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ALTERATION OF ΔΨ-DEPENDENT AMINO ACID TRANSPORTS IN *STREPTOCOCCUS PNEUMONIAE* BY THE ANTITUMORAL DRUG SOAz

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Abstract—Interactions of the antitumoral drug SOAz with natural and model membranes are described. Biological studies were carried out with the bacterium *Streptococcus pneumoniae* taken as a model system. They reveal that SOAz is able to reduce $\Delta\psi$ and the $\Delta\psi$-dependent amino acid transports without being cytotoxic for the bacteria. With respect to model membranes, leakage studies carried out with Na^+ and K^+ loaded lipid vesicles demonstrated that SOAz exhibits no ionophore activity. In contrast, the drug is shown to decrease the surface potential of monolayers of acidic phospholipids but without penetrating within the film. The possibility that SOAz might alter the $\Delta\psi$ part of the proton motive force by decreasing the outside surface potential of the bacterial membrane is discussed.

Interest in inorganic ring systems as anticancer drugs, initially mentioned by Cernov *et al.* [1], was enhanced in the recent past by the finding that the aziridinocyclophosphazenes $N_3P_3Az_6$ and $N_4P_4Az_8$ (Az = Aziridinyl) [2, 3] and mainly aziridinocyclodiphosphathiazenes $(NPAz_2)_2$ $(NSOX)$ (X = F: SOF; X = Ph: SOPHi and X = Az: SOAz) [4–6] were active on a large series of experimental neoplasms. SOAz (Fig. 1) was found to be the most active through the series and its high therapeutic index made its development as an anticancer drug possible.

As SOAz was found to be non-mutagenic for various bacterial systems [7] and showed no significant nephro-, hepato- or cardiotoxicity and controllable hematotoxicity in preliminary tests with dogs and monkeys, it was of interest to explore the actual mode of action of SOAz just as clinical trials [8] were starting.

Recently, we have demonstrated that, *in vitro*, SOAz interacts only slightly and very slowly with DNA molecules without altering their secondary structure [9]. This result, which appears to be in contrast with those reported so far for many anticancer drugs (most of them are known to strongly and rapidly interact with DNA molecules) suggests that DNA is not the primary target of SOAz in malignant cells.

As cytoplasmic membranes are the first site of interaction of any drug with cells, we were interested in checking whether SOAz is capable of damaging membrane functions. This investigation was performed with the bacterium *Streptococcus pneumoniae* which has already proved to be a very good model system for elucidating certain aspects of the cytotoxicity of an antitumoral drug such as methotrexate [10, 11].

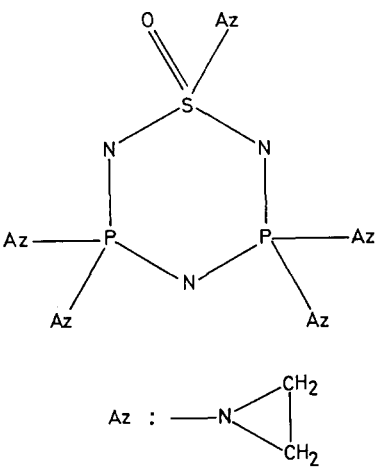

Fig. 1. The SOAz molecule.

Data presented in this report clearly establish that SOAz is able to alter $\Delta\psi$ and the $\Delta\psi$-dependent amino acid transports of the bacterium without presenting any ionophore activity.

MATERIALS AND METHODS

Chemicals. (^{14}C)Amino acids were obtained from Commissariat à l'Energie Atomique (France). Valinomycin, dicyclohexylcarbodiimide (DCCD) and sodium arsenate were of analytical grade. SOAz was synthesized as previously reported [12].

Phosphatidylcholine was extracted and purified from egg yolk [13]. Phosphatidic acid was obtained from phosphatidylcholine by enzymatic digestion with phospholipase D [14]. Phosphatidylglycerol was

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extracted and purified from the lipids of the bacterium *Micrococcus luteus* [15]. Dimyristoylglycerophosphoglycerol was purchased from Medmark (Germany). Salts were of analytical grade. Ultrapure water from an industrial source (Motorola, Toulouse, France) was used for the monolayer experiments.

