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Mechanism of energization of uptake of the fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation [DMP$^+$] into an *acrA* strain of *Escherichia coli*

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The mechanism of uptake of the fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP$^+$) into cells and vesicles of the *acrA* strain AS-1 of *Escherichia coli* was examined. Uptake was energized by substrate oxidation and discharged by uncouplers. Uptake was enhanced by the presence of tetraphenylphosphonium cation, tetraphenylboron anion and tributyltin chloride, which may inhibit the efflux system for DMP$^+$. Uptake was inhibited by 5-methoxyindole-2-carboxylic acid (MIC). By the use of ionophores with right-side-out vesicles loaded with monovalent cations it was shown that DMP$^+$ uptake could be driven both by the establishment of a membrane potential across the vesicle membrane and by a H^+/DMP$^+$ antiport system. Attempts to demonstrate the latter mechanism in everted membrane vesicles were unsuccessful.

Introduction

The fluorescent dye 2-(4-dimethylaminostyryl)-1-ethylpyridinium cation (DMP$^+$) was introduced by Bereiter-Hahn to measure the metabolic state of mitochondria in situ [1]. He concluded that changes in the fluorescence intensity of the dye were not due to ion movements or pH changes. Subsequently, Rafael [2] and Mewes and Rafael [3] concluded that the dye responded to the transmembrane potential of mitochondria. Midgley and coworkers [4,5] examined the response of DMP$^+$ in bacterial cells. They found that the dye was taken up by the cells but was extruded on addition of glucose. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) blocked the extrusion process. Extrusion was blocked by tetraphenylphosphonium cation (TPP$^+$) also. It was proposed that TPP$^+$ competed with DMP$^+$ for a lipophilic cation extrusion system. This hypothesis was further strengthened by subsequent studies [6]. It was suggested that efflux of the lipophilic cations was driven by the transmembrane proton electrochemical gradient, possibly by cation/proton antiport. The efflux system for lipophilic cations has been cloned from *Staphylococcus aureus* and expressed in *E. coli* [7].

No studies have been carried out to examine the mechanism of uptake of DMP$^+$ by *E. coli*. The strain used by Midgley was not suitable for such studies since it possessed an intact outer membrane. In the present paper we describe studies on the mechanism of DMP$^+$ uptake using an *acrA* strain of *E. coli* in which the outer membrane is permeable to lipophilic cations [8,9]. We show that uptake is driven by the proton electrochemical gradient.

Materials and Methods

Bacterial strains

E. coli W3110, a wild-type *E. coli* K-12 strain, and AS-1, an *acrA* mutant derived from W3110, were obtained from Dr. Yasuo Imae, Nagoya University, Japan.

Growth of cells

Bacterial cultures were grown to stationary phase at 37°C with aeration from a 1% (v/v) inoculum on

Abbreviations: Bu_3SnCl, tributyltin chloride; CCCP, carbonyl cyanide *m*-chlorophenyhydrazone; DMP$^+$, 2-(4-dimethylaminostyryl)-1-ethylpyridinium; MIC, 5-methoxyindole-2-carboxylic acid; PMS, phenazine methosulfate; TPP$^+$, tetraphenylphosphonium.

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Penassay Broth (Difco) medium. The cells were harvested by centrifugation and washed once by centrifugation from 50 mM potassium phosphate buffer, pH 7.5.

DMP^+ fluorescence with intact cell preparations

The fluorescence of DMP^+ was measured at 22°C with an SLM-Aminco SPF-500C spectrofluorometer, using an excitation wavelength of 467 nm and an emission wavelength of 557 nm. Washed cell pellets obtained by harvesting 40 ml of cultures were resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5). 50 μl of these cell suspensions were added to 2 ml of 50 mM potassium phosphate buffer (pH 7.5) in cuvets of 1 cm pathlength. The resulting suspensions contained 1 mg of cell protein. Assays were started by the addition of 12.5 μM DMP^+. Energy sources and inhibitors were used at the concentrations indicated in the legends to the figures.

