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Uptake of Protoporphyrin IX by Isolated Rat Liver Mitochondria

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Rat liver mitochondria accumulate protoporphyrin IX from the suspending medium into the inner membrane in parallel with the magnitude of the transmembrane K^+ gradient (K^+_{in}/K^+_{out}). Only protoporphyrin IX taken up in parallel with the transmembrane K^+ gradient is available for haem synthesis. Coproporphyrins (isomers I and III) are not taken up by the mitochondria. The results support the suggestion by Elder & Evans [(1978) *Biochem. J.* **172**, 345–347] that the porphyrin to be taken up by the inner mitochondrial membrane belongs to the protoporphyrin(ogen) IX series. Protoporphyrin IX at concentrations above 15 nmol/mg of protein has detrimental effects on the structural and functional integrity of the mitochondria. The relevance of these effects to the hepatic lesion in erythropoietic protoporphyria is discussed.

The last three reactions in the biosynthesis of haem, i.e. the decarboxylation of coproporphyrinogen III to protoporphyrinogen IX (Sano & Granick, 1961), the oxidation of protoporphyrinogen IX to protoporphyrin IX (Poulson, 1976) and the insertion of ferrous iron into protoporphyrin IX (Jones & Jones, 1969) take place within the mitochondrion. It has been generally assumed that coproporphyrinogen III is taken up by the mitochondria and transformed to protoporphyrin IX before reaching the ferrochelatase on the matrix side of the inner membrane (Jones & Jones, 1969). More recently Elder & Evans (1978) and Grandchamp *et al.* (1978) have shown that the distribution of coproporphyrinogen III oxidase (EC 1.3.3.3) coincides with that of the intermembrane-space enzyme adenylate kinase.

Thus the porphyrin species to be taken up by the mitochondrial inner membrane is either protoporphyrin IX or protoporphyrinogen IX rather than coproporphyrinogen III.

The transport of iron across the mitochondrial inner membrane depends on metabolic energy (Romslo & Flatmark, 1973). The mechanism(s) responsible for the transfer of porphyrins to the ferrochelatase is less well understood. Reports from our laboratory have shown that mitochondria accumulate deuteroporphyrin in part by a mechanism insensitive to metabolic inhibitors, and in part by a mechanism which depends on the transmembrane K^+ gradient and metabolic energy (Koller & Romslo, 1978). Only that deuteroporphyrin taken up in parallel with the transmembrane K^+ gradient and metabolic energy is processed by the ferrochelatase (Koller & Romslo, 1978). Taken together with the localization of the ferrochelatase to the matrix side of the inner membrane (Jones & Jones, 1969), the results suggest that the energy-independent uptake of deuteroporphyrin represents binding to the outer surface of the mitochondria, whereas the energy-dependent uptake represents accumulation of deuteroporphyrin within the mitochondrial inner membrane in the vicinity of the ferrochelatase. From this point of view, deuteroporphyrin reacts with the mitochondria very similarly to other metabolites that are transported to within the inner membrane (Azzone & Massari, 1973). Another important observation (Koller & Romslo, 1978) was that deuteroporphyrin is a very potent uncoupler of oxidative phosphorylation. Thus at concentrations above 8–10 nmol/mg of protein deuteroporphyrin IX uncouples oxidative phosphorylation, induces leakage of endogenous K^+ and swelling and disruption of the mitochondria (Koller & Romslo, 1978).

From a physiological point of view it would be of importance to know whether protoporphyrin IX, the physiological precursor of haem in mammalian cells (Granick & Sassa, 1971), is accumulated by similar mechanisms and if uncommitted protoporphyrin IX in the medium has any detrimental effects on the mitochondria.

In the present study it is shown that rat liver

Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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mitochondria accumulate protoporphyrin IX from the suspending medium essentially as reported for deuteroporphyrin IX (Koller & Romslo, 1978). Only protoporphyrin IX taken up in parallel with the transmembrane K^+ gradient is available for haem synthesis in intact mitochondria. At concentrations above 15nmol/mg of protein, protoporphyrin IX has detrimental effects on the mitochondria. Coproporphyrin (isomers I and III) is not taken up by the mitochondria.

