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Excretion of glutamate from *Corynebacterium glutamicum* triggered by amine surfactants

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Corynebacterium glutamicum **is used for the industrial production of glutamate. Excretion of the amino acid may be induced by various means. We have analyzed the characteristics of glutamate excretion induced by two amine surfactants, dodecylammonium acetate (DA) and dodecyltrimethylammonium bromide (DTA). Addition of these surfactants induced an immediate efflux of internal glutamate. It also induced a perturbation of the energetic parameters of the cell (decrease of $\Delta\tilde{\mu}_{H}$, decrease of the internal ATP concentration). The efflux was not the result of these perturbations: glutamate is taken up by the cells via an ATP-dependent unidirectional active transport system and no efflux took place as a consequence of an artificial decrease of the energetic parameters. In addition, amine surfactants also induced an excretion of other species, in particular potassium. We have tested the possibility that the effluxes result from a permeabilization of the lipid bilayer by analyzing the interactions between the surfactants and liposomes.**

Introduction

Several strains of *Corynebacterium* and *Brevibacterium* that synthesize glutamate in large amounts are used for the industrial production of the amino acid.

Glutamate efflux from the cells can be induced by various treatments. These include, biotin limitation, and/or addition of various neutral or positively charged surfactants.

Biotin limitation has been shown to decrease the activity of fatty acid synthase which results in a decrease of the amount of phospholipids and in an increase of the saturated/unsaturated acyl chains ratio in the membrane [1–3]. Addition of a neutral surfactant, poly(oxyethylene glycol) acylated by saturated fatty acids, also modifies the membrane lipid composition, the saturated fatty acid of the surfactant being incorporated into the membrane phospholipid, which leads to an increase in the saturated/unsaturated acyl chains ratio [4,5].

It has first been proposed that the efflux of glutamate under biotin limitation was the result of an increased permeability of the membrane due to its lipid alteration [1]. Later, it has been advocated that the efflux, either under biotin limitation or in the presence of the neutral acylated surfactant, was the result of the inversion of the corresponding influx system [6,7]. More recently, it has been convincingly shown that in fact, efflux under biotin limitation is mediated via a special efflux transport system which becomes active under biotin limitation [3,8].

Some industrial processes of glutamate production are based on the addition of a cationic amine surfactant, either alone [9], or in conjunction with the poly(oxyethylene glycol) surfactant [10]. Little is known about the mechanism of efflux triggered by the cationic amine surfactant.

In this paper, we characterize the influx of glutamate which, as described in Ref. 11, takes place via an

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexyl carbodiimide; DA, dodecylammonium acetate; DTA, dodecyltrimethylammonium bromide; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; TPP^+, tetraphenylphosphonium ion; TCA, trichloroacetic acid.

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unidirectional ATP-dependent active transport system. We analyze the effects of the addition of dodecylammonium acetate (DA), a protonable amine surfactant similar to the one used in the industrial secretion of glutamate, on the energetics of *Corynebacterium glutamicum* and on the efflux of glutamate. We show that dodecyltrimethylammonium bromide (DTA), a quaternary positively charged surfactant, also elicits an efflux of glutamate. As a whole, our data indicate that the efflux of glutamate by a cationic surfactant is the result neither of a modification of the membrane lipid composition, nor of an inversion of the influx system, nor of a perturbation of the energetics of the cell. Efflux of glutamate in the presence of any one of the two surfactants may either be facilitated by a membrane protein and/or take place passively via the lipids. In order to determine if the later hypothesis is plausible, we have followed the efflux, induced by the surfactants, of organic solutes entrapped in liposomes.

Materials and Methods

Growth of bacteria

Cells from an industrial strain of *Corynebacterium glutamicum* were grown in erlen flasks on a rotary shaker (260 rpm) in the following medium (per liter): 40 g sucrose; 44.4 ml soy bean hydrolysate neutralized by KOH; 1.3 g $MgSO_4 \cdot 7H_2O$; 1.3 g $(NH_4)_2SO_4$; 2.1 g H_3PO_4; 200 μg thiamin; 500 μg biotin. The pH of the medium was adjusted to 7.4 with ammonium hydroxide. Cells were grown at 30°C to an OD_{650} of 1, harvested, washed once, and resuspended at an OD_{650} of 50 (corresponding to 10 mg cell dry weight per ml) in 25 mM Pipes (pH 7.4) supplemented with 200 mM sorbitol and 150 mM NaCl (assay medium). Cells were kept on ice and used within 4 h following the preparation.

