moderately selective inhibition of diamide entry.

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