Uptake of safranine by cardiac mitochondria

Competition with calcium ions and dependence on anions

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Some characteristics of the energized uptake of safranine by rat heart mitochondria were studied. When monitored by changes in differential absorbance (between 524 and 554 nm) of a whole suspension in safranine-containing medium, the changes seen are not linearly related to the quantity of the safranine moving. It happens coincidentally that the changes observed are nearly linearly related to the logarithm of the ratio between the accumulated safranine and its residual concentration in the medium; this explains why the changes of absorbance have been found by other authors to be linearly related to the logarithms of the ratio of internal/external concentrations of such other cations as are permeable. The uptake process appears to compete for energy with Ca^{2+} uptake and vice versa. Energized safranine uptake has an anion requirement, which is seen when movement of endogenous P_i has been inhibited; the small residual safranine uptake obtained when energy is provided in the presence of mersalyl may be attributable to internal P_i . However, a limited anion-independent energized uptake of safranine, in exchange for internal K^+ , may be elicited in the presence of nigericin. Adding ATP to the energized system in the presence of an inhibitor of P_i movement elicits an additional uptake of safranine that is oligomycin-sensitive and that probably arises on account of generation of internal P_i by hydrolysis of the entering ATP.

The uptake of the substituted quaternary ammonium base safranine by mitochondria is energized in a manner similar to the process of Ca^{2+} uptake. It follows then that competition for the energy is likely to occur. However, Ruthenium Red, which inhibits Ca^{2+} uptake (Moore, 1971), does not directly affect the movement of the dye (see, e.g., Åkerman, 1978); therefore the two cations do not compete at the level of the Ca^{2+} carrier. The total safranine accumulation has been related to the number of fixed negative charges at the membrane (Zanotti & Azzone, 1980); these would be the likely sites at which competition between the cations takes place. Colonna *et al.* (1973) observed that the competitive effect of Ca^{2+} accumulation on that of the dye was diminished in the presence of excess of acetate, which suggested that the Ca^{2+} has been removed from the membrane sites into the interior of the mitochondrion. Of the two cations only Ca^{2+} appears to move in this way, since accumulation of the dye does not cause swelling, showing that the dye is not in an osmotically active state (Colonna *et*

al. 1973). Detailed studies of the stacking of the dye confirmed this conclusion (Zanotti & Azzone, 1980).

Recently, considerable application has been made of the safranine uptake to measure what is referred to as the 'membrane potential' of mitochondria in various conditions (Åkerman & Wikström, 1976; Åkerman, 1978; Kauffman & Lardy, 1980). It is not clear why the degree of attachment of the dye to sites on the membrane should measure a potential difference across the membrane, and for this reason we have measured the distribution of the dye between the medium and the mitochondria as well as the changes of absorbance of the suspension. It is the distribution of K^+ or Rb^+ in presence of valinomycin that is commonly used to deduce the 'membrane potential', so it is pertinent to enquire how changes of dye absorbance in the suspension relate to its distribution between medium and mitochondria. Kinetic measurements can confirm the competition between dye and Ca^{2+} for energy during uptake, but the extent of energy-linked binding of safranine to

membrane sites (as studied by Zanotti & Azzone, 1980) can be shown to depend also on competition with Ca^{2+} and the presence of permeant anions.

Methods

Rat heart mitochondria were prepared as described previously (Harris *et al.*, 1979). The final wash was in medium free from chelating agent other than 0.1% serum albumin, and the final suspension was in mannitol (300 mM) with 10 mM-Tris/HCl buffer, pH 7.2. The movements of safranin were followed by observing the absorbance changes of suspensions in a dual-wavelength spectrophotometer (Aminco DW2) between the wavelengths 524 and 554 nm; the records were taken with absorbance decrease reading upwards, because as the dye moves into the particles the suspensions become less absorbent. The safranin was used at initial concentrations of 10–20 μM in the suspensions in a medium containing 120 mM-KCl, 20 mM-Tris/HCl buffer, pH 7.2, and 3% poly(ethylene glycol) (average mol.wt. 6000), with other additions specified. Ca^{2+} movements were followed as described previously (Harris, 1977, 1979a), with Arsenazo III as indicator and with the wavelength pair 685 and 665 nm.

