8-Anilidonaphthalene-1-sulphonate Interaction with Whole and Disrupted Mitochondria: A Re-evaluation of the Use of Double-Reciprocal Plots in the Derivation of Binding Parameters for Fluorescent Probes Binding to Mitochondrial Membranes

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It is shown that 8-anilidonaphthalene-1-sulphonate is a permeant anion of whole mitochondrial membranes. It is also shown experimentally and algebraically that plots of reciprocal fluorescence against reciprocal membrane concentration, at a fixed 8-anilidonaphthalene-1-sulphonate concentration, are straight lines even when more than one binding site is involved.

Whether or not 8-anilidonaphthalene-1-sulphonate is a permeant anion of mitochondria and submitochondrial particles is a crucial factor in the interpretation of the changes in fluorescence that are observed on energization. The interpretation of Skulachev (1970) requires that it is. The interpretation of Azzi (1969), Azzone (1972) and Azzone & Massari (1973) requires that it is not. The hypothesis of Radda and his co-workers (Barrett-Bee & Radda, 1972; Barrett-Bee *et al.*, 1972; Radda & Vanderkooi, 1972), involving four membrane states, energized or unenergized and aplectic or symplectic, seems to be independent of the permeability properties of 8-anilidonaphthalene-1-sulphonate. Evidence has been published which indicates that it is a permeant anion of mitochondrial membranes (Jasaitis *et al.*, 1973), submitochondrial particles (Layton *et al.*, 1974) and erythrocyte membranes (Radda & Smith, 1973; Fortes & Hoffman, 1974). The following results, obtained by using a different technique, support this evidence that it is a permeant anion of mitochondrial membranes.

Materials and methods

Mitochondria were prepared in a 250mM-sucrose medium buffered with 5mM-Hepes* (potassium salt) at pH7.55. Liver mitochondria were isolated from decapitated albino Wistar rats (150–200g). The isolation essentially followed that of Selwyn *et al.* (1970). Frozen-thawed mitochondria were prepared by freezing at -10°C and thawing at $+10^{\circ}\text{C}$; the cycle was repeated twice. Membranes were separated from matrix proteins by centrifugation at 40000g for 15 min. The membranes were washed by recentrifugation in a small volume of the isolation medium. The supernatant from this was added to the matrix protein fraction and was centrifuged at 100000g for 60 min to remove membrane fragments. This procedure was not intended to remove all the matrix pro-

* Abbreviation: Hepes 2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonic acid.

teins from the membrane fraction but to prepare matrix protein without membrane contamination and to minimize loss of either membranes or matrix protein. The membranes were made up to the same volume as the frozen-thawed mitochondria from which they were prepared. The matrix protein was made up to four times this volume. This allowed samples of the matrix protein and/or membrane fractions to be added to the assay medium in the same proportion as in the original mitochondria. Osmotically shocked mitochondria were prepared by diluting whole mitochondria in 5mM-Hepes buffer at pH7.55, then adding 2.5M-sucrose–5mM-Hepes buffer at pH7.55 to bring the sucrose concentration back to 250mM. Light-scattering studies (N. Gains, unpublished work) showed that after this treatment the mitochondria were not osmotically active. At the maximum concentration of mitochondria used, the osmotic concentration, before the sucrose concentration was brought to 250mM, was 12.5mosm. Whole mitochondria, but none of the other preparations, showed a decrease in 8-anilidonaphthalene-1-sulphonate fluorescence on energization with 1mM-succinate.

Fluorescence was measured in a front-faced fluorimeter constructed in this laboratory. The contents of the cuvette were continuously stirred and were maintained at 30°C . The light-source was a Bausch and Lomb 40 W quartz-iodide lamp. An image of the lamp element was focused through a Wratten 18A filter on to the cuvette so that it occupied the whole of the front face. The maximum intensity of the exciting light was at 380nm. The emitted light was analysed by a Bausch and Lomb monochromator (no. 33-86-02) at 480nm with a band width of 10nm.