Measurement of $\Delta\psi$, ΔpH and cytoplasmic volume

$\Delta\psi$ was determined from the distribution of [^{3}H]TPP$^+$ according to Ref. 12. [^{3}H]TPP$^+$ (10 GBq/mmol, 10 μM final concentration) was added to 1 ml of the cell suspension (OD_{650} = 2, i.e., 0.4 mg cell dry weight per ml) in the assay medium supplemented with sucrose (4 mg/ml) as an energy source. 100 μl of the cell suspension were filtered on Whatman GF/F glass fiber filters. The filters were washed twice with 4 ml assay medium and counted for radioactivity. A steady-state level of [^{3}H]TPP$^+$ radioactivity was reached in 10 min. TPP$^+$ accumulation was corrected for unspecific binding by substracting a blank obtained under identical conditions, except that the cells were pretreated with the protonophore CCCP (20 μM final concentration).

ΔpH was estimated from the distribution of [^{14}C]benzoate according to Ref. 12 and using centrifugation. 1 ml of a cell suspension (OD_{650} = 2) in the assay medium supplemented with sucrose (4 mg/ml) as an energy source was incubated 5 min with [^{14}C]benzoate (10 μM, 19.7 MBq/mmol) in the presence of ^3H_2O (0.11 MBq/ml). After centrifugation, the pellet and the supernatant were counted for radioactivity. [^{14}C]benzoate accumulation was corrected for unspecific binding by substracting a blank obtained under identical conditions, except that the cells were pretreated with the protonophore CCCP (20 μM final concentration).

The cytoplasmic volume was determined according to Ref. 13 using ^3H_2O (0.11 MBq/ml) and [^{14}C]inulin (6 MBq/ml). The exact volume depended on the experimental conditions (presence or absence of surfactant). In the absence of surfactant, 1 mg cell dry weight corresponded to an internal cell volume of 2.7 μl.

ATP measurement

ATP was measured by bioluminescence using a Biocounter M2010 (Lumac) and a Boehringer kit assay (Mannheim, FRG). 10 μl of the bacterial suspension (0.4 mg cell dry weight/ml) were diluted with 90 μl of dimethylsulfoxide to lyse the cells. 4.9 ml of sterile water were added and 100 μl of this suspension was assayed for ATP measurements.

Determination of the potassium content of the cells

The variations in the potassium content of cells were determined by measuring the changes of the potassium concentration in the external medium (K^+_{out}) with a potassium-valinomycin-selective electrode, associated with a protected calomel reference containing a secondary salt bridge filled with a solution of 2 M NaCl [14]. To estimate the total potassium content of the bacteria, the cation was released from the cells by osmotic shock: cells were diluted 20-fold in ice-cold distilled water; they were then filtered on a 0.45 μm filter (Acrodisc, Gelman Sciences). The amount of released potassium was measured in the filtrate with the potassium electrode. Internal K^+ was calculated from the value of external K^+ and expressed in nmol/mg cell dry weight.

Determination of the glutamate content of the cells

To estimate the total glutamate content of the bacteria, the amino acid was released from the cells either by cold osmotic shock as described above, or by addition of TCA 10%. The amount of glutamate released was then estimated enzymatically (Boehringer kit assay, Mannheim, FRG). Both techniques of glutamate release led to similar values of internal glutamate. This was taken as an indication that the release of glutamate was complete.

Glutamate uptake

The cells (0.4 mg cell dry weight /ml) were incubated at 30°C in the assay medium supplemented with sucrose (4 mg/ml) as energy source, chloramphenicol (0.1 mg/ml) and when needed, the ionophores or inhibitors to be tested. Uptake was initiated by adding [^{14}C]glutamate (10 μM, 0.6 GBq/mmol). Aliquots (100 μl) of the cell suspension were filtered at given intervals on Whatman filters (GF/F); the filters were washed twice with 4 ml of the assay medium and counted for radioactivity.

Glutamate efflux from bacteria upon addition of the surfactants

Glutamate efflux was determined in two different ways:

(1) The cells (0.4 mg cell dry weight /ml) were incubated at 30°C for 10 min in the assay medium supplemented with sucrose (4 mg/ml) as energy source, and chloramphenicol (0.1 mg/ml). Cells were then allowed to accumulate [^{14}C]glutamate (10 μM, 0.6 GBq/mmol) during 15 min. They were then centrifuged 10 min at 4300 $\times g$ and resuspended in the same buffer without glutamate at an OD_{650} of 2. Efflux of [^{14}C]glutamate was initiated by addition of the surfactants. Aliquots (1 ml) of the cell suspension were filtered at given intervals on Whatman filters (GF/F). The filters were then counted for radioactivity without further washing. The amount of efflux was calculated from the radioactivity remaining associated with the cells.