Preparation of membrane vesicles

Right-side-out membrane vesicles were prepared by osmotic lysis of spheroplasts. Spheroplasts were prepared by the method of Witholt et al. [10], and were converted to right-side-out vesicles by osmotic lysis in distilled water. The vesicles were collected by centrifugation at $144000 \times g$ for 2 h, washed and resuspended in an appropriate buffer as indicated in the figure legends. In some experiments right-side-out vesicles were loaded with K^+ or Na^+ by resuspending the vesicles in 0.5 M potassium or sodium phosphate buffers at pH 8.0 and incubating the suspension at 40°C for 30 min. The suspension was cooled on ice and $MgSO_4$ was then added to 10 mM prior to collecting the 'loaded' vesicles by centrifugation at $30000 \times g$ for 10 minutes. The vesicles were washed once and resuspended in 0.4 M sucrose containing 10 mM $MgSO_4$ (pH 6.4) or in an appropriate buffer as indicated in the figure legends.

Everted membrane vesicles were prepared by French press treatment of cell suspensions as previously described [11]. Vesicles were washed once and resuspended in 50 mM potassium phosphate, pH 7.5.

Aliquots of right-side-out or everted membrane vesicle suspensions were added to DMP^+ fluorescence assay systems to give a final protein concentration of 0.5–1.0 mg/ml. DMP^+ fluorescence was measured as described for intact cell experiments.

Preparation of rat liver mitochondria

Rat liver mitochondria were prepared by the method of Johnson and Lardy [12] and resuspended in 0.25 M sucrose. 100 μl of this suspension was added to the DMP^+ fluorescence assay systems as described in the legends to the figures.

Determination of protein

Protein was measured by the method of Lowry et al. [13] using bovine serum albumin as a standard.

Results

Experiments with intact cells

In the experiments described in this paper we have followed the uptake and efflux of DMP^+ from increases or decreases in the fluorescence intensity of the dye. The work of Midgley and coworkers indicates that this is a satisfactory procedure [4–6]. Furthermore, we have examined spectroscopically the possibility that DMP^+ interacts directly with the various reagents used in our experiments. No evidence for interactions was obtained.

Fig. 1 compares the response of intact cells of the parent strain W3110 with that of its *acrA* mutant AS-1. These cells were grown to the stationary phase and had a low rate of endogenous respiration. In a similar manner to that described by Midgley and coworkers [4–6], addition of D-lactate or glucose to a cell suspen-

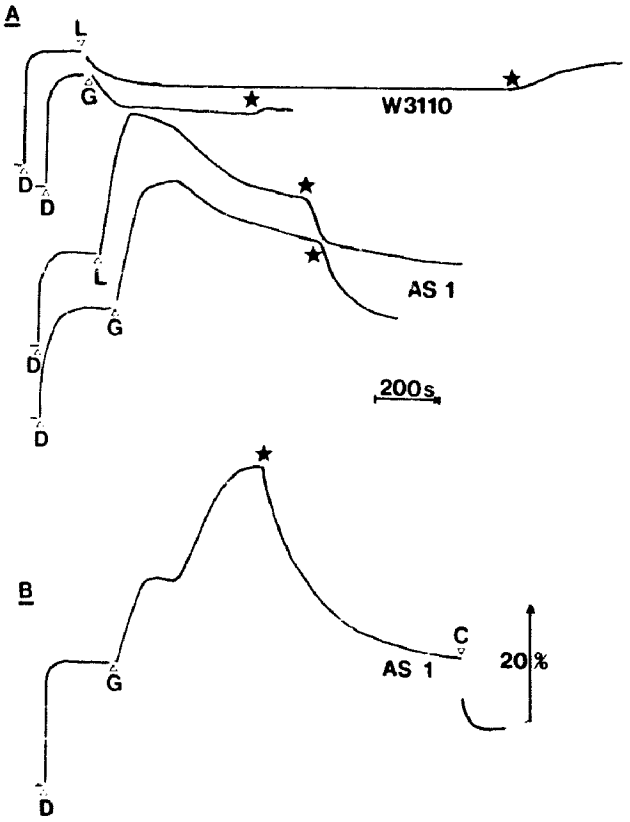

Fig. 1. Effect of substrates on the fluorescence of DMP^+ in cell suspensions of W3110 and AS-1. The procedure is described in Materials and Methods. Additions to the cell suspensions were as follows: D, DMP^+ (12.5 μM); L, D-lactate (10 mM); G, D-glucose (10 mM). The dissolved oxygen is exhausted at the star. In (B), CCCP(C) was added to 12.5 μM. Changes in fluorescence intensity are shown on the vertical axis. The arrow indicates 200 s on the time scale.