Monolayer studies. The surface potential ΔV was determined with the apparatus described in [16]. The film surface pressure was measured with a platinum plate connected to a torsion balance of our fabrication. Experimental conditions were identical to those in [16]. Phosphatidylglycerol was spread from chloroform/methanol 5/1 (v/v) solutions. Reproducibility of π and ΔV determinations (carried out at 20°) were ± 0.2 mN/m and ± 15 mV respectively.

Drug–lipid interactions were studied as follows: a concentrated water solution (10^{-1} M, pH 5) of the drug was injected stepwise under films of phosphatidylglycerol compressed at an initial surface pressure π of 13 mN/m ($A = 0.75$ nm^2). π and ΔV were measured at equilibrium, after 1 min stirring of the subphase at each step. In order to ensure a good ΔV recording, the subphase was 50 mM NaCl or 50 mM KCl, at pH 5. At this pH and ionic strength, the lipid can be regarded as fully ionized [17] whereas SOAz is still chemically stable [9].

Assays of ionophoric properties. Multilamellar liposomes (MLV) were prepared either with pure egg-phosphatidylcholine supplemented with 10% phosphatidic acid, or with an equimolecular mixture of pure egg-phosphatidylcholine and dimyristoylglycerophosphoglycerol. Liposomes were loaded either with 100 mM NaCl or 100 mM KCl; then, the preparation was dialyzed and used in 200 mM sucrose. The buffer was 100 mM Tris–HCl, pH 7.5.

Ion-leak was followed with specific glass electrodes, with a Ag/Pt reference electrode. Valinomycin was used to test the method with potassium ions.

Bacterial strain and media. The *Streptococcus pneumoniae* strain used in this study derives from the strain R36A [18]. Bacteria were routinely grown to the late log phase in a peptone (1% v/v) yeast extract (0.4% w/v), NaCl (0.85% w/v) pH 7.8 supplemented with glucose 0.4% (w/v) glutamine (0.3 mM) and asparagine (30 μM) [11].

Transport was assayed in A medium [19] which contains 120 mM NaCl, 52 mM NH_4Cl, 7.5 mM KCl, 20 mM NaH_2PO_4, 40 mM Trizma base, 0.4% (w/v) glucose pH 7.55.

Toxicity determination was achieved in plate test assays using freshly grown bacteria diluted and plated in growth medium supplemented with 2% w/v agar, 0.5% blood and various amounts of SOAz. The plates were incubated at 37° for 12 hr and the number of colonies in the plates containing SOAz was compared to a control without SOAz.

Transport assays. Ten ml of freshly grown bacteria were centrifugated at 4°, 3000 *g*, for 10 min. The pellet, resuspended in 10 ml of A medium (see above), was centrifugated again under the same conditions. Then, the bacteria were resuspended in A medium containing 0.4% glucose at a concentration equivalent to 3 mg of protein/ml. They were kept on ice before use.

For transport measurements, bacteria were diluted 50-fold in A medium 0.4% w/v glucose maintained at room temperature, and allowed to glycolyse for 2 min before the addition of the (^{14}C) amino acid. Then 0.1 ml aliquots were removed at desired times, filtered on glass fibre filters (Whatman GF/B) and rinsed with 3 × 3 ml of A medium. The radioactivity retained by the cells on the filter was determined by liquid scintillation. Blank values were estimated in experiments performed at 4° or with de-energized bacteria.

$\Delta\psi$ (interior negative) was measured by means of the radioactive lipophilic cation ^{14}C-tetraphenylphosphonium (TTP^+) and using the same filtration procedure as described above. In our experimental conditions, steady state accumulation of TPP^+ was obtained for 2–5 min accumulation time at 20°. Blank values were determined with valinomycin (2 μM) treated bacteria in the presence of 50 mM KCl. They represent 5–10% of the signal values.