(2) Cells were suspended in the assay medium at an OD_{650} of 2. Efflux of endogenous glutamate was initiated by addition of the surfactant. Aliquots (3 ml) were filtered on Acrodisc 0.45 μm (Gelman Sciences) at given intervals. Glutamate was measured in the filtrate using enzymatic Boeringher kit assay.

Preparation of liposomes

Large unilamellar vesicles (LUV) with entrapped solutes were prepared by reverse phase evaporation [15] using Azolectine (type II-S, Sigma) and trace amounts of [^{14}C]phosphatidylcholine (3.75 MBq/mmol). The solutes to be entrapped ([^{3}H]glutamate (5 μM, 0.74 GBq/mmol) or [^{3}H]lysine (5 μM, 0.74 GBq/mmol)) were added to the incubation medium (25 mM Pipes, 100 mM KCl (pH 7.4)) prior to the formation of liposomes. The vesicle suspension was then sequentially extruded through 0.8, 0.4 and 0.2 μm polycarbonate membranes in order to obtain a uniform size distribution [16,17], and purified by gel filtration on a 15 $\times$ 58 mm Sephacryl S100 column (Pharmacia) to remove nonencapsulated material. The liposomes were kept at 4°C in buffer and used within a week following their preparation. The radioactivity lost during that time was not significant.

The vesicles for fusion studies were prepared identically except that the entrapped solute was omitted and the fluorescent probes NBD-PE and Rh-PE were added to the lipids at a ratio of 1.5 mol% each.

The lipid concentration was calculated from the specific activity of lipids.

Release of the liposome content

Efflux of the radioactive compounds from the liposomes was determined by separating the entrapped from the excreted solute by gel filtration on Sephacryl S100 (Pharmacia).

Liposomes (0.37 mM final lipid concentration) were incubated 10 min in 25 mM Pipes, 100 mM KCl (pH 7.4), in the absence or presence of various concentrations of surfactant. 1 ml of the suspension was layered on the top of the gel (10 ml), prepared in a disposable chromatography column (Econo-Pak, Bio-Rad). The liposomes were eluted with the same buffer and the eluate fractions were counted for radioactivity.

Determination of the detergent bound to the cells or liposomes

The concentrations of dodecylammonium chloride (DA) or dodecyltrimethylammonium bromide (DTA) in the incubation medium were determined with a selective-membrane-electrode associated with a protected calomel reference electrode containing a secondary salt bridge filled with a solution of 2 M NaCl [18]. The DA or DTA electrode potential was calibrated with DA or DTA solutions of known concentrations prepared in buffer. The amount of surfactant bound to the cells or liposomes was calculated from the difference between the concentration measured in the external medium and the known quantity introduced.

Fusion of liposomes

The NBD/Rh resonance energy transfer assay was used to monitor membrane intermixing in the presence of the surfactant [19]. Two populations of liposomes were prepared: labeled vesicles, containing 1.5 mol% of each probe, and unlabeled vesicles. Labeled vesicles were mixed with unlabeled vesicles at a 1:3 ratio, at a total lipid concentration of 0.37 mM in 1.5 ml of 25 mM Pipes/100 mM KCl (pH 7.4). Fluorescence measurements were made in an SFM 25 spectrofluorimeter (Kontron Instruments). The excitation wavelength was 450 nm (excitation of NBD-PE) and the emission wavelength was 520 nm (emission of Rh-PE).

Fusion was also estimated by turbidity measurements at a wavelength of 280 nm and by size distribution of the liposomes, determined by dynamic light scattering in a Coulter 4N MD submicron particle analyser.

Chemicals

[^{3}H]TPP$^+$, [^{3}H]glutamate and [^{3}H]lysine were from Amersham International (Buckinghamshire, UK). ^3H_2O, [^{14}C]benzoate, [^{14}C]inulin and [^{14}C]glutamate were from C.E.A (Seclay, France). DA and DTA were from Aldrich Chimie (Strasbourg, France). NBD-PE and Rh-PE were from Molecular Probes (Eugene, USA).

Results

Effect of $\Delta\tilde{\mu}_H$ and internal ATP on glutamate influx and efflux

The internal glutamate concentration of cells which have been grown and resuspended as described in Materials and Methods was equal to 550 nmol/mg cell dry weight. Upon addition of [^{14}C]glutamate in the external medium (final concentration: 10 μM) there was an accumulation of the radioactive solute within the cell. The time course of accumulation is displayed in Fig. 1. The accumulation reached a stable maximum within 20 min.