sion of W3110 resulted in a decrease in the fluorescence intensity of DMP^+ as it was expelled from the cell. Additionally, we observed that the dye was reaccumulated when the dissolved oxygen in the cuvet had been exhausted. By contrast, these substrates caused uptake of DMP^+ into cells of AS-1. Uptake ceased and dye was lost from the cells on exhaustion of oxygen in the medium. Addition of 25 μM CCCP abolished oxidation-dependent efflux of DMP^+ in W3110 and oxidation-dependent uptake of this dye in AS-1 (see accompanying paper, Ref. 14). We conclude that both oxidation-dependent uptake and efflux are energy-coupled reactions. The initial phase of fluorescence increase on addition of DMP^+ to the cell suspension appears to have a non-energy-coupled component as well as an energy-coupled component since addition of CCCP after decay of the oxidation-dependent uptake resulted in further loss of DMP^+ but did not cause complete abolition of the fluorescence response (Fig. 1B). Furthermore, addition of CCCP to the cell suspension in the presence of DMP^+, but prior to the addition of substrate, resulted in some quenching. This suggested that metabolism of endogenous substrates maintained a certain level of fluorescence enhancement. Titration of the initial phase of fluorescence in the presence of CCCP gave a half-saturation value of 0.1 μmol DMP^+/mg protein. The fluorescence response following addition of glucose to AS-1 shows biphasicity in the results shown in Fig. 1B. This behaviour was variable, being shown by some but not all cell suspensions. The explanation of this phenomenon has not been determined.

Midgley and coworkers showed that efflux of DMP^+ was blocked by competition with TPP^+ [4–6]. In agreement with this hypothesis, addition of TPP^+ to a respiring cell suspension of AS-1 resulted in a marked increase in fluorescence intensity which slowly decayed on exhaustion of oxygen in the cuvet (Fig. 2). the rate of decay was slower in the presence of TPP^+ than in its absence. This is consistent with TPP^+ blocking the efflux pathway by competition with DMP^+. However, the mechanism for the TPP^+ effect may be more complex than proposed by Midgley. Addition of tributyltin chloride (Bu_3SnCl) and tetraphenylboron anion also increased the uptake of DMP^+ (Fig. 3). Bu_3SnCl catalyzes chloride–hydroxyl ion exchange to uncouple proton electrochemical gradients [15]. However, it affected the uptake of DMP^+ in the absence of chloride ions. Thus it is unlikely that it is acting as an uncoupler. Moreover, its effect was blocked by the uncoupler CCCP (Fig. 3). It is possible that Bu_3SnCl and the tetraphenylboron anion exert their effects by interacting with a lipophilic site on the lipophilic cation efflux system.

5-Methoxyindole-2-carboxylic acid (MIC) is an inhibitor of lipoamide dehydrogenases in animal cells

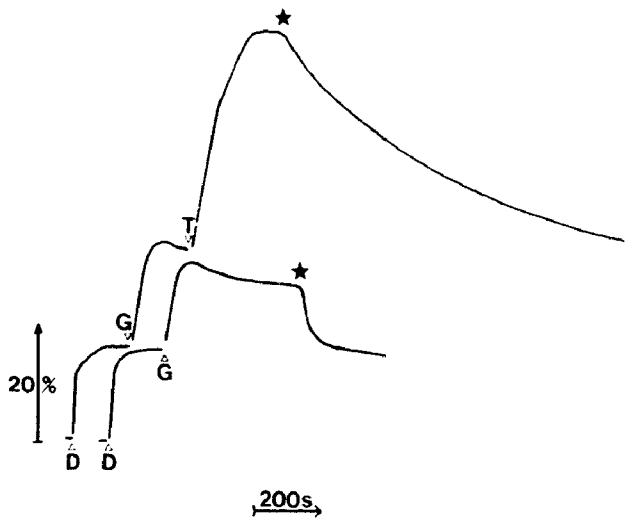

Fig. 2. Effect of TPP^+ on DMP^+ fluorescence in a cell suspension of AS-1. The procedure is described in Materials and Methods. Symbols and concentrations are as in Fig. 1. T, TPP^+ added to 0.125 μM.