Inhibitors were used as follows: sodium arsenate, 20 mM, was added to a phosphate-free A medium. Arsenate medium was further used for all the steps of the assay: wash of the cells, resuspension transport, wash of the filter. Valinomycin, 0.6 μM, and SOAz were added in the bacterial suspension kept on ice 10 min before the assay. Dicyclohexylcarbodiimide (DCCD) 0.1 mM was added at room temperature in glycolysing bacteria 3 min before the addition of the (^{14}C) amino acid. The amino acid concentrations and specific activities are given in the legends of the corresponding tables or figures.

Table 1. Effect of SOAz compared to that of DCCD, arsenate and valinomycin on amino acid transports of *S. pneumoniae*

	Arsenate (20 mM)	Valinomycin (0.6 μM)	DCCD (0.1 mM)	SOAz (10 μM)
(^{14}C)Isoleucine (10^{-5} M)	38	57	70	36
(^{14}C)Glutamine (10^{-6} M)	—	82	58	54
(^{14}C)Asparagine (10^{-4} M)	92	11	0	8
(^{14}C)Lysine (10^{-5} M)	90	3	5	0

Figures in this table represent the inhibition of amino acid transports, expressed as % of the control accumulation measured without drug in the assay medium. Steady state accumulation was determined in the standard uptake medium (pH 7.65) containing 7.5 mM KCl. Five minutes incubation were required for isoleucine and ten minutes for asparagine, glutamine and lysine to reach the steady state accumulation. The radioactivity of the uptake medium was adjusted to 0.25 μCi/ml.

With respect to accumulation, calculations were achieved by assuming that 1 ml of bacteria suspension with an optical density of 1 at 540 nm represents an internal water volume of 1 μl, as measured with (^{14}C) hydroxymethylinulin and (^{3}H) H_2O [20]. $\Delta\psi$ was determined from the accumulation factor of ^{14}C-TPP$^+$, using the Nernst equation [21].

RESULTS

SOAz interaction with biological membranes

Specificity of SOAz inhibition on amino acid transport. SOAz is not toxic for *Streptococcus pneumoniae* growth up to a concentration of 10^{-1} M. In order to determine its interaction with biological membranes, we investigated its effect on several amino acid transport systems. It must be reminded here that *Streptococcus pneumoniae* cells are devoid of respiratory systems, and that it is currently admitted that such bacteria rely on the hydrolysis of ATP by their F_1,F_0-ATPase to establish and maintain their electric transmembrane potential ($\Delta\psi$) and pH gradient (ΔpH) [22]. The effects of the classical glycolysis inhibitor arsenate, the F_1,F_0-ATPase inhibitor DCCD as well as the K^+-ionophore valinomycin which are usually used to depress the transmembrane potentials, were checked in parallel. A significant reduction of isoleucine and glutamine accumulation was observed in arsenate, DCCD, valinomycin and SOAz-treated bacteria while lysine and asparagine transports were selectively reduced in arsenate treated cells (Table 1). This shows that in our experimental conditions isoleucine and glutamine accumulation relied on the proton current across the membrane since they are reduced by the ATPase inhibitor DCCD, and more precisely upon $\Delta\psi$, since valinomycin inhibited their uptake. Asparagine and lysine uptake require glycolytic ATP or a derivative but are independent of the proton current since their transports were abolished by arsenate but barely affected by valinomycin or DCCD. As shown in Table 1, SOAz mimics valinomycin and DCCD effects on amino acid transport.

Characteristics of SOAz inhibition on isoleucine transport. Isoleucine is accumulated in *Streptococcus pneumoniae* by a single transporter whose K_T is approx 1 μM and V_i of transport is 10 nmoles/min per mg protein [23]. The kinetic of isoleucine uptake in bacteria treated with 10 μM SOAz (Fig. 2) showed that the V_i of uptake shifts from 4.5 nmoles/min per mg proteins to 2.25 nmoles/min per mg proteins while the accumulation factor decreased from 175 in the control to 90 in SOAz treated bacteria.