We have verified that during the time course of the experiment, the radioactive glutamate taken up by the cell was not transformed. Cells which had accumulated radioactive glutamate for 20 min were centrifuged, resuspended in a medium devoid of glutamate and subjected to an osmotic downshock by a 20-fold dilution in cold distilled water. This resulted in a complete release of the radioactive solutes in the external medium. Thin-layer chromatography revealed that over 99% of the radioactivity was in the form of glutamate.

The kinetics of glutamate influx is hyperbolic with a V_{max} and a K_T of 5 nmol/mg cell dry weight per min and 0,1 mM, respectively. These values are close to those reported for the same transport system in the closely related *Brevibacterium* strain [20]. On the other hand, the K_T is significantly larger than that reported in Ref. 21.

The uptake of glutamate is unidirectional: as shown in Fig. 1, when cells were allowed to accumulate [^{14}C]glutamate, centrifuged and resuspended in a medium devoid of glutamate, there was no efflux of internal glutamate.

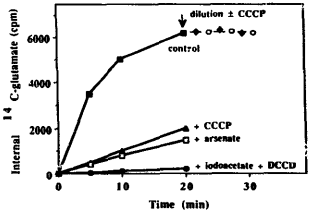

Fig. 1. Transport of glutamate (influx and efflux). Cells were incubated 40 min in the assay medium as described in Materials and Methods without further addition (control) (■), or in the presence of 20 μM CCCP (▲), 2 mM arsenate (□), 300 μM DCCD+1 mM iodoacetate (●). [^{14}C]Glutamate (10 μM final concentration) was then added and uptake was determined over a 20-min period as described in Materials and Methods. Two samples of the control experiment which had accumulated [^{14}C]glutamate for 20 min were centrifuged and resuspended in the same assay medium devoid of glutamate, respectively, in the absence of CCCP (dilution − CCCP, ○) and in the presence of CCCP (20 μM final concentration) (dilution + CCCP, ◆). [^{14}C]glutamate content was then followed over a 10-min period.

We present in Table I the values of the different energy parameters ($\Delta\psi$, ΔpH and internal ATP content) under various conditions at pH 7.4. In the absence of ionophores and inhibitors, $\Delta\psi$ was 190 mV (interior negative) and ΔpH was 30 mV (i.e., 0.5 unit, interior alkaline) resulting in a total $\Delta\tilde{\mu}_H$ of 220 mV. The internal ATP concentration was 32 nmol/mg cell dry weight. The ATP concentration is some five times higher than that usually reported in *Escherichia coli* [22]. This is probably the result of our growth conditions which favor high glutamate synthesis. Addition of 20 μM CCCP, which renders the membrane permeable to protons, immediately collapsed both components of $\Delta\tilde{\mu}_H$; concomitantly, the internal ATP content decreased as a function of time reaching a value of 11

TABLE I

Energetic parameters of Corynebacterium glutamicum under various experimental conditions

$\Delta\psi$, ΔpH and ATP concentration were determined as described in Materials and Methods. ATP concentration was determined after a 40-min incubation in the presence of ionophores and inhibitors. The internal volume required for the calculation of $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_H$ was determined as described in Materials and Methods. In the absence and presence of 170 μM surfactant (DA) it was equal to 2.7 and 1.9 μl/mg cell dry weight, respectively.

	Control	+CCCP (20 μM)	+Arsenate (2 mM)	+DCCD (300 μM) +iodoacetate (1 mM)	+DA (170 μM)
$\Delta\psi$ (mV)	190	0	–	–	98
ΔpH (mV)	30	0	–	–	48
$\Delta\tilde{\mu}_H$ (mV)	220	0	–	–	146
pH_{in}	7.9	7.4	–	–	8.2
ATP (nmol/mg)	32	11	4	0.4	13

nmol/mg cell dry weight. 40 min after addition of the protonophore. Addition of arsenate, which competes with phosphate for ATP synthesis decreased the internal ATP to 4 nmol/mg cell dry weight. Finally, the incubation of the cells with iodoacetate (an inhibitor of glycolysis) and DCCD (an inhibitor of the membrane H^+-ATPase) eliminates all sources of ATP synthesis. In their presence, the internal ATP concentration was practically nil after 40 min incubation.