Results

Changes of absorbance and their relation to dye uptake

In typical experiments the dye, dissolved in ethanol, was added to the basic medium to give an initial differential absorbance of approx. 0.5, and mitochondria were added at a concentration of 1 mg of protein/ml. Fig. 1(a) shows that with no added substrate the oxidation of endogenous material and use of internal ATP leads to a temporary dye uptake followed by spontaneous release. Addition of the uncoupler trifluoromethyltetrachlorobenzimidazole (1.5 μM) then slightly lessens the dye remaining in the particles, whereas addition of ADP (50 μM) causes a slight re-uptake (possibly reflecting myokinase activity). If, after the dye has moved first in, then out, a substrate (β -hydroxybutyrate, 3 mM) is added, there is a limited re-uptake. If the substrates pyruvate and malate are added initially to the suspension (3 mM each), the initial dye uptake is maintained at a high value and is then slightly increased in response to ATP (50 μM). In each of the conditions at the ends of records the suspensions were quickly (1 min) separated in a bench centrifuge (Coleman Micro-fuge) and the absorbances of the supernatants were measured in the same cuvette. The maximum dye

Fig. 1. (a) Composite of records of the changes of absorbance of mitochondrial suspensions supplemented with safranin in response to energization and de-energization, and (b) the relation between the differential absorbances of whole suspensions and those of the separated supernatants

(a) The mitochondria (Mit) (1 mg of protein) were added to 1 ml of medium containing 18.6 μM -safranin. Other additions, as indicated, were pyruvate (Pyr), malate (Mal), β -hydroxybutyrate (HO-But), ATP, ADP or 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB). The differential absorbance ($A_{524} - A_{554}$) is plotted with upward deflexion registering a diminished absorbance because this corresponds to an uptake of the safranin into the particles. The calculation of the amounts of dye moved into the mitochondria in the stable conditions of absorbance was made from subsequent measurements of the absorbances of separated supernatants (see the text). (b) The samples taken in experiments like those illustrated in (a) provided pairs of readings of absorbance of the fluid before and after separation of the particles. The results were obtained for two initial safranin concentrations (sigmoid curves labelled 10 and 18.6 μM). The straight line ruled through points is the natural logarithm of the ratio between the amount of dye moved into mitochondria (1 mg of protein) and the dye concentration remaining in the medium as given by (initial absorbance of medium - absorbance of supernatant)/(absorbance of supernatant), with the absorbance of the supernatant read from the 18.6 μM initial concentration curve. Note that the amounts so obtained for the numerator relate to 1 mg of protein.

uptake, corresponding to the minimum absorbance (after ATP addition), left $2.6\mu\text{M}$ -safranin in the medium, so the difference, $18.6 - 2.6 = 16\text{ nmol/mg}$, had entered the mitochondria. With respiration uncoupled (or with respiratory inhibitor) the dye concentration in the medium was $14.6\mu\text{M}$, so non-energized adsorption on the protein had removed 4 nmol of dye from the medium; hence the energy-linked dye uptake was $16 - 4 = 12\text{ nmol/mg}$. In Fig. 1(b) the supernatant absorbances are plotted against those of the suspensions for the stable conditions illustrated in Fig. 1(a), and for other stable conditions. With the wavelength pair in use (554 and 524 nm) the coloured particles contribute to the total absorbance, so that as the dye moves into the particles the observed differential absorbance of the suspension does not change as much as corresponds to the dye moved into the particles. With a lower initial concentration of dye the correction is still important, but is less than with the higher concentration. A technical point is that diluted solutions of the dye in aqueous media gradually lost their absorbance, so it was preferred to inject the ethanolic safranin into the medium at the time of starting each experiment.