As shown in Fig. 3, isoleucine accumulation decreased when the concentration of SOAz in the uptake medium increased from 0.1 nM to 10 μM. A plateau was observed in the SOAz concentration range 10 μM to 0.1 M. This reduction of isoleucine accumulation can be explained by a decrease of the transmembrane potential $\Delta\psi$. Indeed, this parameter, which ranged around 130 ± 5 mV in control cells, in agreement with previous determinations [24] was found to drop by 19 ± 3 mV after addition of SOAz at a final concentration of 0.1 μM in the uptake medium. $\Delta\psi$ decreased by about 40–50 mV for SOAz concentrations of 10^{-5} M and 10^{-4} M. Note

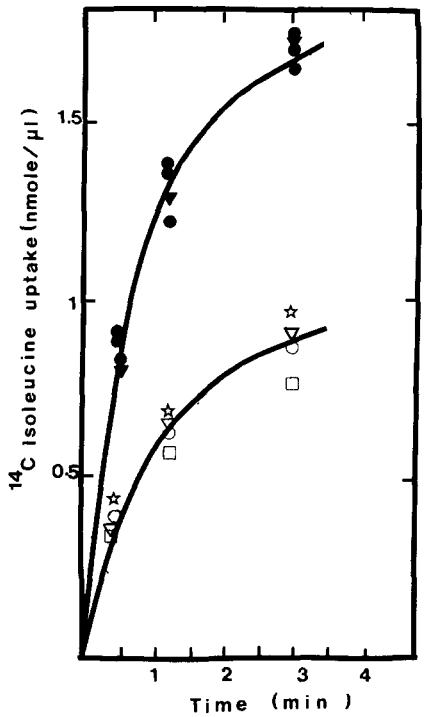

Fig. 2. Effect of SOAz on the kinetics of isoleucine accumulation in *S. pneumoniae*. Bacteria were grown in the absence (●, ☆, ○) or in the presence (▽,▼) of SOAz (10 μM) and washed as described in Materials and Methods. (^{14}C)isoleucine (10 μM; 0.25 μCi/ml) uptake was measured for SOAz concentrations in the uptake medium: 0.01 mM (△, □); 0.1 mM (○); 1 mM (☆).

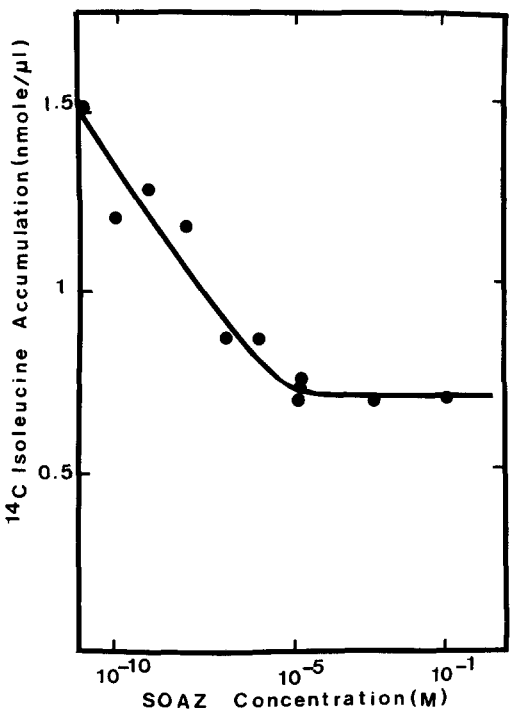

Fig. 3. Inhibition of isoleucine accumulation in *S. pneumoniae* by increasing SOAz concentration in the uptake medium. (^{14}C)isoleucine (10 μM; 0.25 μCi/ml) accumulation was measured after 3 min incubation.