The effect of $\Delta\tilde{\mu}_{H}$ and internal ATP on glutamate influx are shown in Fig. 1. Addition of CCCP or arsenate prior the addition of [^{14}C]glutamate inhibited its transport only partially; prior incubation of the cells with both iodoacetate and DCCD led to a complete inhibition of glutamate influx.

Once glutamate has been accumulated to its maximal level, the collapse of $\Delta\tilde{\mu}_{H}$ induced by addition of CCCP did not result in a significant efflux of glutamate (Fig. 1).

Influx and efflux of glutamate in the presence of DA

The commercial surfactant used for the industrial excretion of glutamate from the cells consists of a mixture of aliphatic primary amines with predominantly saturated dodecyl chains. In the following we have used a pure synthetic surfactant, dodecylammonium chloride (DA) which affects glutamate influx and efflux, and the energetic parameters of the cells, in the same way as the commercial surfactant.

Interestingly, at low DA concentration (up to 15 μM) there is a 3-fold stimulation of the initial rate of uptake of glutamate (data not shown). True rates of influx could no longer be determined at higher DA concentration due to the subsequent efflux of glutamate which takes place in the presence of the surfactant (see below).

Addition of DA to cells results in an efflux of glutamate. The net efflux was followed either by determining the concentration of endogenous glutamate in the external medium or by first accumulating [^{14}C]glutamate and then determining the amount of [^{14}C]glutamate remaining inside the cells (see Materials and Methods). We have obtained similar results in both cases.

Fig. 2 displays the time course of endogenous glutamate efflux as a function of DA concentration. As already mentioned, in the absence of DA there was no efflux of internal glutamate (control). The efflux of glutamate increased with increasing amount of DA added. 56 μM of DA totally depleted the cells of glutamate within 20 min.

DA affects the energetic parameters of the cells. 10 min after the addition of a concentration of DA which results in a rapid and complete efflux of glutamate (170 μM), $\Delta\psi$ had decreased from 190 mV to 98 mV, while ΔpH had increased from 30 to 48 mV ($\Delta\tilde{\mu}_{H}$ = 146 mV)

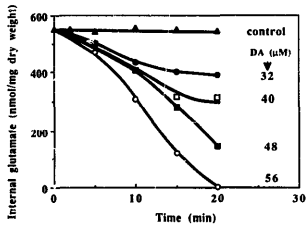

Fig. 2. Efflux of internal endogenous glutamate induced by DA. Cells were suspended at an OD_{650} of 2 (0.4 mg/ml) in the assay medium. Efflux of glutamate was initiated by addition of various concentrations of DA. Internal endogenous glutamate was followed as a function of time as described in Materials and Methods.

(Table I). In a parallel experiment, the internal ATP concentration determined 40 min after the addition of DA (170 μM) had decreased from 32 to 13 nmol/mg cell dry weight (Table I). The decrease in ATP was not the result of cell lysis; indeed, we could not detect ATP in the external medium.

The changes in the energetic parameters are not responsible for the observed efflux. Indeed, addition of CCCP which results in a total collapse of $\Delta\tilde{\mu}_{H}$ and a similar decrease of ATP does not induce any efflux.

DA elicits K^+ efflux independently of glutamate efflux

Addition of DA led to a rapid efflux of internal K^+ initially present inside the cell at a concentration 1800 nmol/mg cell dry weight) (Fig. 3). The initial rate of efflux was dependent on the concentration of DA. For

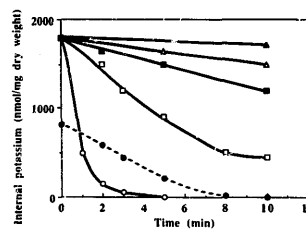

Fig. 3. K^+ efflux induced by DA. Cells were suspended at an OD_{650} of 2 (0.4 mg/ml) in the assay medium. DA was added at various concentrations: 16 μM (▲), 32 μM (△), 48 μM (■), 65 μM (□), 160 μM (○), and the internal K^+ was determined as a function of time as described in Materials and Methods. In a parallel experiment, cells were first depleted of glutamate and K^+ by a cold osmotic shock as described in the text. They were then allowed to accumulate K^+ to an internal concentration of 800 nmol/mg. DA (65 μM) was then added and the internal K^+ was determined as a function of time (●).

a given concentration of DA, it was larger than the initial rate of glutamate efflux by a factor of 5 to 10. This suggests that K^+ and glutamate efflux elicited by DA are not directly coupled. This conclusion is strengthened by the fact that K^+ efflux was also observed independently of glutamate efflux: cells were first depleted of K^+ and glutamate by a cold osmotic shock and then allowed to accumulate K^+ again in the presence of an external energy source but in the absence of glutamate (Fig. 3). Under these conditions, they retain about 800 nmol/mg cell dry weight K^+ which were excreted upon addition of 65 μM DA.