Materials and Methods

Rat liver mitochondria were prepared as previously described (Koller & Romslo, 1978). The functional integrity of the mitochondria was tested by measuring the respiratory-control ratio with ADP, with succinate as the substrate (Romslo & Flatmark, 1973). Only preparations with respiratory-control ratios greater than 4.0 were used.

The accumulation of porphyrins by the mitochondria was determined by preincubating mitochondria (approx. 2mg of protein) in a final volume of 1ml for 2min at 25°C with 0.25M-sucrose in 10mM-Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid] buffer, pH 7.40. Further additions were as described in the legends to Figures and Tables. The reaction was initiated by adding the appropriate porphyrin. The concentration of ethanol in the protoporphyrin-IX experiments (see below) was kept below 1% (v/v). At timed intervals, 0.5ml samples were withdrawn and the accumulation was terminated by adding 2vol. of ice-cooled incubation buffer containing 4% (w/v) poly(vinyl alcohol) followed immediately by centrifugation in an Eppendorf micro-centrifuge (type 3200) at 13000*g* for 2min. Poly(vinyl alcohol) was included to improve the solubility of protoporphyrin IX. With incubation periods less than 4min, 4% (w/v) poly(vinyl alcohol) has no effect on the respiratory activity of the mitochondria. However, if the incubation period is prolonged beyond 4min, poly(vinyl alcohol) induces loose coupling. For this reason poly(vinyl alcohol) was included only in the stop-buffer (see above). Note also that poly(vinyl alcohol) has no effect on the fluorescence of the porphyrins. After centrifugation, HCl was added to samples of the supernatants to give final concentrations of 3M-HCl (protoporphyrin IX) or 0.1M-HCl (deutero- and copro-porphyrins I/III) (Smith, 1975), and the amount of porphyrin was determined fluorimetrically as previously described (Koller & Romslo, 1978). The decrease in the amount of porphyrins in the supernatant has been shown to parallel the uptake of porphyrins by the mitochondria when determined in the pellet (Koller & Romslo, 1978). The excitation and emission wavelengths were: protoporphyrin IX 405 and 605nm, deuteroporphyrin IX 393 and 593nm and coproporphyrins I/III 396 and 596nm.

The spectral shift of safranine and swelling of the mitochondria were determined as previously described (Koller & Romslo, 1978).

K^+ was determined by flame photometry. Haem was determined by the pyridine haemochrome method (Porra & Jones, 1963). Protein was determined by using the Folin–Ciocalteau reagent (Flatmark *et al.*, 1971).

Chemicals

ADP, Hepes (A grade), CCCP and valinomycin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Poly(vinyl alcohol) was the product of Wacker-Chemie G.m.b.H. (München, Germany). Safranine was purchased from Merck A.G. (Darmstadt, Germany). Porphyrins were obtained from Porphyrin Products (Logan, UT, U.S.A.). Protoporphyrin IX was dissolved in 10mM-NaOH/20% (v/v) ethanol to a concentration of 1.5mM; deuteroporphyrin IX (crystalline dihydrochloride) and coproporphyrins I and III (crystalline dihydrochloride) were dissolved in 10mM-NaOH to a concentration of 2.5mM. The purity of the porphyrins was better than 95% as assessed by t.l.c. on silica gel after esterification as previously described (Koller & Romslo, 1978). Other chemicals were of highest purity commercially available. Double-quartz-distilled water was used throughout.