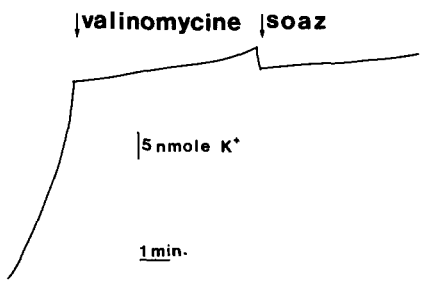

Fig. 4. Potassium leak in multilamellar vesicles (egg-phosphatidylcholine/dimyristoylglycerophosphoglycerol 1/1 mixture). Arrows indicate addition of SOAz and of valinomycin in the suspension medium at final concentrations of 10^{-4} M and 10^{-6} M respectively.

that in this latter case and owing to technical reasons, determination of $\Delta\psi$ became very difficult and poorly accurate.

Then, we checked whether the effect of SOAz on *Streptococcus pneumoniae* was reversible or not. Bacteria were grown during 2 hr (four generation time) in a medium containing the drug at a concentration of 10 μM. When such bacteria were washed (see Methods) and tested for isoleucine uptake, no difference was observed as compared with non-treated bacteria (Fig. 2). In treated and non-treated bacteria, an influence of SOAz on isoleucine transport was observed only when the drug was present in the uptake medium (Fig. 2). This shows that the effect of SOAz on isoleucine transport is reversible.

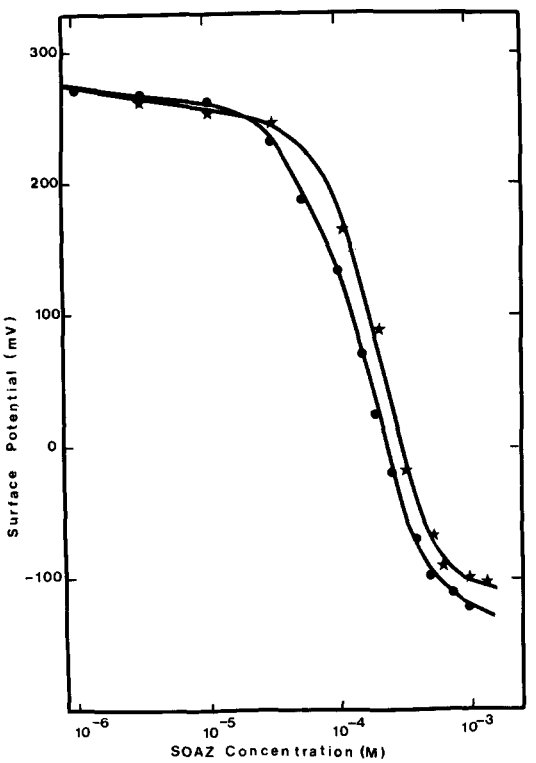

Fig. 5. Changes in surface potential for phosphatidylglycerol in monolayers as a function of SOAz concentration in the subphase. The subphase was: 50 mM NaCl (★) or 50 mM KCl (●), at pH 5.

SOAz interaction with artificial membranes

Lipid vesicles studies. When SOAz was added to a suspension of multilamellar vesicles loaded with potassium or sodium, it was not possible to detect any ion leakage for drug concentrations lower than 10^{-4} M. At this concentration, SOAz provoked a non-specific leakage of both Na^+ and K^+ at a rate 8–10 times slower than that recorded with 1 μM valinomycin in K^+ loaded vesicles. Figure 4 illustrates a typical experiment carried out in the presence of K^+.

It can be concluded that SOAz does not exhibit ionophoric properties towards Na^+ and K^+ and that ion leakages are likely to be excluded in the drug concentration range ($<10^{-5}$ M) where a clear-cut effect of the drug on isoleucine transport and $\Delta\psi$ could be observed.

Monolayer studies. The polar lipids in the membrane of *Streptococcus pneumoniae* are mainly phosphatidylglycerol and diphosphatidylglycerol [10]. In this study, phosphatidylglycerol monolayers were used as mo lel membranes to test the effect of SOAz on lipids. Changes in the surface potential ΔV vs SOAz concentration in the subphase are shown in Fig. 5. Data obtained in the presence of NaCl and KCl were very similar. For both cations, one can observe a large decrease of ΔV (~350 mV) for drug concentration varying from 3.10^{-5} M to 10^{-3} M. No change in surface pressure was detected. These results indicate that SOAz interacts with phosphatidylglycerol polar head groups at the water–lipid interface without penetrating within the lipid molecules in the film.