The energy-dependent part of the dye uptake, if expressed as a ratio between the content in nmol/mg of protein and nmol/mg of medium, is of the order of thousands, in contrast with ratios for the singly charged K^+ ion of 50–100. The discrepancy is not surprising in view of the dye being lipophilic, but it points to negative charges being made available in the lipid phase or at the membrane surface in response to a combination of energy supply and appropriate anions. The qualitative result agrees with the proposal by Åkerman & Wikström (1976) and later work (Åkerman, 1978; Brand *et al.*, 1978) that safranin uptake measures a 'membrane potential', but, since we are dealing with high accumulations, presumably in the lipid phase (see Zanotti & Azzone, 1980), it seems most appropriate to equate the dye uptake to the exposure of negative charges in a particular region of the structure. If conditions permit other cations such as Ca^{2+} or K^+ or H^+ to accumulate in a part of the structure alternatively accessible to these negative charges, then the cations will compete, and one can displace another just as if a single locus were involved; the only difference is between the accumulation ratios holding between the contents of the respective phases and the medium. In fact, as shown in Fig. 1(b) by the straight line, the change of differential absorbance of the suspension happens to be related linearly to the logarithm of the safranin accumulation ratio calculated from the changes of dye concentration in the supernatants. It can be verified that for an initial concentration of, say, $10u$ that $10 - x$ representing the diminution of absorbance is

linear to better than 10% with the function $\log[x/(10 - x)]$ for x between 3 and 7 u , with x the amount moved from medium to a non-absorbent sink.

Effects of competition by Ca^{2+} and its inhibition by Ruthenium Red on the uptake of safranin

Uptake of Ca^{2+} (Chance, 1965) and of safranin (Colonna *et al.*, 1973) are each energy-dependent, and it seemed likely that competition would arise between the two cations for respiration-dependent accumulation. Fig. 2 illustrates a pair of experiments in which the re-uptake of safranin after a preliminary discharge was followed without and with Ruthenium Red present to inhibit active Ca^{2+} uptake (Vasington *et al.*, 1972). At the start of the experiment there was an uptake, on account of endogenous substrates, which was discharged with rotenone ($2\mu\text{g/mg}$ of mitochondrial protein). Succinate then initiated re-uptake, which is shown with an expanded time scale. With Ruthenium Red present the absence of uptake of endogenous Ca^{2+} as competitor for energy led to more rapid dye uptake. That the difference is due to endogenous Ca^{2+} is borne out by a series of experiments (not illustrated) in which previous additions of Ca^{2+} were made to the 1 ml of suspension carrying 1 mg of protein before restoration of respiratory activity with succinate. The half-times for the dye uptake were: with no addition, 15 s; with 5 nmol of CaCl_2 , 24 s; with 10 nmol of CaCl_2 , 34 s.

Fig. 2. Consequence of inhibiting Ca^{2+} uptake on the rate of safranin uptake

Two experiments are shown. In Expt. A P_i at 1.5 mM and Ruthenium Red (RR) ($1\mu\text{M}$) were added, in Expt. B only P_i . Other additions, as indicated, were rotenone (Rot) and succinate (Succ). The safranin in the medium was $15.4\mu\text{M}$, the dye retained in absence of substrate was 5.7 nmol/mg and the energy-linked uptake was 7.2 nmol/mg which together diminished the external concentration to $2.5\mu\text{M}$. Protein was 1 mg/ml .

Fig. 3. Slowing of Ca^{2+} uptake by safranin

The three experiments were performed with the use of Arsenazo III as indicator of the Ca^{2+} concentration in the medium by using the wavelength pair 685 and 665 nm. On addition of the Ca^{2+} at $2\mu\text{M}$ to the suspension supplemented with P_i (1.5 mM) and succinate (3 mM), the Ca^{2+} uptake is shown by the return of the trace towards the initial value of absorbance. With $10\mu\text{M}$ - or $20\mu\text{M}$ -safranin (Saf) present the rates of Ca^{2+} removal were retarded. The protein was 0.4mg/ml , 2.5ml of suspension being used. The Arsenazo III in the medium was sufficient to give an A_{520} value of 1.6.