Results and Discussion

Deuteroporphyrin IX (and coproporphyrins I and III) at low concentrations is freely soluble in the incubation medium at pH 7.40. Protoporphyrin IX, however, revealed a variable degree of precipitation. Thus, when protoporphyrin IX at concentrations of 10–30μM was suspended in the incubation medium, more than 80% was precipitated when centrifuged at 13000*g* for 2min. Therefore, to minimize precipitation of protoporphyrin IX not bound to the mitochondria during centrifugation, we had to increase the hydrophobicity of the separation medium, taking care not to disrupt the mitochondria. Poly(vinyl alcohol) at 4% (w/v) was found to be an effective solvent of protoporphyrin IX; it decreased the non-specific precipitation by 80–85% and at the same time it had no effect on the mitochondria or on the fluorescence. Poly(vinyl alcohol) was therefore included in the buffer used to terminate the reaction (see the Materials and Methods section).

From a transport point of view, it is of importance to know whether protoporphyrinogen of protoporphyrin IX is the species to be transported across the inner membrane to the ferrochelatase. As shown by Elder & Evans (1978) and Grandchamp

et al. (1978), coproporphyrinogen oxidase is localized to the intermembrane space, with a minor fraction loosely associated with the inner membrane. Protoporphyrinogen thus formed on the cytosol side of the inner membrane is further processed by protoporphyrinogen oxidase. According to Poulson (1976), this enzyme is a hydrophilic protein associated with the inner mitochondrial membrane, and as such is possibly localized to its outer surface.

According to Nishida & Labbe (1959), Labbe *et al.* (1963) and Porra & Jones (1963), protoporphyrinogen is converted into haem at a much slower rate than is protoporphyrin. These results, however, are difficult to interpret in terms of transport, because the experiments were run at 38°C for several hours (Nishida & Labbe, 1959) or in the presence of detergents (Labbe *et al.*, 1963, Porra & Jones, 1963) under conditions where the integrity of the mitochondria is largely lost.

Thus as yet it cannot be stated with confidence whether protoporphyrinogen or protoporphyrin is the species to be transported across the inner membrane. However, at the oxygen concentrations prevailing in the liver cells (Drabkin, 1975), protoporphyrinogen is readily oxidized unless protective reducing mechanism(s) exists. Therefore the oxidized species has been chosen as the model compound in the present study.

Protoporphyrin IX is slightly negatively charged at physiological pH. An anion of this size would not penetrate the inner mitochondrial membrane unless mediated by some mechanism(s) other than passive diffusion. Such a mechanism has been described for mitochondrial accumulation of deuteroporphyrin IX (Koller & Romslo, 1978). As shown in Table 1, protoporphyrin IX, but not coproporphyrins I and III, are taken up by isolated rat liver mitochondria. The uptake of protoporphyrin IX proceeds by mechanism(s) sensitive to CCCP and the combined effect of CCCP plus valinomycin (Table 2). Thus, in the presence of CCCP the uptake is decreased by approx. 35%, and with CCCP plus valinomycin the uptake is decreased by a further 20%. We can define a CCCP-plus-valinomycin-sensitive uptake of protoporphyrin IX (i.e. the uptake in the absence of inhibitors minus the uptake in the presence of inhibitors), which, as shown in Fig. 1, has a $V_{max.}$ of approx. 6nmol/30s per mg of protein and a K_m of 15μM. Furthermore, as reported for deuteroporphyrin IX (Koller & Romslo, 1978), the uptake of protoporphyrin IX is a very rapid process, with saturation reached within 30s, the shortest interval tested (Fig. 2). The patterns of the progress curves with respect to both concentration (Fig. 1) and time (Fig. 2) for the energy-independent binding of protoporphyrin IX and for the energy-dependent accumulation of protoporphyrin IX are similar.

The time progress curves for the synthesis of deutero- and proto-haem are shown in Fig. 3. The steady-state rate of deuterohaem synthesis is approx. 4 times that of protohaem. Similar results were reported by Jones & Jones (1969, 1970).

In the presence of CCCP plus valinomycin the syntheses of deutero- and proto-haem are both

Table 1. *Uptake of porphyrins by isolated rat liver mitochondria*

Mitochondria (approx. 2mg of protein) were incubated as described in the Materials and Methods section. Porphyrin was added and the reaction terminated at 30s (for further details see the Materials and Methods section). The results represent means and ranges (in parentheses) of four parallel experiments.