Finally, the efflux of K^+ was not a direct consequence of the decrease of $\Delta\psi$. Addition of 65 μM DA resulted in a relatively small decrease of $\Delta\psi$ (from 190 to 120 mV) yet the efflux of K^+ was rapid (170 nmol/mg cell dry weight per min (see Fig. 3). The same decrease of $\Delta\psi$ brought about by the addition of CCCP did not result in a significant efflux of K^+ (data not shown).

Efflux of glutamate in the presence of DTA; binding of DA and DTA to the cells

DA is a weak base with a pK of 10.6 and therefore exists in solution essentially in a protonated form at physiological pH. However, once inserted into the hydrophobic core of the membrane it may undergo a protonation-deprotonation cycle. We have therefore determined the effect on glutamate efflux of dodecyltrimethylammonium bromide (DTA), a quaternary non protonable amine, which carries a positive charge whatever the pH. The effects are qualitatively similar to those reported for the efflux induced by DA. However, quantitatively, DTA is less effective than DA in inducing an efflux of glutamate (see Fig. 4).

The different efficiencies of DA and DTA in inducing an efflux of glutamate may be related, at least in part, to their difference in cell binding which was estimated using electrodes specific for each of the surfactants (see Materials and Methods). The binding, which reflects the association of the surfactant to both the cytoplasmic membrane and the cell wall, increases linearly with increasing surfactant concentration (Fig. 5). Under our conditions (0.4 mg cell dry weight/ml), binding of DA was 40% of the total DA concentration while binding of DTA was 5% of the total DTA concentration.

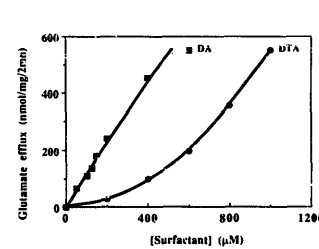

Fig. 4. Comparison of [^{14}C]glutamate efflux induced by DA and DTA. Cells were first allowed to accumulate [^{14}C]glutamate as described under Materials and Methods. Various concentrations of DA and DTA were then added. 1 ml of the suspension was filtered 2 min later. Efflux was determined as described in Materials and Methods.

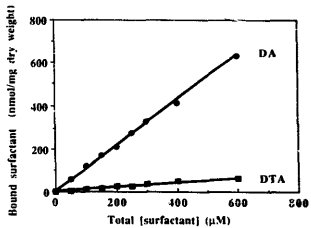

Fig. 5. Binding of DA or DTA to cells. The amount of surfactant bound to the cell (0.4 mg cell dry weight/ml) as a function of the total external surfactant concentration was determined as described in Materials and Methods.

We have also determined the binding of DA and DTA to liposomes (0.37 mM lipid final concentration): under these conditions, binding of DA and DTA was, respectively, 100% and 30% of the total concentration (data not shown). Thus, the difference observed in whole cells is the result, at least in part, of a larger binding of DA relative to DTA, to the lipids of the cytoplasmic membrane.

Interactions of DA and DTA with liposomes

Efflux of glutamate in the presence of any one of the two surfactants may either be facilitated by a membrane protein and/or take place passively via the lipids. In order to determine if the second possibility is plausible we have analyzed the interactions between liposomes and the surfactants.

Large unilamellar vesicles (LUV) composed of azolectin and trace amounts of [^{14}C]phosphatidylcholine were prepared by reverse phase evaporation as described in Materials and Methods, with or without entrapped organic solutes ([^{3}H]glutamate or [^{3}H]-lysine).

We observed that addition of DA, in the concentration range which elicits an efflux of glutamate from the cells, promotes the fusion of the liposomes. The fusion was determined by following the changes in the turbidity of the suspension, in the size of the liposomes and

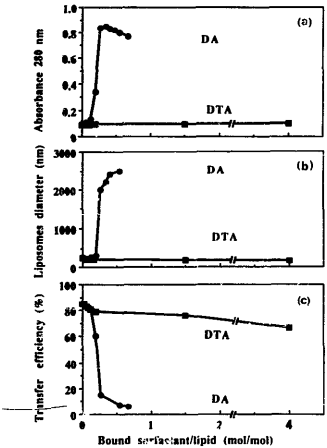

Fig. 6. Interactions of DA and DTA with liposomes. Liposomes were prepared as described in Materials and Methods. DA or DTA was then added. The parameter to be followed stabilized within 5 min after addition of the surfactant. (a) Turbidity followed by absorption measurements at 280 nm; (b) size of the vesicles followed by dynamic light scattering in a Coulter 4N MD submicron particle analyser; (c) energy transfer between NBD-PE and Rh-PE (see Materials and Methods). In all experiments, the final lipid concentration was 0.37 mM.

in the energy transfer. The data are presented in Fig. 6.