The converse effect of the prior presence of safranin on the rate of Ca^{2+} uptake is illustrated in Fig. 3. There is a slower uptake of Ca^{2+} when the dye is present. The results are consistent with a mutual competition for energy supply during the uptake process. This competition differs from that described by Colonna *et al.* (1973) because our kinetic experiments were performed in presence of 1.5mM-P_i . Another competition manifest by the steady-state uptake of safranin obtainable being Ca^{2+} -dependent is best seen if movement of leaked endogenous P_i is stopped, as is described below. It is this competition between the dye and Ca^{2+} that is seen when no penetrant anion has been added and is, for example, relieved by acetate addition (Colonna *et al.*, 1973).

In the absence of added anion, and with mersalyl present to inhibit movement of leaked endogenous P_i , the addition of succinate to the suspension with safranin and rotenone present elicited only a limited dye uptake. This result was obtained both in KCl- and mannitol (300 mM)-based media. As shown in Fig. 4(a), in the presence of EGTA to remove adventitious Ca^{2+} there is the greatest uptake of

Fig. 4. (a) Consequence of Ca^{2+} removal or addition on safranin uptake as limited by energization and availability of co-anion, and (b) an example of the oscillatory response to succinate that some preparations displayed

(a) The mitochondria (Mit) (1mg of protein/ml) were added to a medium containing 25nmol of mersalyl (Mers)/ml and other additions mentioned below. The dye taken up was discharged with rotenone (Rot) at $2\mu\text{g/ml}$ and respiration was restored by adding succinate (Succ) at 1.5mM . Incubation medium A contained $100\mu\text{M}$ -EGTA to chelate external Ca^{2+} , medium B contained $1\mu\text{M}$ -Ruthenium Red to inhibit active re-uptake of Ca^{2+} , medium C had no further addition, and to medium D was added $5\mu\text{M}$ - CaCl_2 at the time the dye was discharged. It can be seen that removal of the Ca^{2+} (Expt. A) or inhibition of its re-uptake (Expt. B) allows more safranin to enter on re-energization, whereas additional Ca^{2+} (Expt. D) lessens the safranin uptake. In each case removal of mersalyl by dithiothreitol (DTT) ($250\mu\text{M}$) followed by P_i (1.5mM) initiates a rapid safranin uptake. The safranin concentration in the medium was initially $18.4\mu\text{M}$, and was diminished to $13.4\mu\text{M}$ by the de-energized mitochondria and to $2.6\mu\text{M}$ by the provision of succinate and P_i , so the energy-linked uptake was 10.8nmol/mg with a final external concentration of $2.6\mu\text{M}$. (b) This experiment was performed with $10\mu\text{M}$ -safranin initially in the medium and 25nmol of mersalyl/mg of protein. The uptake on account of endogenous substrates was 6.8nmol/mg , thus lowering the external safranin concentration to $3.2\mu\text{M}$. Of the uptake 0.8nmol/mg was not discharged with rotenone (Rot); the re-uptake with succinate (Succ) was at maximum an additional 2nmol/mg . In a similar experiment with $1\mu\text{g}$ of oligomycin/mg of protein the response was the same.

safranin in response to the succinate; use of Ruthenium Red to inhibit active uptake of the adventitious Ca^{2+} is slightly less effective (curve B); the control made without additions is shown as curve C, and the depressant consequence of adding Ca^{2+} is seen by comparing curve D with the others. In all cases the removal of the inhibition by mersalyl of P_i movement by dithioerythritol allowed a considerable P_i -related uptake of the safranin. It was noticed that as the possibility of Ca^{2+} movement increased from Expt. A to Expt. D so the stability of the final uptake of dye diminished. This may be on account of increasing dissipation of energy of Ca^{2+} cycling.

Anion-dependence of safranin uptake

Comparison, for a particular mitochondrial preparation, between the uptakes of dye induced by succinate without and with mersalyl present showed that about half the dye moving when mersalyl was omitted was attributable to the possibility of movement of such leaked endogenous P_i as was present; this quantity differed between preparations (for example, values measured were $5\text{--}10\mu\text{M}$) and increased with aging. With some preparations the response to succinate addition took the form of a damped oscillation (Fig. 4b), which was found to be insensitive to oligomycin.