	Uptake (nmol of porphyrin/mg of protein)
Deuteroporphyrin IX (12.5μM)	5.2 (4.9–5.5)
Protoporphyrin IX (13.0μM)	3.2 (2.8–3.4)
Coproporphyrin I (20.0μM)	0.3 (0.1–0.5)
Coproporphyrin III (20.0μM)	0.5 (0.2–0.8)

Table 2. *Relationship between the endogenous concentration of K^+ and the uptake of protoporphyrin IX*

Mitochondria (approx. 2mg of protein/ml) were incubated as described in the Materials and Methods section. The incubation medium was supplemented with 10mM-KCl. The uptake of protoporphyrin IX and the K^+ concentration of the pellet were determined as described in the Materials and Methods section. The results, which are the means and the ranges (in parentheses) from six parallel experiments, are given as a percentage of that obtained in the absence of ionophores. The mean 100% values were 4.57nmol of protoporphyrin IX/mg of protein and 150nmol of K^+/mg of protein. The concentration of protoporphyrin IX was 16μM.

	Protoporphyrin IX uptake (% of control)	Mitochondrial potassium content (% of control)
CCCP (5μM)	67.3 (65.7–78.2)	80.0 (75.7–85.5)
Valinomycin (0.5μg/mg of protein)	96.9 (82.0–110.2)	93.2 (90.7–109.5)
CCCP (5μM) plus valinomycin (0.5μg/mg of protein)	46.8 (44.2–59.4)	46.7 (41.5–55.6)

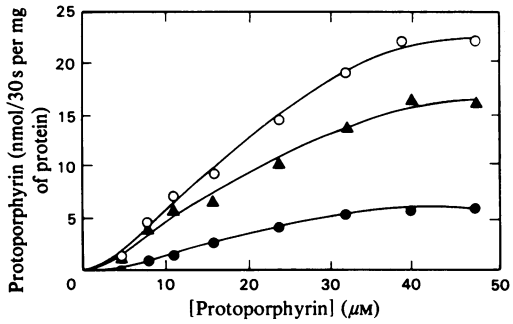

Fig. 1. *Effect of protoporphyrin concentrations on the uptake of protoporphyrin IX*

Mitochondria (approx. 2mg of protein) were incubated as described in the Materials and Methods section in the absence (○) and in the presence (▲) of CCCP (5μM) plus valinomycin (5μg/mg of protein). The CCCP-plus-valinomycin-dependent uptake (●) is calculated from the uptake in the absence minus the uptake in the presence of inhibitors.

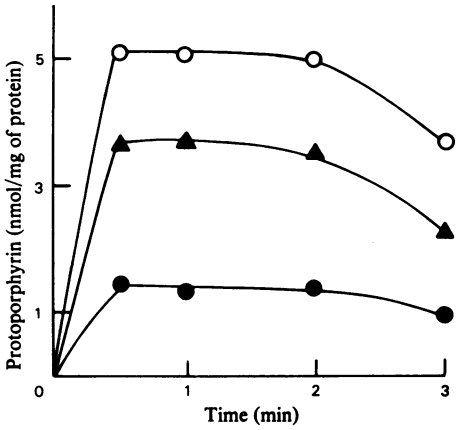

Fig. 2. *Time progress curve for the uptake of protoporphyrin IX*

Mitochondria (approx. 2mg of protein) were incubated as described in the Materials and Methods section in the absence (○) and in the presence (▲) of CCCP (5μM) plus valinomycin (0.5μg/mg of protein). The reaction was initiated by the addition of 12μM-protoporphyrin IX. At timed intervals the reaction was terminated as described in the Materials and Methods section. The CCCP-plus-valinomycin-dependent uptake (●) is calculated from the uptake in the absence minus the uptake in the presence of inhibitors.

markedly inhibited. The degree of inhibition, however, varies both with the porphyrins and with the time of incubation. Below 30min incubation the