The turbidity at 280 nm of the liposome suspension increased drastically over a small range of DA addition (between 50 and 100 μM, i.e., between 0.13 and 0.26 mol of DA bound per mol of lipid) (Fig. 6a)

The increased turbidity paralleled an increase in the size of the liposomes determined by dynamic light scattering (see Materials and Methods). The diameter of the vesicles increased from a mean value of 200 nm (in the absence of DA) to 1800 nm (100 μM DA, i.e., 0.26 mol of DA bound per mol of lipid) (Fig. 6b).

Finally, the fusion of the vesicles was confirmed by fluorescence energy transfer. A population of liposomes containing fluorescent lipids (1.5 mol% NBD-PE and 1.5 mol% Rh-PE) was mixed with liposomes devoid of fluorescent probes. The close proximity of the two probes within a single liposome resulted in an efficient energy transfer. Addition of DA led to a large decrease in the energy tranfer indicating a dilution of the fluorescent probes due to the fusion of liposomes containing the dyes with liposomes devoid of the dyes. The range of DA addition leading to the change in energy transfer coroborates with that leading to a change in the turbidity of the suspension and in the size of the liposomes (from 50 to 100 μM, i.e., 0.13 to 0.26 mol of DA bound per mol of lipid) (Fig. 6c).

In contrast to DA, DTA did not promote a fusion of the vesicles. For up to 5 mM (4 mol of DTA bound per mol of lipid) there was no significant change in turbidity, in size or in energy transfer (Fig. 6). At higher DTA concentrations (larger than 9 mM) a micellisation of the liposomes occurred leading to a decrease of the turbidity (see Fig. 6) and in a decrease in the efficiency of energy transfer (data not shown).

The fusion of liposomes induced by the addition of DA precludes an analysis of the efflux of solutes entrapped within the liposomes. Such studies were performed using DTA, the non-fusogenic surfactant.

Large unilamellar vesicles with entrapped [^{3}H] glutamate or [^{3}H]lysine (see Materials and Methods) were incubated 10 min with a given concentration of DTA, then eluted over a Sephacryl S100 column.

From the elution profiles it is possible to determine, for a given DTA concentration, the amount of solute remaining inside the liposomes relative to that initially present (% solute in). The data are presented in Fig. 7. A complete permeabilization of the liposomes towards both glutamate and lysine is achieved for a DTA concentration of 7.5 mM. It takes place in the concentration range leading to a micellisation of the liposomes as determined by turbidity.

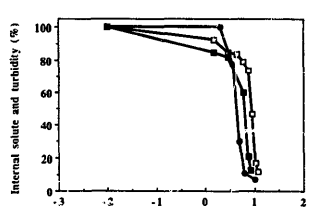

Fig. 7. Glutamate and lysine efflux from liposomes as a function of DTA concentration; comparison with the micellisation of the liposomes. Liposomes (0.37 mM final lipid concentration) were incubated 10 min in 25 mM Pipes, 100 mM KCl (pH 7.4) in the presence of various concentrations of DTA (mM). 1 ml of the suspension was eluted with the same buffer on a 15×53 mm Sephacryl S100 column allowing a separation of liposomes and free organic solutes. The amount of glutamate (■) and lysine (●) remaining inside the liposomes in the presence of DTA were compared to controls obtained in the absence of DTA. The turbidity (□) was determined on aliquots of the liposome suspension.

Discussion

Corynebacterium glutamicum displays an active transport system for glutamate. Initially, it has been proposed that this uptake takes place via $\Delta\tilde{\mu}_{H}$ dependent secondary active transport system [6]. More recently, it has been concluded that the uptake of glutamate takes place via an ATP-dependent primary active transport system [11]. Although this type of transport has first been characterized in Gram-negative bacteria, it has recently been shown to exist in Gram-positive bacteria as well [23,24]. Our data are in agreement with this second type of mechanism. First, complete dissipation of $\Delta\tilde{\mu}_{H}$ by addition of CCCP inhibits only partially the uptake, this inhibition being probably the consequence of the decrease in ATP; second, the uptake is unidirectional as expected for the ATP dependent primary active transport, and once it has taken place, there is no efflux upon addition of CCCP or dilution, as would be expected in the case of a glutamate/H^+ symport.