DISCUSSION

The above results clearly establish that, in our experimental conditions, SOAz can alter $\Delta\psi$ and consequently the amino acid transports which are $\Delta\psi$-dependent, i.e. those of isoleucine and of glutamine. SOAz has no effect on the transport of asparagine and of lysine which are likely to be primary transport processes, independent of $\Delta\psi$. The effect of SOAz appears to be very similar to that of DCCD and of valinomycin.

As a first possibility, this suggests that SOAz could interfere with transmembrane ionic currents, for example by interacting with the F_1,F_0-ATPase which is the major agent responsible for the establishment of the proton motive force $\Delta\mu H^+$ in streptococci [22].

As another possibility, SOAz could act as a ionophore. In fact, the present investigation clearly demonstrates that the effect of the drug on isoleucine transport is reversible and that it does not easily penetrate lipid membranes. It does not provoke any leakage of Na^+ and K^+ loaded lipid vesicles in the concentration range used for transport assays and $\Delta\psi$ determinations. There is no change in the lipid molecular packing in monolayers even for SOAz concentrations as high as 1 mM. In contrast, it is of interest to observe that the drug is able to strongly affect the surface potential of phosphatidylglycerol monolayers. As in our experimental conditions SOAz can be regarded as a neutral molecule, these changes in surface potential might result from

dipole–dipole interactions, including water molecules, between SOAz and the lipid polar head groups at the water–lipid interface. Such a possibility is strongly supported by the remarkable water solubility of SOAz [25]. It has been reported that SOAz can be detected in intracellular material only after several days of incubation with cells (TAIHO Pharmaceutical Co. Ltd., Osaka, Japan, private communication), an observation which is not in contradiction with the reversibility of the effect of SOAz on isoleucine transport. In these conditions, if one assumes that SOAz concentration is higher outside than inside the living cell, a decrease in the outside surface potential compared to the inside potential would bring about a decrease in the transmembrane potential $\Delta\psi$ and therefore a decrease in the $\Delta\psi$-dependent amino acid transports, as it is observed.

The effect of SOAz on amino acid transport in the bacteria and on the surface potential of phosphatidylglycerol in monolayers occurs over different drug concentration ranges. This is not really surprising as direct correlation between a living cell and the very simple model membrane systems monolayers is difficult to achieve.

In particular, the water–membrane interface is certainly much more complex than the water–monolayer interface. As a consequence, the exact drug concentration at the membrane surface for a given drug concentration in the external medium is difficult to assess, and differences in the drug accessibility toward the membrane and monolayer surfaces cannot be excluded. In this respect, a limitation of the drug accessibility towards the membrane surface might explain the apparent saturation effect observed in Fig. 3 for the effect of SOAz on isoleucine transport.

In any case, a noticeable decrease of 30 mV in the monolayer surface potential is already observed for a drug concentration of 10^{-5} M in the subphase. This value correlates well with the 40 mV decrease in $\Delta\psi$ measured for *Streptococcus pneumoniae* for the same drug concentration.

To conclude, it is worthwhile pointing out that, like the antitumoral drugs adriamycin [24], ellipticines [26–28] and methotrexate [10], SOAz provides a novel example of an anticancer drug which is able to interact with cytoplasmic membranes.