8-Anilidonaphthalene-1-sulphonate (magnesium salt) was from Eastman Kodak Co., Rochester, N.Y., U.S.A. Hepes was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Rotenone was from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other reagents were of AnalaR grade.

Results and discussion

Fig. 1 is a plot of reciprocal fluorescence against reciprocal mitochondrial concentration. The line is drawn through three sets of data, for 8-anilino-naphthalene-1-sulphonate binding to whole, frozen-thawed and osmotically shocked mitochondria. This shows that physically damaging mitochondria does not result in an increase in the number of 8-anilino-naphthalene-1-sulphonate-binding sites. If this compound is a permeant anion of mitochondrial membranes (Layton *et al.*, 1974) this observation requires no further assumptions. If it is not, then it must be assumed either that there are no binding sites for it on the matrix proteins and on the inner surface of the inner membrane, or that if there are then when it is bound to them it does not fluoresce. This seems unlikely as it binds, with an increase in fluorescence, to submitochondrial particles (Azzi *et al.*, 1969), which are usually assumed to be inside-out with respect to mitochondria. However, if it can be shown that it binds to mitochondrial matrix proteins with an increase in fluorescence then it must permeate mitochondrial membranes.

The lower line in Fig. 2, which is a plot of fluorescence against fluorescence $\times [8\text{-anilino-naphthalene-1-sulphonate}]^{-1}$, shows that 8-anilino-naphthalene-1-sulphonate binds, with a fluorescence enhancement, to mitochondrial matrix proteins. This result could be an artifact caused by contamination of the supernatant by membrane fragments, but this is unlikely as the shape of the curve for 8-anilino-naphthalene-1-sulphonate binding to the matrix proteins differs markedly from those of its binding to the membrane fraction (middle curve) or to whole mitochondria (upper curve).

The conclusion drawn from these experimental results, that 8-anilino-naphthalene-1-sulphonate is a

Fig. 1. Double-reciprocal plot of 8-anilino-naphthalene-1-sulphonate fluorescence when bound to whole, osmotically shocked and frozen-thawed mitochondria

The assay medium contained 250mM-sucrose, 5 μ g of rotenone/ml, 5mM-Hepes buffer, pH7.55, and 2 μ M-8-anilino-naphthalene-1-sulphonate. Δ , Whole mitochondria; $\blacktriangle$, osmotically shocked mitochondria; $\bullet$, frozen-thawed mitochondria.

permeant anion of mitochondrial membranes, is supported by the work of Layton *et al.* (1974). These authors report that sonication does not increase the fluorescence of an 8-anilino-naphthalene-1-sulphonate-submitochondrial particle suspension.

In the above discussion it has been assumed that if 8-anilino-naphthalene-1-sulphonate permeates mitochondrial membranes it does so as an anion. There is no experimental basis for this assumption for mitochondria, but as the pK of the sulphonate group is likely to be about 1 and that of the amino group 3–5 (March, 1968) permeation in an uncharged or neutral form would be insignificant.

If energization creates a transmembrane potential which is negative inside mitochondria (Mitchell & Moyle, 1965a), then 8-anilino-naphthalene-1-sulphonate bound on the inside will tend to be extruded. Similarly, if energization of submitochondrial particles creates a transmembrane potential positive inside (Mitchell & Moyle, 1965b), then there seems no reason why the sulphonate would not be actively accumulated. This has been proposed by Jasaitis *et al.* (1971), and opposed by Radda & Vanderkooi (1972). This opposition is based on the assumption that the amount of 8-anilino-naphthalene-1-sulphonate accumulated inside the submitochondrial particles would measurably deplete the external concentration. Azzone & Massari (1973) agree with this

Fig. 2. Titration of whole mitochondria, washed mitochondrial membranes and matrix protein with 8-anilino-naphthalene-1-sulphonate