Addition of DA modifies the influx of glutamate. At low concentrations, there is a stimulation of influx. This may be due to an increased fluidity of the membrane upon insertion of the surfactant; indeed it has been shown that the turnover of numerous membrane transport system increases with increasing fluidity [25].

Addition of DA or DTA results in an efflux of glutamate. This efflux is not a consequence of the perturbation of the energetics of the cell (CCCP does not elicit a significant efflux). Also, it is unlikely that the observed efflux is the result of the inversion of the influx transport system as the ATP-dependent transport system is unidirectional.

As mentioned in the introduction, the efflux of glutamate from *Corynebacterium glutamicum* may be induced in a variety of ways: addition of cationic aliphatic amines, as reported here, addition of a polyoxyethylene glycol, biotin limitation.

Biotin limitation or addition of polyoxyethylene glycol, a neutral saturated surfactant, to cells in culture does not result in the immediate release of glutamate. The efflux takes place after a perturbation of the metabolism which results in a profound modification of the phospholipid/protein ratio of the membrane and of the fatty acid composition of the phospholipids (more saturated) [1–5]. This behaviour contrasts with that observed upon addition of the positively charged saturated surfactants, DA or DTA. Their simple insertion within the bilayer of the membrane results in an immediate efflux of glutamate. This suggests that the positive charge of the surfactant may play a major role in the efflux process.

It has been proposed that efflux, as a consequence of biotin limitation, occurs via a special efflux carrier system different from the one reponsible for influx [3,8]. During this efflux, the internal glutamate concentration remains at or above 120 mM (threshold value). Furthermore, this efflux is inhibited by an inhibition of respiration [8]. The efflux which we report here does not follow these patterns. First, there is no effect of respiration on efflux: addition of cyanide which blocks respiration does not inhibit the efflux induced by the surfactants (data not shown); second, for a large enough concentration of surfactant the efflux goes to completion; finally, under biotin limitation, the efflux system is very specific for glutamate while we report here a large non-specific leakage.

Addition of the cationic surfactant results, in addition to the efflux of glutamate initially present inside the cell at a concentration of 550 nmol/mg cell dry weight, in a large efflux of K^+ initially present inside the cell at a concentration of 1800 nmol/mg cell dry weight. The effluxes are not coupled since a K^+ efflux is observed even if the cells were previously depleted of glutamate. Since the efflux of K^+ is much larger than that of glutamate, another ion must be moving across the membrane to maintain electroneutrality. Finally, the membrane has also become more permeable to H^+ since a decrease in the $\Delta\tilde{\mu}_{H}$ is observed. In addition, another amino-acid, aspartic acid, is excreted upon addition of an amine surfactant (data not shown). However, it should be mentioned that the permeabilization is not complete: the functioning of the respiratory chain is able to maintain a substantial $\Delta\tilde{\mu}_{H}$ and some metabolites are not excreted at all (we could not detect ATP outside the cell).

The effluxes may be mediated either non specifically via a protein component of the membrane (a pore or a channel), or be the result of a general permeabilization of the lipid core of the membrane. We have attempted to determine if the second hypothesis is plausible by following the efflux of organic solutes from liposomes induced by the addition of the surfactants.

DA induces a fusion of the liposomes which precludes such an analysis. DTA does not induce fusion of the liposomes. Nevertheless, we have not observed a notable efflux of solutes entrapped within the liposomes at concentration of DTA below that leading to their micellisation. Thus, the hypothesis of a general permeabilization of the lipid core cannot be directly demontrated using the liposome assay. However, it should be emphasized that a fusion event, such as that induced by DA requires that the bilayer of the two membranes which come in contact be disrupted. The nature of the intermediate structures is largely unknown; yet, various mechanisms propose the formation of transient hydrophilic pores [26]. Although the addition of DA to whole cells clearly does not result in membrane fusion, it may lead to the appearance of lipidic pores through which various solutes and ions may diffuse down their concentration gradient.

The difference in behaviour of DTA and DA in liposomes may reside, at least in part, in the nature of the positive charge. DTA a quaternary amine is always positively charged. This may prevent the distribution of the surfactant between the two leaflets of the bilayer; as a consequence, DTA remains preferentially associated with the external leaflet. In contrast, DA is a primary amine able to undergo a protonation/deprotonation step. This would enable this detergent, like most weak acids or bases, to equilibrate between the two leaflets.

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