The inhibition of safranin uptake in the presence of mersalyl can be overcome by provision of a penetrant anion other than phosphate. Thus, if

Fig. 5. Safranin uptake with acetate as co-anion. With mersalyl (Mers) (25 nmol/mg of protein) and Ruthenium Red (RR) ($1\mu\text{M}$) to inhibit respectively uptake of P_i and of Ca^{2+} , the limited safranin uptake by mitochondria (Mit) in response to succinate was increased on addition of acetate at 12.5 mM . The initial safranin concentration was $19.2\mu\text{M}$ and protein was 1 mg/ml . After rotenone addition the safranin content was 6.4 nmol/mg ; with succinate (Succ) (1.5 mM) plus acetate this increased to 15.2 nmol/mg , leaving $4\mu\text{M}$ safranin in the medium.

instead of dithiothreitol and P_i an addition of acetate is made, then more dye moves in than followed the succinate addition (Fig. 5), though the quantity moving is less than when dithiothreitol and P_i have been added.

These experiments show the similar anion requirements for uptake of Ca^{2+} and of safranin. The anion requirement for Ca^{2+} has been illustrated previously (Harris, 1978). The same anions (P_i , acetate) increase the uptake of K^+ in response to valinomycin (Harris *et al.*, 1966).

Safranin uptake and K^+ movement

Dye uptake, like that of Ca^{2+} (Åkerman & Wikström, 1976), can also be obtained by discharge of internal K^+ with valinomycin (Rossi *et al.*, 1967; Scarpa & Azzone, 1970; Fiskum *et al.*, 1979). This exchange can utilize the energy supply stored in the gradient of K^+ concentration set up between mitochondrial interior and a low- K^+ medium. With this procedure an energy-linked uptake of 11.4 nmol of safranin/mg of protein was obtained with the heart mitochondria suspended in a mannitol medium leaving $4.6\mu\text{M}$ external safranin; the external K^+ concentration at this time was 1.65 mM and the internal K^+ content was 87 nmol/mg of protein. The K^+ content in $\mu\text{mol/g}$ of protein (which may correspond to $\mu\text{mol/ml}$ of internal water; see Harris & van Dam, 1968) divided by the K^+ concentration in $\mu\text{mol/ml}$ is 53, whereas the accumulation of lipid-soluble dye if expressed in the same way is about 2500. [In Fig. 1(b) the dye ratios are between contents per mg and the external concentration.] Of course, if the concentration in the mitochondrial lipid were calculable, a still higher value would be obtained.

An alternative to the passive interchange between internal K^+ and external safranin is to use nigericin to mobilize the K^+ in respiring mitochondria, effectively translating the self-limiting energized safranin-proton exchange into an active interchange between safranin and K^+ . This has been illustrated for Ca^{2+} uptake (Estrada-O. *et al.*, 1972; Harris, 1978), and Fig. 6 shows the phenomenon obtained with safranin. As indicated by Åkerman & Wikström (1976), the consequence of nigericin addition resembles that of adding P_i , though the effects, as shown, are partially additive. It is worth noting that the induction by nigericin of uptake by the respiring particles proceeds as well in a medium with 120 mM-KCl as with a low- K^+ medium, so the K^+ concentration gradient is made unimportant by the respiratory activity.

Potential by ATP of safranin uptake, previously limited by anion availability

When safranin uptake in the presence of respiratory substrate is limited by the addition of

Fig. 6. Restoration of safranin uptake with nigericin when P_i movement has been inhibited

With mersalyl (Mers) (25 nmol/mg of protein) and Ruthenium Red (RR) ($1 \mu\text{M}$), as in Fig. 5, the limited dye uptake by mitochondria in response to succinate (Succ) (1.5 mM) was enhanced by nigericin (Nig) (15 ng/mg of protein). A further uptake of safranin was then obtained in response to a combination of dithiothreitol (DTT) ($250 \mu\text{M}$) and P_i (1.5 mM). The medium contained initially $19.6 \mu\text{M}$ -safranin, and the total uptake after both nigericin and P_i was 17.7 nmol/mg of protein, which diminished the external concentration to $1.9 \mu\text{M}$. The safranin uptake remaining after antimycin inhibition of respiration was 4 nmol/mg of protein, so the energy-linked uptake was 13.7 nmol/mg .

mersalyl, a considerable uptake is obtained in response to adding ATP, and the effect is more pronounced when Ruthenium Red has been used to inhibit re-uptake of external Ca^{2+} (Fig. 7). The additional uptake obtained with ATP after succinate is partly of a transient nature. It decays less rapidly when Ruthenium Red is present, and the uptake can be restored with more ATP. Addition of oligomycin after ATP leads to loss of about 75% of the extra safranin, and the residual extra safranin is similar in quantity to that which enters in response to ATP added after oligomycin.