REFERENCES

1. V. A. Cernov, V. B. Litkina, S. I. Sergievskaya, A. A. Kropacheva, V. A. Pershina and L. E. Sventsitkaya, *Farmakol. Toksikol.* **22**, 365 (1959).
2. J.-F. Labarre, S. Cros, J.-P. Faucher, G. François, G. Lévy, C. Paoletti and F. Sournies, in *Proc. 2nd Int. Symp. on Inorganic Ring Systems (IRIS), Göttingen*, p. 44. Gesellschaft Deutsches Chemiker (1978).
3. J.-F. Labarre, J.-P. Faucher, G. Lévy, F. Sournies, S. Cros and G. François, *Eur. J. Cancer* **15**, 637 (1979).
4. J.-F. Labarre, F. Sournies, J. C. Van de Grampel and A. A. Van der Huizen, ANVAR French Patent 79-17336, 4 July (1979) (World Extension on 4 July, 1980).
5. J.-F. Labarre, F. Sournies, S. Cros, G. François, J. C. Van de Grampel and A. A. Van der Huizen, *Cancer Letters* **12**, 245 (1981).
6. K. Kitazato, S. Takeda and N. Unemi, *J. Pharm. Dyn.* **5**, 803 (1982).
7. N. Yajima, K. Kondo and K. Morita, Proc. 10th Meeting of the Environmental Mutagen Society, Tokyo, p. 648 (1981).
8. S. Nasca, D. Jezekova, P. Coninx, E. Garbe, Y. Carpentier and A. Cattan, *Cancer Treatment Rep.* **66**, 2039 (1982).
9. M. Manfait and J.-F. Labarre, *Adv. Mol. Relax. Interact. Proc.* **21**, 117 (1981).
10. M.-C. Trombe, M.-A. Lanéelle and G. Lanéelle, *Biochim. biophys. Acta* **574**, 290 (1979).
11. M.-C. Trombe and A.-M. Sicard, *J. Bacteriol.* **121**, 766 (1975).
12. J. C. Van de Grampel, A. A. Van der Huizen. A. P. Jekel, D. Wiedijk, J.-F. Labarre and F. Sournies, *Inorg. Chim. Acta* **53**, L169 (1981).
13. M. B. Lees, in *Methods in Enzymology* Vol. III, p. 331. Academic Press, New York (1957).
14. D. Papahadjopoulos, K. Jacobson, S. Nir and T. Isac, *Biochim. biophys. Acta* **311**, 330 (1973).
15. M. Welby, M.-A. Lanéelle and G. Lanéelle, *Biochimie* **58**, 377 (1976).
16. M. M. Sacré, E. M. El Mashak and J.-F. Tocanne, *Chem. Phys. Lipids* **20**, 305 (1977).
17. F. Lakhdar-Ghazal, J.-L. Tichadou and J.-F. Tocanne, *Eur. J. Biochem.* **134**, 531 (1983).
18. G. Tiraby, M. Fox and H. Bernheimer, *J. Bacteriol.* **121**, 608 (1975).
19. A.-M. Sicard, *Genetics* **50**, 31 (1974).
20. I. R. Booth, W. J. Mitchell and W. A. Hamilton, *Biochem. J.* **182**, 687 (1979).
21. H. Rottenberg, *Methods Enzymol.* **55**, 547 (1979).
22. F. M. Harold, Membrane and Energy Transduction in Bacteria, *Curr. Top. Bioenerg.* **6**, 83 (1977).
23. M.-C. Trombe, M. Coste and A.-M. Sicard, in *Transformation 1978* (Eds. S. N. Glover and L. O. Butler), p. 191. Cotswold Press, Oxford (1979).
24. M.-C. Trombe, G. Lanéelle and A.-M. Sicard, submitted for publication.
25. J. Galy, R. Enjalbert and J.-F. Labarre, *Acta Cryst. C*, in press (1984).
26. E. Goormaghtigh, P. Chatelain, J. Caspers and J. M. Ruysschaert, *Biochim. biophys. Acta* **597**, 1 (1980).
27. E. M. El Mashak and J.-F. Tocanne, *Eur. J. Biochem.* **105**, 593 (1980).
28. F. Tercé, J.-F. Tocanne and G. Lanéelle, *Eur. J. Biochem.* **125**, 203 (1982).
29. F. Tercé, J.-F. Tocanne and G. Lanéelle, *Eur. J. Biochem.* **133**, 349 (1983).
30. F. Tercé, J.-F. Tocanne and G. Lanéelle, *Biochem. Pharmac.* **32**, 2189 (1983).