and bacteria [16,17]. Richarme [17] has shown that it inhibited binding protein-dependent transport in *E. coli* without affecting lactose permease or the phosphotransferase system. MIC blocked uptake of DMP^+ driven by oxidation of glucose, D-lactate, succinate, and formate in the presence of TPP^+ (Fig. 4). An effect on

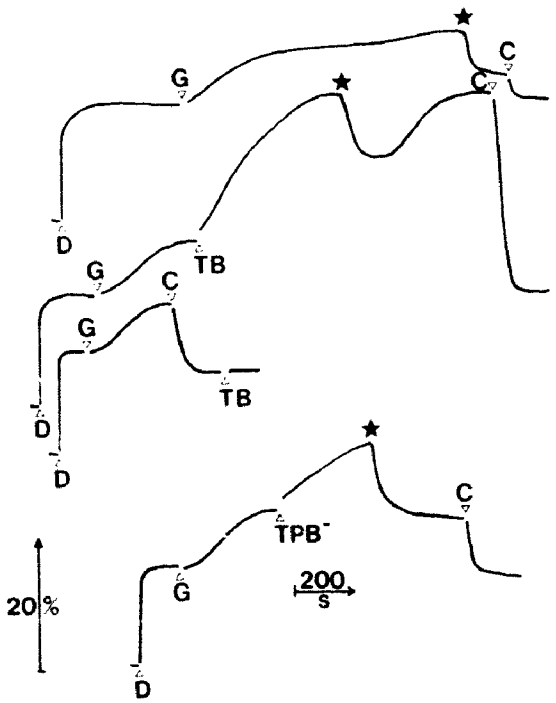

Fig. 3. Effect of Bu_3SnCl and tetraphenylboron on DMP^+ fluorescence in a cell suspension of AS-1. The procedure is described in Materials and Methods. Symbols and concentrations are as in Fig. 1. TB, Bu_3SnCl added to 1.25 μM; TPB^-, tetraphenylboron added to 1.25 μM.

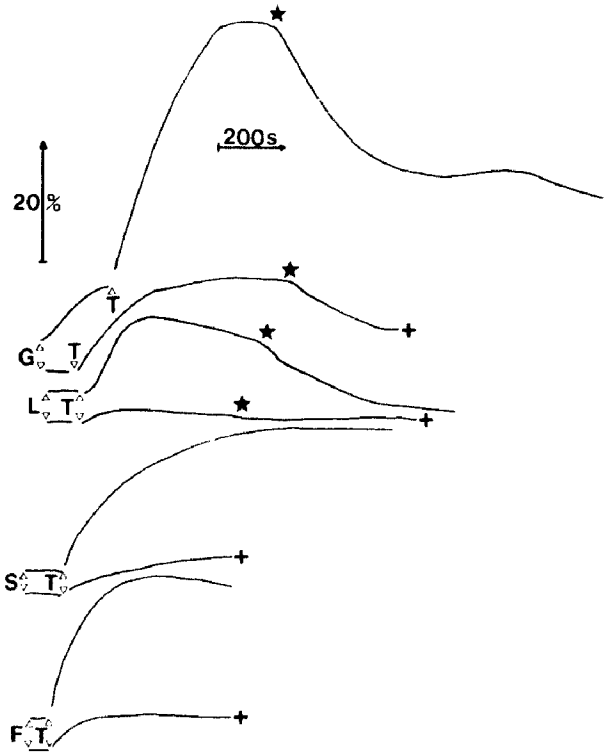

Fig. 4. Effect of 5-methoxyindole-2-carboxylic acid (MIC) on TPP^+-induced DMP^+ fluorescence changes in a cell suspension of AS-1. The initial fluorescence level was attained in the presence of 12.5 μM DMP^+. Substrates were added to a final concentration of 10 mM. G, D-glucose; L, D-lactate; S, succinate; F, formate. T, TPP^+ added to 0.125 μM. The ▽ symbol indicates that MIC (4.7 mM) was present in the system. Dissolved oxygen was exhausted at the star.

lipoamide dehydrogenase is unlikely to be the mechanism of action of MIC on DMP^+ uptake in our experiments.