In this case, the increase in 'membrane potential' over that seen with succinate alone is most probably not due simply to proton translocation driven by the ATPase. The generation of internal P_i by ATP hydrolysis, under conditions where efflux of P_i is blocked by mersalyl, can contribute to safranin uptake in two ways. Firstly, internal buffer is now available to support respiration-driven proton-safranin exchange. Secondly, and probably more importantly, internal hydrolysis of externally added ATP^{4-} with subsequent export of ADP^{3-} and retention of P_i is in itself an electrogenic process (and one that is uncoupler-insensitive). Under analogous conditions, potassium phosphate can be accumulated (Baum, 1977). It is therefore to be expected that, under the present conditions, safranin would accumulate along with the internally generated phosphate.

Fig. 7. Response of the safranin uptake to ATP when P_i movement has been inhibited

Two experiments are shown. The suspensions were supplemented with mersalyl (Mers) at 25 nmol/mg of protein and to medium A Ruthenium Red (RR) at $1 \mu\text{M}$ was also added. The initial safranin concentration was $15.4 \mu\text{M}$. The initial uptake is not shown. It was discharged with rotenone (Rot), and a limited re-uptake was obtained in response to succinate (Succ) with, as before, a greater uptake if simultaneous movement of Ca^{2+} had been inhibited with Ruthenium Red (RR). On addition of ATP at $50 \mu\text{M}$ the extra uptake was in each case about 4.1 nmol of safranin/mg of protein, and this subsequently diminished, the more rapid decay being when Ca^{2+} was still free to move. Addition of oligomycin (Oligo) at $2 \mu\text{g/mg}$ of protein led to a discharge to a safranin content slightly greater than that induced by succinate alone. If the oligomycin was added before the ATP (not shown) the response to ATP was diminished to about 2.2 nmol of dye/mg.

Discussion

We can explain the linear relation found by Åkerman & Wikström (1976) between the change of absorbance of the whole suspension and the logarithm of the ratio between the internal and external concentrations of ions rendered permeant. Safranin, like the other ions in a coupled system in which one ion gradient can feed energy to another, will accumulate in the structure, so that its potential energy, proportional to $\ln[\text{safranin}]_{\text{in}}/[\text{safranin}]_{\text{out}}$, is linearly related to logarithms of the other ion ratios. Since the safranin accumulates in a different phase, presumably lipid, the logarithm of the safranin ratio will differ from the logarithms of the other ions by a constant depending on the energy difference between safranin in the medium and safranin dissolved in the lipid phase, but a linear relation will still hold. Now it happens that the sigmoid relationship between absorbance of the separated medium and that of a safranin-containing suspension has the property of making changes in the latter almost linearly related to the logarithm of the ratio [safranin moved into the mitochondria]/[safranin remaining in the medium].

This point is illustrated by the straight line drawn on Fig. 1(b). For this reason the safranine absorbance changes seen in the whole-suspension carrying a permeant anion can be expected to reflect changes of potential energy of the system as measured by the concentration ratios of other permeant cations.

However, the reason for making these measurements was not primarily to study the details of the functioning of safranine as a membrane probe. This has recently been very thoroughly documented by Zanotti & Azzone (1980). Instead, we have been concerned to examine the relationship between safranine accumulation and the availability of energy supply, of other cations and of anions.

Evidently the transfer of charges to the lipid requires a continuous energy supply, since safranine stimulates respiration (Colonna *et al.*, 1973), a fact that we have confirmed. In contrast, Ca^{2+} , though requiring energy for uptake, can be held in the mitochondria after energy supply is stopped, with very low leakage provided that the conditions are chosen correctly (Harris, 1979b).