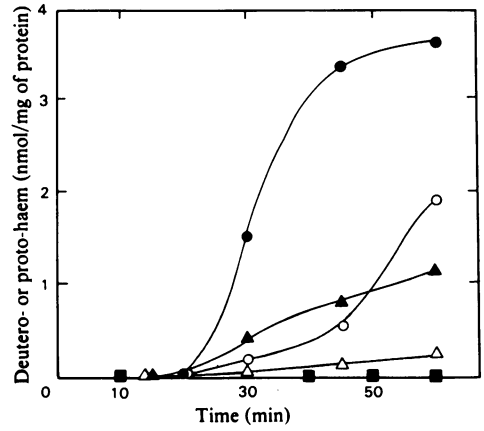

Fig. 3. *Time course of deuterohaem synthesis in the absence (●) and in the presence (○) of CCCP (5μM) plus valinomycin (5μg/mg of protein) and of protohaem synthesis in the absence (▲) and in the presence (△) of CCCP (5μM) plus valinomycin (0.5μg/mg of protein)*

Mitochondria (approx. 17mg of protein) were incubated at 30°C in 5ml of the incubation medium (see the Materials and Methods section), supplemented with 10mM-succinate and 50μM-$FeCl_3$. CCCP and valinomycin were added 2min after $FeCl_3$. The reaction was initiated with the addition of 20μM-porphyrin and the amount of haem formed was assayed as pyridine haemochrome. ■, Haem synthesis with coproporphyrins (isomer I or III) (100nmol).

synthesis of deutero- and proto-haem is inhibited by approx. 80%. Beyond 30min incubation the inhibitory effect of CCCP plus valinomycin on the synthesis of deuterohaem reaches a plateau. The synthesis of protohaem, however, remains inhibited. The difference may be ascribed to a time-dependent disruption of the mitochondria and the elimination of the permeability barrier to the ferrochelatase by the larger amounts of deuteroporphyrin IX accumulated compared with protoporphyrin IX (see Fig. 1, and Koller & Romslo, 1978).

The amount of protohaem synthesized during 60min incubation (Fig. 3) is significantly less than the amount of protoporphyrin IX accumulated (Figs. 1 and 2). However, the rate of haem synthesis as determined from the time interval 15–45min (Fig. 3) is about 0.05nmol/min per mg of protein, or very close to the value of 0.047nmol/min per mg of protein as reported by Jones & Jones (1969). Thus, rather than the accumulation of protoporphyrin IX, the ferrochelatase reaction is the rate-limiting step in the synthesis of haem. This conclusion is supported also by the delay between porphyrin accumulation (Fig. 2) and initiation of haem synthesis (Fig. 3).

Coproporphyrins (isomers I and III) were not

substrates for the ferrochelatase in the present system (Fig. 3), nor did they have any effect on the metabolism of the mitochondria even at concentrations more than 30 nmol/mg of protein.

The uptake of deuteroporphyrin IX has been shown to depend on a transmembrane K^+ gradient (Koller & Romslo, 1978). This relationship applies to the uptake of protoporphyrin IX as well (Fig. 4). When the exogenous concentration of K^+ is increased, the inhibitory effect of CCCP plus valinomycin on the uptake process decreases. At a K^+ concentration of 100 mM the addition of CCCP plus valinomycin has no effect on the uptake of protoporphyrin IX. The importance of the transmembrane K^+ gradient to the uptake is evident also from the results given in Table 3. When mitochondria are suspended in 100 mM-K^+ the synthesis of haem is less than one-third of that in a K^+-free medium, and with ultrasonically treated mitochondria K^+ at concentrations of 0–100 mM has no effect on the synthesis of haem. Note that in a medium of 100 mM-K^+ the mitochondria are still tightly coupled. Apparently the effect of CCCP is an indirect one, mediated by blocking the energy-dependent K^+ pump (Lehninger *et al.*, 1967).