The assay medium contained 250mM-sucrose, 2 μ g of rotenone/ml, 5mM-Hepes buffer at pH7.55 and: $\square$, whole mitochondria, 0.424mg/ml; $\blacksquare$, washed membranes, 0.340mg/ml, plus matrix protein, 0.074mg/ml; $\circ$, washed membranes, 0.340mg/ml; $\triangle$, matrix protein, 0.074mg/ml. Concentrations of matrix and membrane protein fractions correspond to their respective concentrations in the whole mitochondria.

assumption. However, the total internal water space of a suspension of submitochondrial particles at 1 mg/ml may be only one-thousandth of that outside, in which case a fiftyfold accumulation of the sulphonate inside the submitochondrial particles might not be detected if there was a combined error of 5% in measuring the free and the bound sulphonate.

The curved lines in Fig. 2 show that there are at least two 8-anilino-naphthalene-1-sulphonate-binding sites in mitochondria and mitochondrial membranes. Fluorescence lifetime studies (Brocklehurst *et al.*, 1970) show that there are probably two of these binding sites in submitochondrial particles. The line in the double-reciprocal plot (Fig. 1) for 8-anilino-naphthalene-1-sulphonate binding to whole and disrupted mitochondria is, as far as can be judged, straight. Radda (1971, p. 114) has used the straightness of the plot of reciprocal fluorescence against reciprocal submitochondrial particle concentration as a criterion for the correctness of his assumption that all the bound sulphonate molecules have the same fluorescence enhancement. Other authors (Harris, 1971; Layton *et al.*, 1974) have used the curvature of this plot as a criterion of the presence of more than one 8-anilino-naphthalene-1-sulphonate-binding sites. The use of these criteria is not necessarily valid either in practice, as is shown in Fig. 1, or in theory, as is shown from the derivation of suitable equations.

A plot of reciprocal fluorescence against reciprocal mitochondrial or submitochondrial particle concentration at a fixed 8-anilino-naphthalene-1-sulphonate concentration is analogous to a plot of reciprocal rate against reciprocal substrate concentration where one enzyme is acting on two substrates (a and b) in a fixed proportion. Dixon & Webb (1964, p. 92) give the following equation for this, as:

$$v_0 = \frac{V_a \frac{[a]}{K_m^a} + V_b \frac{[b]}{K_m^b}}{1 + \frac{[a]}{K_m^a} + \frac{[b]}{K_m^b}}$$

In terms of fluorescent-probe binding to two sites in a membrane this equation may be rewritten as follows, assuming that the probe concentration is low compared with the concentration of its binding sites:

$$f = \frac{\frac{pa[M][P]}{K_a} + \frac{qb[M][P]}{K_b}}{1 + \frac{a[M]}{K_a} + \frac{b[M]}{K_b}}$$

or in the reciprocal form:

$$\frac{1}{f} = \frac{K_a K_b}{[M][P](pK_b a + qK_a b)} + \frac{bK_a + aK_b}{[P](pK_b a + qK_a b)}$$

where f is fluorescence, $[M]$ is the membrane concentration, a is the number of sites of site A per unit mass

of membrane, b is the number of sites of site B per unit mass of membrane, $[P]$ is the concentration of the probe used, p is the fluorescence of probe bound to site A, q is the fluorescence of probe bound to site B, K_a is the dissociation constant of site A, K_b is the dissociation constant of site B.

The last equation shows that a plot of reciprocal fluorescence against reciprocal mitochondrial or submitochondrial particle concentration will be linear for two binding sites. From the derivation of this equation and a similar one for three sites it can be seen that the linearity is independent of the number of sites or of the quantum yield of the bound 8-anilino-naphthalene-1-sulphonate. If the existence in mitochondria or submitochondrial particles of two or more sites, with differing quantum yields for bound sulphonate, cannot be ruled out, then the changes in maximum fluorescence value observed on energization (Brocklehurst *et al.*, 1970; Barrett-Bee & Radda, 1972) cannot with certainty be attributed to changes in membrane conformation. These changes in the derived maximum fluorescence value and in the binding may equally well be explained by a change in a transmembrane potential causing a redistribution of 8-anilino-naphthalene-1-sulphonate between the sites.