Experiments with vesicles and mitochondria

DMP^+ was taken up in right-side-out membrane vesicles of AS-1 (and W3110) driven by oxidation of ascorbate with PMS (or D-lactate, result not shown). Uptake was increased if TPP^+ was present to block efflux (Fig. 5A). Succinate drove the uptake of DMP^+ in rat-liver mitochondria. DMP^+ effluxed on exhaustion of oxygen. Similar data were obtained by Bereiter-Hahn [1]. By contrast with membrane vesicles of *E. coli* addition of TPP^+ blocked uptake of the dye or caused immediate efflux (Fig. 5B). This is consistent with the discharge of the transmembrane potential of the mitochondrion and agrees with the proposal of Mewes and Rafael [3] that the transmembrane potential is the driving force for DMP^+ uptake in mitochondria. Furthermore, the results suggest that an active efflux system for lipophilic cations, which can be competitively inhibited by TPP^+, does not occur in mitochondria in contrast with *E. coli*.

Right-side-out membrane vesicles were loaded with potassium ions by incubation with 0.5 M potassium phosphate, pH 8, at 40°C [18]. DMP^+ uptake by the vesicles was measured following addition to a potassium-free choline chloride buffer at pH 8. There was little uptake until valinomycin was added (Fig. 6). Addition of nigericin (or CCCP) caused more rapid efflux of the dye. Uptake of DMP^+ on addition of valinomycin did not occur if the external medium contained potassium phosphate buffer. Surprisingly, addition of nigericin to potassium phosphate-loaded vesicles in choline chloride buffer also resulted in uptake of DMP^+. Valinomycin caused efflux. In the first experiment, DMP^+ uptake must be driven in response to the transmembrane potential, negative inside, formed on addition of valinomycin. In the second experiment, nigericin would acidify the vesicle interior by electroneutral K^+/H^+ exchange. Uptake of DMP^+ could then be driven by H^+/DMP^+ antiport. The presence of both valinomycin and nigericin would be expected to discharge the ion gradients driving uptake.

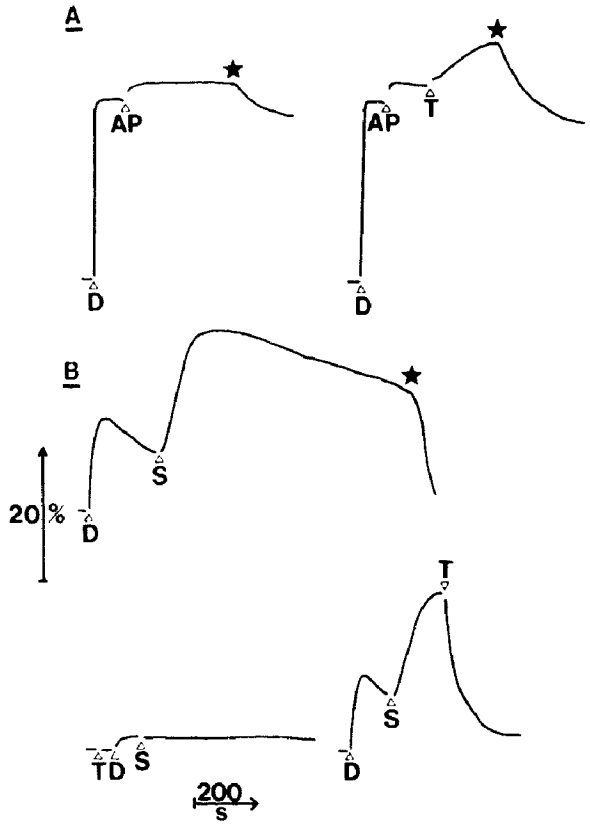

Fig. 5. Effects of substrate and TPP^+ on DMP^+ fluorescence in a suspension of right-side out vesicles of AS-1 (A) and rat-liver mitochondria (B). Vesicles prepared as described in Materials and Methods were resuspended in sodium phosphate, pH 8, and assayed in 50 mM sodium phosphate buffer (pH 8). Mitochondria were assayed in 10 mM Hepes-KOH buffer (pH 7.5), containing 0.3 M KCl. The following additions were made as indicated: AP, ascorbate (10 mM) and PMS (5 μM); T, TPP^+ (12.5 μM); S, succinate (10 mM); D, DMP^+ (12.5 μM). Dissolved oxygen was exhausted at the star.