However, not only is a supply of energy required, but it is quite clear that for maximal uptake of safranine two other conditions must apply. Firstly, there must be no competing cations, such as Ca^{2+} , that can respond to energetically generated anionic sites, albeit that the locus of final accumulation of the cation might be different from that of safranine. Secondly, it is evident that, even when the cationic species is stacked and osmotically inactive, as in the case of safranine, an appropriate anion must be provided if net cation uptake is to occur. Limited energy-dependent safranine-proton exchange can be achieved without anion involvement; and nigericin can permit more extensive energized exchange between safranine and K^+ , albeit that charge is now effectively transferred from the aqueous to the membranous phase. However, such uptakes are restricted energetically by the availability of H^+ and K^+ for exchange, so further safranine uptake must necessarily be in association with anion. Therefore, for maximal transduction of respiratory energy into a safranine 'signal' (Brand *et al.*, 1978), not only must endogenous Ca^{2+} uptake be suppressed (with, say, EGTA), but anion (such as P_i or acetate) must be available, possibly together with nigericin.

The concept of energized separation of fixed charges can include the requirement that its occurrence is dependent for energetic reasons on the supply of mobile ions of both signs to occupy the fixed sites. This would explain why mobile anions are

needed if safranine (or Ca^{2+}) uptakes are to attain their maximum values in response to energization. The energy requirement for salt accumulation becomes formally identical with the sum of the electrical and pH difference components used in the Mitchell formulation when electroneutrality is maintained in the latter.

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References

- Åkerman, K. E. (1978) *Biochim. Biophys. Acta* **502**, 359–366
- Åkerman, K. E. & Wikström, M. (1976) *FEBS Lett.* **68**, 191–197
- Baum, H. (1977) In *The Molecular Biology of Membranes* (Fleischer, S., Hatefi, Y., MacLennan, D. H. & Tzagoloff, A., eds.), vol. 1, pp. 243–262, Plenum Press, New York and London
- Brand, M. D., Harper, W. G., Nicholls, D. G. & Ingledew, W. J. (1978) *FEBS Lett.* **95**, 125–129
- Chance, B. (1965) *J. Biol. Chem.* **240**, 2729–2747
- Colonna, R., Massari, S. & Azzone, G. F. (1973) *Eur. J. Biochem.* **34**, 577–585
- Estrada-O., S., Cespedes, C. & Calderon, E. (1972) *J. Bioenerg.* **3**, 361–375
- Fiskum, G., Reynafarje, B. & Lehninger, A. L. (1979) *J. Biol. Chem.* **254**, 6288–6295
- Harris, E. J. (1977) *Biochem. J.* **168**, 447–456
- Harris, E. J. (1978) *Biochem. J.* **176**, 983–991
- Harris, E. J. (1979a) *Biochem. J.* **178**, 673–680
- Harris, E. J. (1979b) *Biochem. Soc. Trans.* **7**, 770–775
- Harris, E. J. & van Dam, K. (1968) *Biochem. J.* **106**, 759–766
- Harris, E. J., Cockrell, R. C. & Pressman, B. C. (1966) *Biochem. J.* **99**, 200–213
- Harris, E. J., Al-Shaikhaly, M. & Baum, H. (1979) *Biochem. J.* **182**, 455–464
- Kauffman, R. F. & Lardy, H. A. (1980) *J. Biol. Chem.* **255**, 4228–4235
- Moore, C. (1971) *Biochem. Biophys. Res. Commun.* **42**, 298–305
- Rossi, C. S., Azzi, A. & Azzone, G. F. (1967) *J. Biol. Chem.* **242**, 951–959
- Scarpa, A. & Azzone, G. F. (1970) *Eur. J. Biochem.* **12**, 328–335
- Vasington, F. D., Gazotti, P., Tiozzo, R. & Carafoli, E. (1972) *Biochim. Biophys. Acta* **256**, 43–54
- Zanotti, A. & Azzone, G. F. (1980) *Arch. Biochem. Biophys.* **201**, 255–265