Neither the experiment described above nor the re-interpretation of the double-reciprocal-plot data disproves the hypothesis of Radda and his co-workers (Barrett-Bee & Radda, 1972; Barrett-Bee *et al.*, 1972; Radda & Vanderkooi, 1972) of energy-induced changes in membrane conformation. But the possibility of 8-anilino-naphthalene-1-sulphonate redistribution on energization, from one category of sites to another, provides an alternative which is compatible with the observations of Radda and his co-workers on the changes in quantum yield, binding, lifetimes, polarization, mean 8-anilino-naphthalene-1-sulphonate-to-tryptophan distances (Brocklehurst *et al.*, 1970) and relative $^2\text{H}_2\text{O}$ -to- H_2O quenching (Ballard *et al.*, 1972) which occur on energization to 8-anilino-naphthalene-1-sulphonate bound to submitochondrial particles.

The permeability of mitochondrial membranes to 8-anilino-naphthalene-1-sulphonate also means that those hypotheses which assume that it is not true are untenable.

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- Azzi, A. (1969) *Biochem. Biophys. Res. Commun.* **37**, 254–260
 Azzi, A., Chance, B., Radda, G. K. & Lee, C. P. (1969) *Biochemistry* **62**, 612–619
 Azzone, G. F. (1972) *J. Bioenerg.* **3**, 95–103
 Azzone, G. F. & Massari, S. (1973) *Biochim. Biophys. Acta* **301**, 195–226

- Ballard, S. G., Barker, R. W., Barrett-Bee, K. J., Dwek, R. A., Radda, G. K., Smith, S. G. & Taylor, J. A. (1972) in *Proceedings of a Symposium on the Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E. & Siliprandi, N., eds.), pp. 257–275, Academic Press, London and New York
- Barrett-Bee, K. & Radda, G. K. (1972) *Biochim. Biophys. Acta* **267**, 211–215
- Barrett-Bee, K., Radda, G. K. & Thomas, N. A. (1972) *Proc. FEBS Meet. 8th* 231–252
- Brocklehurst, J. R., Freedman, R. B., Hancock, D. J. & Radda, G. K. (1970) *Biochem. J.* **116**, 721–731
- Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd edn., Longmans London,
- Fortes, P. A. G. & Hoffman, J. F. (1974) *J. Membrane Biol.* **16**, 79–100
- Harris, R. A. (1971) *Arch. Biochem. Biophys.* **267**, 436–445
- Jasaitis, A. A., Kuliene, V. V. & Skulachev, V. P. (1971) *Biochim. Biophys. Acta* **234**, 177–181
- Jasaitis, A. A., La Van Chu & Skulachev, V. P. (1973) *FEBS Lett.* **31**, 241–245
- Layton, D. G., Symmons, P. & Williams, W. P. (1974) *FEBS Lett.* **41**, 1–7
- March, J. (1968) *Advanced Organic Chemistry*, p. 250, McGraw-Hill, New York
- Mitchell, P. & Moyle, J. (1965a) *Nature (London)* **208**, 147–151
- Mitchell, P. & Moyle, J. (1965b) *Nature (London)* **208**, 1205–1206
- Radda, G. K. (1971) *Curr. Top. Bioenerg.* **4**, 81–126
- Radda, G. K. & Smith, D. S. (1973) *Biochim. Biophys. Acta* **318**, 197–204
- Radda, G. K. & Vanderkooi, J. (1972) *Biochim. Biophys. Acta* **265**, 509–549
- Selwyn, M. J., Dawson, A. P., Stockdale, M. & Gains, N. (1970) *Eur. J. Biochem.* **14**, 120–126
- Skulachev, V. P. (1970) *FEBS Lett.* **11**, 301–308