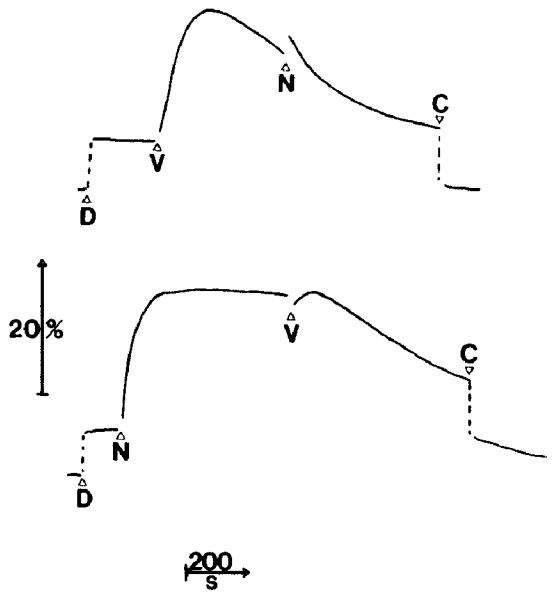

Fig. 6. Effect of the ionophores valinomycin and nigericin on the DMP^+ fluorescence in a suspension of K^+-loaded, right-side-out vesicles. The vesicles were prepared at pH 8 as described in Materials and Methods. The assay system contained 50 mM choline chloride buffer, pH 8.0. V, valinomycin (1.25 μg/ml); N, nigericin (1.25 μg/ml); C, CCCP (12.5 μM).

Further verification of the two routes of energization of uptake of DMP^+ into right-side-out membrane vesicles was obtained using non-loaded or Na^+-loaded vesicles. Addition of valinomycin to vesicles prepared in a K^+-free buffer at pH 8 and suspended in potassium phosphate buffer, pH 8, induced efflux of DMP^+ in response to the transmembrane potential, positive internally (Fig. 7A). Monensin, which catalyzes the

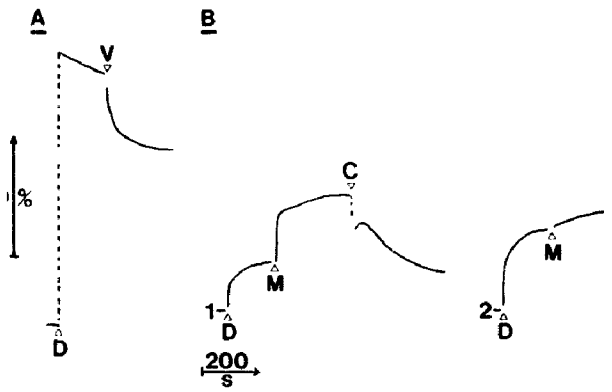

Fig. 7. Effect of ionophores on DMP^+ fluorescence in a suspension of K^+-free (A) or Na^+-loaded (B) right-side-out vesicles. Vesicles were prepared as described in Materials and Methods. In (A), valinomycin (V, 1.25 μg/ml) was added to K^+-free vesicles prepared at pH 8, suspended in sodium phosphate buffer, pH 8, and assayed in 50 mM potassium phosphate buffer, pH 8. In (B1), monensin (M, 50 μM) was added to Na^+-loaded vesicles prepared at pH 8 and assayed in 0.4 M sucrose/10 mM $MgSO_4$ (pH 6.4) (trace 1). In (B2), K^+-loaded vesicles were assayed in 50 mM sodium phosphate buffer (pH 8) (trace 2). L, DMP^+ (12.5 μM); C, CCCP (12.5 μM).

electroneutral exchange of Na^+ and H^+, caused DMP^+ uptake in right-side-out vesicles loaded with sodium phosphate, pH 8, and assayed in Na^+-free buffer (Fig. 7B1). The uncoupler CCCP caused efflux of the DMP^+. Uptake of DMP^+ did not occur if K^+-loaded vesicles were treated with monensin in sodium phosphate buffer (Fig. 7B2). These results are consistent with a mechanism in which monensin causes acidification of the vesicle interior by mediating exchange of H^+ for Na^+. DMP^+ would then be taken up into the vesicles by H^+/DMP^+ antiport.

