Inhibition of Mitochondrial Phosphate Transport by *p*-Diazobenzenesulphonate

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The impermeant protein-modifying reagent *p*-diazobenzenesulphonate inhibits phosphate transport in rat liver mitochondria. The rate of inhibition is increased by the presence of oxidizable substrate, and decreased by uncoupling agents. These results are compared with the results on inhibition of phosphate transport by *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide (Klingenberg *et al.*, 1974).

p-Diazobenzenesulphonate is a protein-modifying reagent to which most membrane systems are impermeable. In the case of erythrocytes (Berg, 1969; Bender *et al.*, 1971; Carraway *et al.*, 1971) it appears to react only with proteins located on the exterior of the membranes of intact cells, because its specificity is much broader when applied to membrane fragments. *p*-Diazobenzenesulphonate has also been used by Dilley *et al.* (1972) to study the asymmetry of chloroplast membranes and by Schneider *et al.* (1972) and Tinberg *et al.* (1974) to study that of mitochondrial membranes.

It is known that *p*-diazobenzenesulphonate reacts with a wide variety of functional groups in proteins (Howard & Wild, 1957), including histidine, tyrosine, amino and thiol residues. The reactivity of *p*-diazobenzenesulphonate with thiol groups suggested that it might inhibit phosphate transport in rat liver mitochondria, which is known to be sensitive to a variety of thiol reagents (Tyler, 1969; Fonyo, 1968). This seems to be the case. At low concentrations (10 nmol/mg of protein) it inhibits the passive swelling of mitochondria suspended in iso-osmotic ammonium phosphate.

Klingenberg *et al.* (1974) have found that, for the impermeant maleimide derivative *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide, the sensitivity of the phosphate carrier to inhibition alters depending on the preincubation conditions, being influenced by, for example, respiratory substrates, uncouplers, ionophores and respiratory inhibitors. Their results can be rationalized in terms of the reorientation of the phosphate carrier between the inside and the outside of the membrane, rendering it more or less accessible to attack by *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide. On a simple reorientation model the variation in reactivity of the carrier should be independent of the nature of the inhibitor used as long as the inhibitor is impermeant. The present paper reports the variations in the inhibition of phosphate transport by *p*-diazobenzenesulphonate under various metabolic conditions.

Materials and methods

Materials. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and valinomycin were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Rotenone, antimycin A and dithiothreitol were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] was from Hopkin and Williams Ltd., Romford, Essex, U.K.

Preparation of mitochondria. Rat liver mitochondria were prepared as described by Selwyn *et al.* (1970). Protein concentration was measured by the biuret method (Gornall *et al.*, 1949), after clarification with Triton X-100.

Preparation of *p*-diazobenzenesulphonate. This was carried out as described by Dilley *et al.* (1972). The resulting solution of *p*-diazobenzenesulphonate was assayed by colorimetric titration against a standard solution of 2-naphthol.

Light-scattering measurement. For measurement of passive swelling in ammonium phosphate (Chappell & Crofts, 1966), 1–5 mg of mitochondrial protein (10–50 μ l of suspension) was added to 0.6 ml of a preincubation medium containing 200 mM-sucrose, 20 mM-sucrose, 20 mM-KCl, 0.7 mM-EDTA and 5 mM-Hepes-KOH, pH 7.6, contained in a rapidly stirred vessel maintained at 20°C. Other additions were present as indicated. After 30 s *p*-diazobenzenesulphonate was added and the reaction stopped after the required time by the addition of 20 μ l of 20 mM-dithiothreitol. Samples (0.2 ml) of the preincubation mixture, containing mitochondria, were then added to 2.5 ml of 83 mM-ammonium phosphate, pH 7.6, in a stirred cell in the light-scattering apparatus described by Selwyn *et al.* (1970). The output from the apparatus was connected to a Vitatron log recorder, by using a scale expansion of $E = 0.01 \text{ cm}^{-1}$, with the chart running at $10 \text{ cm} \cdot \text{min}^{-1}$. Since the traces were non-linear, the rate of swelling was taken as ΔE between 3 s and 12 s (Klingenberg *et al.*, 1974).

Results and discussion

Osmotic swelling in ammonium phosphate (Chappell & Crofts, 1966) is in many ways the most satisfactory method of measuring phosphate entry, since a high activity of the phosphate carrier is obtained. Fig. 1(a) shows the dependence of the inhibition in this system on *p*-diazobenzenesulphonate concentration. Complete inhibition is obtained at approx. 10 nmol of *p*-diazobenzenesulphonate/mg of protein, a similar value to that found for organomercurials (Tyler, 1969). This value is independent of the absolute protein concentration used. By using dithiothreitol to quench the reaction, it is possible (Fig. 1b) to follow the kinetics of the inhibition of phosphate transport by *p*-diazobenzenesulphonate, although the rate of reaction is rather high and the range of *p*-diazobenzenesulphonate and protein concentrations that can be used is consequently rather restricted. Fig. 1(b) shows that the inclusion of succinate in the preincubation mixture increases the rate of inhibition, whereas carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in the preincubation decreases it considerably. In the absence of rotenone, β -hydroxybutyrate has been found to have a similar effect to that of succinate, the stimulation in this case being abolished by rotenone. These results are the precise opposite to those found by Klingenberg *et al.* (1974) for the impermeant inhibitor *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide. In that case, the presence of oxidizable substrate depressed the rate of inhibition, whereas carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone potentiated it.