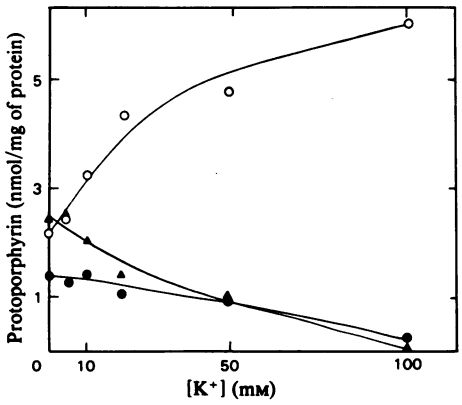

Fig. 4. *Effect of increasing concentrations of K^+ on the CCCP-dependent* (●), *CCCP-plus-valinomycin-dependent* (▲) *and the CCCP-plus-valinomycin-independent* (○) *uptake of protoporphyrin IX*

Mitochondria (2 mg of protein/ml) were incubated as described in the Materials and Methods section at increasing concentrations of KCl. The CCCP-dependent uptake was calculated from the difference between the uptake in the absence minus the uptake in the presence of CCCP. The CCCP-plus-valinomycin-dependent uptake was calculated as described in the legend of Fig. 1. The osmolality of the incubation medium was kept constant at 280 mosmol/kg by varying the concentration of sucrose.

The extent of stacking of safranine on binding to the mitochondria closely parallels the magnitude of the transmembrane electrochemical potential gradient under conditions of small variations in the transmembrane pH-gradient (Åkerman & Wikstrøm, 1976). In a K^+-free medium addition of valinomycin to mitochondria in suspension induces a large increase in the stacking of safranine (Fig. 5), concomitantly with the generation of a transient

Table 3. *Comparison of the synthesis of protohaem by intact mitochondria and by sonicated mitochondria in the absence and presence of K^+*

Mitochondria (approx. 4 mg of protein/ml) were incubated in 0.25 mM-sucrose in 10 mM-Hepes buffer, pH 7.40, supplemented with 20 mM-succinate and 75 μM-$FeCl_3$. The reaction was initiated with 25 μM-protoporphyrin IX. A, incubation medium containing no K^+; B, incubation medium supplemented with 100 mM-KCl. In the experiments with high K^+. sucrose concentration was decreased to keep osmolality constant at 280 mosmol/kg. All experiments were carried out at 30°C and haem was determined as pyridine haemochromogen (see the Materials and Methods section).

	Protohaem (nmol/30 min per mg of protein)	
Mitochondria	A	B
Intact	1.29	0.40
Sonicated	2.80	2.90

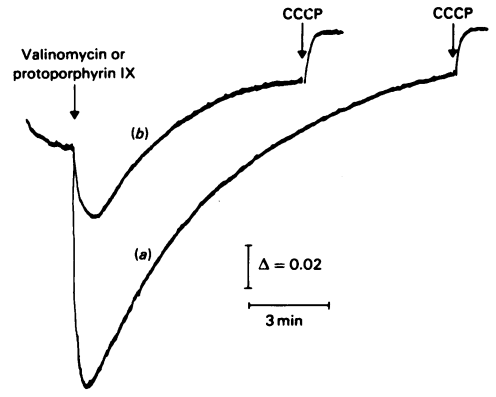

Fig. 5. *Effect of valinomycin and protoporphyrin on the spectral shift of safranine*

Mitochondria (approx. 0.7 mg of protein/ml) were incubated in a K^+-free medium as described in the Materials and Methods section in the presence of 40 μM-safranine. Valinomycin (0.7 μg) (*a*) and protoporphyrin IX (3 μM) (*b*) were added and the change in absorbance, $\Delta (A_{524}-A_{554})$, was recorded.