In a further set of experiments, everted membrane vesicles prepared by French pressing were examined particularly to establish the role of H^+/DMP^+ antiport in the efflux system. Addition of DMP^+ to everted vesicles gave a level of fluorescence intensity which could not be altered by oxidation of NADH (or ascorbate with PMS), hydrolysis of ATP, or by attempting to establish transmembrane H^+ or charge gradients in either direction by means of ionophore addition to pre- or non-preloaded vesicles, as described above for right-side-out vesicles. Addition of TPP^+ to block the efflux system had no effect.

Discussion

As previously documented by Midgley and coworkers [4–6], the reaction of DMP^+ with *E. coli* cells is a balance between the processes of uptake and efflux of the dye. Normal wild-type cells are unsuitable for studies of uptake as the outer membrane imposes a permeability barrier to lipophilic cations [9,19]. This barrier is overcome in *acrA* mutants like strain AS-1 used in our intact cell studies. In AS-1 the rate at which DMP^+ is able to reach the inner membrane is not rate-limiting. Thus, it is possible to measure uptake of the dye. The uptake rate is greater than the rate of efflux so that net accumulation of the dye is observed. That efflux also occurs is indicated by the enhancement in the rate of uptake of DMP^+ when TPP^+ is present. Midgley and coworkers [4–6] have suggested that TPP^+ competes with DMP^+ at the efflux system, thus reducing the rate of extrusion of DMP^+. Bu_3SnCl and the lipophilic anion tetraphenylboron behave similarly to TPP^+ but it is not clear if their mechanism of action is the same as that of TPP^+. Bu_3SnCl did not act through catalyzing Cl^-/OH^- exchange, its normal mode of action [15].

Uptake of DMP^+ in AS-1 is driven by oxidation of substrates such as glucose, D-lactate, succinate, formate and ascorbate (in the presence of the electron carrier PMS). Uptake ceases, and efflux occurs, when oxygen has been exhausted in the system. The uncoupler CCCP abolishes uptake suggesting that the electrochemical gradient of protons set up by respiration is involved in the uptake process. CCCP decreases the

level of DMP^+ to below that produced by substrate oxidation indicating that endogenous metabolism must contribute to the uptake process. The uncoupler does not reduce fluorescence to zero. This implies that a portion of the observed fluorescence can be attributed to passive binding of the dye to the cells.

The use of unloaded or monovalent cation-loaded right-side-out vesicles in the presence of the ionophores valinomycin, nigericin and monensin has shown that uptake into the vesicles can be energized by the transmembrane potential (negative inside) and by H^+/DMP^+ antiport. It is the presence of the latter system which explains why addition of TPP^+ to *E. coli* vesicles does not cause inhibition of DMP^+ uptake such as occurs with mitochondria, where presumably the only means of uptake is the equilibration of the lipophilic DMP^+ across the membrane in response to the membrane potential (negative inside mitochondrion). It is not clear from our experiments if we are measuring as an uptake system the H^+/DMP^+ antiport system postulated by Midgley to be responsible for efflux. Our inability to set up an appropriate efflux system with everted membrane vesicles suggests that the uptake by H^+/DMP^+ antiport that we measure in right-side-out vesicles is not the efflux system. However, it is possible that we cannot measure DMP^+ uptake driven by the acidic interior of the everted membrane vesicle because of its small internal volume. Against this argument is the fact that we can readily measure the formation of an acidified vesicle interior on substrate oxidation or ATP hydrolysis by uptake of the fluorescent probe quinacrine [21] (unpublished results). Nevertheless, it seems unlikely that there would be both a H^+/DMP^+ antiport uptake and efflux system. For example, tetracycline uptake and efflux occur by different mechanisms [22]. Further characterization of the efflux system is needed to establish that it is indeed a H^+/DMP^+ antiport system.

Although uptake of the lipophilic DMP^+ in response to the transmembrane potential can occur through passive diffusion, antiport mechanisms require a carrier. Preliminary attempts to see if the cations lysine, arginine, spermine, putrescine, spermidine and streptomycin would block DMP^+ uptake by competition for the antiport system in intact cells of AS-1 were not successful (results not shown). The identity of the normal substrate for the antiport system used by DMP^+ remains to be identified.

Acknowledgements

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