Fig. 2 shows the effect of various compounds on

the inhibition of phosphate entry by *p*-diazobenzenesulphonate. As with the maleimide derivatives (Klingenberg *et al.*, 1974), phosphate protects against inhibition by *p*-diazobenzenesulphonate. The electron-transfer inhibitors rotenone and antimycin A both decrease the inhibition, antimycin to a greater extent than rotenone, although neither are as effective as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The effect of rotenone and antimycin is again opposite to that found for *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide (Klingenberg *et al.*, 1974). Both nigericin and valinomycin have very little effect on the rate of inhibition. For *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide, valinomycin potentiated the inhibition considerably (Klingenberg *et al.*, 1974).

It has been found that, when *p*-diazobenzene-sulphonate reacts to completion with mitochondria under conditions similar to those of Fig. 1(a), 1 mol of 5,5'-dithiobis-(2-nitrobenzoic acid)-reactive material disappears per mol of *p*-diazobenzenesulphonate. This suggests that, despite the wide chemical reactivity of diazonium salts, thiol groups react preferentially under these conditions. If *p*-diazobenzene-sulphonate inhibits phosphate transport by reaction with the same group or groups as *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide, it would be expected that changes in reactivity due to reorientation of the carrier between the inner and outer faces of the mitochondrial membrane would be similar for both inhibitors. This would also be true if the inhibitors reacted with different groups on the carrier, if the carrier molecule underwent complete translocation from one face to the other. However, on a model where the

Fig. 1. Inhibition of passive swelling of mitochondria in ammonium phosphate by *p*-diazobenzenesulphonate

(a) Effect of *p*-diazobenzenesulphonate concentration. The preincubation mixture contained, in 0.6 ml, 20 mM-KCl, 200 mM-sucrose, 1 mM-EDTA, 1 μg of rotenone, 5 mM-Hepes-KOH, pH 7.6, and 4.5 mg of mitochondrial protein. Incubation with *p*-diazobenzenesulphonate was for 2 min. Other details were as described in the text. (b) Time-dependence of the inhibition. The preincubation mixture was as for (a) except that it contained 0.75 mg of mitochondrial protein. Reaction with inhibitor was started by the addition of 11 nmol of *p*-diazobenzenesulphonate and stopped after the required time with 20 μl of 20 mM-dithiothreitol. ○, No addition; ●, 0.5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone added 15 s before *p*-diazobenzenesulphonate; Δ, 10 mM-succinate added 15 s before *p*-diazobenzenesulphonate.

Fig. 2. Effects of phosphate and some ionophores and inhibitors on the inhibition of swelling of mitochondria in ammonium phosphate by *p*-diazobenzenesulphonate

The preincubation medium was as for Fig. 1(a), with the omission of rotenone. It contained 5 mg of mitochondrial protein. Inhibition was started by addition of 50 nmol of *p*-diazobenzenesulphonate, and the reaction time was 10 s. The following compounds were added 15 s before *p*-diazobenzenesulphonate: (a) no addition; (b) 0.1 μ g of nigericin; (c) 0.1 μ g of valinomycin; (d) 1 μ g of rotenone; (e) 1 μ g of antimycin A; (f) 10 mM-KH₂PO₄-KOH, pH 7.6; (g) 0.5 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; (h) control, dithiothreitol added before *p*-diazobenzenesulphonate.

carrier molecule spanned the membrane and accomplished translocation by rotation about an axis in the plane of the membrane this would not necessarily be the case. Inhibitors that reacted preferentially with opposite sides of the carrier would show opposite changes in reactivity as the carrier reorientated. Until it is established whether or not *p*-diazobenzenesulphonate and *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide react at the same site, this therefore remains a possibility.

It is well established that mitochondrial thiol groups show changes in reactivity depending on the

metabolic state of the mitochondria (e.g. Gautheron, 1973). Apart from any possible effects due to reorientation of carriers, this might reflect, for instance, conformational changes or changes in charge distribution on the outer surface of the inner membrane. Since *p*-diazobenzenesulphonate and *N*-(*N*-acetyl-4-sulphamoylphenyl)maleimide are chemically dissimilar, it is entirely possible that changes of this type in the environment of the inhibitor-binding site might differentially alter its reactivity towards the two reagents. Also, under the conditions used here for *p*-diazobenzenesulphonate, the reaction is second-order. The inhibitor is present at a comparable concentration to that of the reactive thiol groups. There will therefore be competition for the inhibitor between groups on the phosphate-transport system and other reactive groups on the membrane. Changes in the rate of inhibition of phosphate transport could arise as a result of changes in reactivity of groups not directly involved in phosphate transport. It is thus possible that changes in reactivity of impermeant inhibitors might be due to other factors, besides carrier reorientation.

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