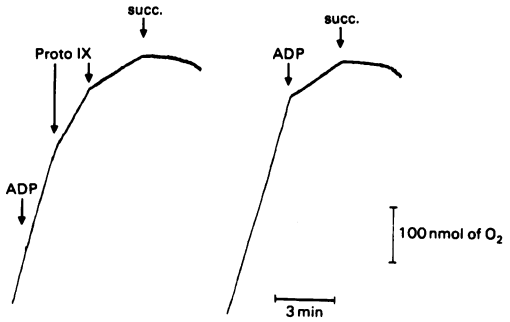

Fig. 6. *Effect of protoporphyrin IX on mitochondrial respiration*

Mitochondria (approx. 2 mg of protein) were incubated at 25°C in a reaction medium containing, in a final volume of 3.0 ml: 50 mM-glucose, 175 mM-sucrose, 5 mM-$MgCl_2$, 10 mM-KCl, 5 mM-KH_2PO_4, 1.67 μM-rotenone and 10 mM-Hepes buffer, pH 7.40. Additions as indicated were: 5 mM-succinate (succ.), 0.83 mM-ADP and 7 + 7 μM-protoporphyrin IX (Proto IX).

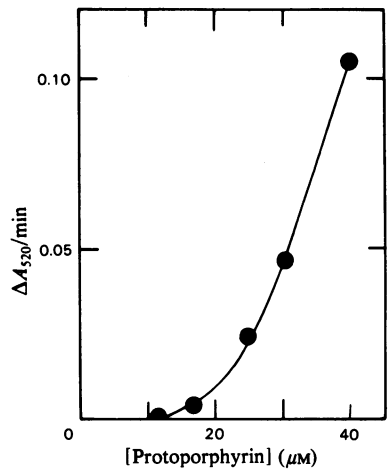

Fig. 7. *Effect of protoporphyrin IX on the swelling of mitochondria*

Mitochondria (approx. 2 mg of protein) were incubated as described in the Materials and Methods section. Protoporphyrin IX was added and the swelling was determined from the steady state rate of the change in turbidity, measured at 520 nm.

electrochemical potential gradient. If protoporphyrin IX (or deuteroporphyrin IX) is added instead of valinomycin, a similar stacking is observed, as would be expected from the effect of the porphyrins on the leakage of K^+ (Koller & Romslo, 1978). The stacking obtained with protoporphyrin IX, however, was significantly less than obtained with valinomycin, and it did not increase in parallel to the amount of protoporphyrin IX added. Thus at concentrations of protoporphyrin IX above 10–15 nmol/mg of protein the initial stacking was masked by a rapid de-stacking and uncoupling. A challenging hypothesis to emerge from these observations is that protoporphyrin IX (at sub-uncoupling concentrations) sets up a transmembrane electrochemical potential gradient, which in turn may drive the porphyrin electrogenically within the membrane. The transmembrane pH-gradient of the total protonmotive force is of minor importance to the uptake process (Table 2).

As for the effect on mitochondrial metabolism, protoporphyrin IX behaves essentially like deuteroporphyrin IX. Thus, at concentrations more than 10–15 nmol/mg of protein, protoporphyrin IX induces loose coupling (Fig. 6) and swelling (Fig. 7) of the mitochondria. Similar results have been reported by Stumpf *et al.* (1979), and it has also been shown that mitochondria isolated from porphyric rats are loosely coupled (Miyahara *et al.*, 1973).

The detrimental effects of protoporphyrin IX on the mitochondria may be of importance in disorders of haem biosynthesis in which uncommitted protoporphyrin IX accumulates within the cell. Patients with erythropoietic protoporphyria have been shown to have diminished ferrochelatase activity and intracellular accumulation of uncommitted protoporphyrin IX (Bottomley *et al.*, 1975; Bonkowsky *et al.*, 1975; DeGoeij *et al.*, 1975; Becker *et al.*, 1977; Bloomer *et al.*, 1977). Above a certain amount, protoporphyrin IX accumulated in the cytosol may uncouple oxidative phosphorylation, increase the loss of endogenous k^+ and disrupt the mitochondria. These mechanisms should be considered in the development of hepatic cirrhosis seen in some patients with erythropoietic protoporphyria (Donaldson *et al.*, 1971; Scott *et al.*, 1973; Cripps & Goldfarb, 1978).

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