Permeability Barrier to Hydrophilic Solutes in *Mycobacterium chelonae*

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In order to define the permeability barrier to hydrophilic molecules in mycobacteria, we used as a model a smooth, β -lactamase-producing strain of *Mycobacterium chelonae*. The rates of hydrolysis of eight cephalosporins by intact and sonicated cells were measured, and the permeability coefficient (P) was calculated from these rates by the method of Zimmermann and Rosselet (W. Zimmermann and A. Rosselet, *Antimicrob. Agents Chemother.* 12:368-372, 1977). P ranged from $(0.9 \pm 0.3) \times 10^{-8}$ (benzothienylcephalosporin) to $(10 \pm 3.3) \times 10^{-8}$ cm/s (cephaloridine); i.e., the P values were lower than those reported for *Pseudomonas aeruginosa* and *Escherichia coli* by 1 and 3 orders of magnitude, respectively. The permeability barrier was shown to reduce drastically the stream of drug molecules entering the cell, allowing the rather low level of β -lactamase (0.1 U/mg of protein with penicillin G) to decrease radically the concentration of the drug at the target; this explains the poor in vitro activities of the β -lactams against *M. chelonae*. We also estimated P for small, hydrophilic molecules (glucose, glycerol, glycine, leucine), by studying their uptake kinetics. The values found, ranging from 15×10^{-8} to 490×10^{-8} cm/s, were consistent again with a very low permeability of *M. chelonae* cell wall. The permeation of cephalosporins was not very dependent on the hydrophobicity of the molecules or on the temperature, suggesting a hydrophilic pathway of penetration for these molecules.

It is commonly believed that the mycobacterial cell wall constitutes an efficient permeability barrier, and several properties of mycobacteria, such as acid fastness, the slow rates of growth, and the natural resistance to a wide range of antibiotics, are often thought to be at least partially related to the poor penetration of solutes across the cell wall (11, 35, 37). It is also well known that the mycobacterial cell wall is extremely rich in lipids, which, principally in the form of very-long-chain fatty acids (mycolic acids), account for 30 to 60% of the weight of the wall (24, 35). Such a thick layer of lipidic material obviously acts as a barrier to penetration of hydrophilic solutes. However, mycobacteria must take up nutrients, which are mostly hydrophilic molecules, from the outside medium. Currently little is known about the mechanism of entry of these molecules through the mycobacterial cell wall.

There are some suggestive and qualitative pieces of evidence on the role of the mycobacterial cell wall as a permeation barrier. For example, the sensitivity to antibiotics is increased when the cell wall is altered by growth in the presence of a detergent, such as Tween 80 (16), by the removal of cell wall material through spheroplasting (10), or by mutations that make the cell surface rough (10). In species that produce β -lactamase presumably located inside the cell wall but outside the cytoplasmic membrane, such as *Mycobacterium smegmatis*, *M. phlei*, and *M. fortuitum*, the enzyme is cryptic, suggesting that the cell wall slows down the permeation of the β -lactam substrates (17). Finally, a low permeability of the cell wall had to be postulated in order to explain the level of aminoglycoside resistance in *M. fortuitum* from the activity of aminoglycoside-modifying enzymes found in the cells (42).

Despite the presence of these indirect data, to our knowledge there has been no direct and quantitative measurement of cell wall permeability in mycobacteria. The purpose of

this study was to carry out such a systematic study and, further, to obtain data that would suggest the nature of pathway(s) through which small, hydrophilic molecules penetrate the cell wall barrier.

MATERIALS AND METHODS

Choice of strain to study. *M. chelonae* PS4770 was selected among the fast-growing mycobacteria isolated from clinical specimens at the Pitié-Salpêtrière Hospital, Paris, France, because its characteristics met the requirements of the Zimmermann and Rosselet method (46) used in this study for measuring permeability (see below). Briefly, the strain, identified as recommended elsewhere (36), was found (i) to grow in Middlebrook 7H9 broth (Difco Laboratories) with little clumping, (ii) to produce an adequate constitutive level of β -lactamase, i.e., 0.1 U/mg of protein as measured in crude cell extract by the microacidimetric method (15, 18), using penicillin G as the substrate, and (iii) to show very little β -lactamase leakage (less than 1% of the total activity), as determined by comparing the β -lactamase activities of culture supernatants, intact cells, and cell extracts, using an iodometric method in gel (20) with penicillin G as the substrate.

Growth conditions and preparation of cell suspensions. Preliminary attempts to grow the strain in Middlebrook 7H9 broth showed that homogeneous suspensions could not be obtained unless a high concentration of detergent was added. In order to minimize the cell aggregation without using detergent, the cells were grown by flooding plates of Middlebrook 7H11 agar supplemented with 10% OADC enrichment medium (Difco). After 3 to 4 days of incubation at 30°C, the confluent growth was harvested by careful stirring in ice-cold distilled water. On an average the cells yielded by an agar plate (30 to 60 mg [wet weight] of cells) gave, after suspension in 1 ml of water, a concentration of 7×10^{10} to 15×10^{10} CFU/ml.

Measurement of the permeability to cephalosporins. The

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permeability coefficient (P) was determined by measuring comparatively the rates of hydrolysis of cephalosporins by intact cells and cell extracts. Cell extracts were prepared by sonication at 0°C for 45 min by 20- to 30-s bursts, using a Soniprep 150 sonicator (32 kHz). The sonication resulted in the disruption of $\geq 95\%$ of the cells, as shown by microscopic examination after acid-fast staining.

The Michaelis constant (K_m) of the *M. chelonae* β -lactamase was determined spectrophotometrically by measuring the rate of hydrolysis at 260 nm (or 482 nm for nitrocefin) at different substrate concentrations, using a Hitachi model 100-40 spectrophotometer, and by Lineweaver-Burk double-reciprocal plotting of the data. Cephaloridine, cephapirin, cefazolin, and cephalothin were from Sigma Chemical Co., nitrocefin was from BBL Microbiology Systems, cephaloram and benzothienylcephalosporin (see reference 29 for chemical structure) were gifts of Eli Lilly & Co., and cephacetrile was a gift of CIBA-GEIGY Corp. Since the purity of some of the cephalosporins tested was unknown, we assumed, as in a previous study (29), that under the experimental conditions used, the complete hydrolysis of a 1 mM solution of any compound decreases by 0.8 the optical density at 260 nm in a cuvette of a 1-mm light path. For nitrocefin, the hydrolysis of a 1 mM solution is known to increase the optical density at 482 nm by 1.59 (32). The K_m values (in micromolar) were as follows: cephaloridine, 216; cephapirin, 31; cephacetrile, 88; cefazolin, 88; cephalothin, 22; cephaloram, 35; benzothienylcephalosporin, 37; and nitrocefin, 45.

The rate of hydrolysis by intact cells was measured spectrophotometrically by the method described by Nikaido and Rosenberg (29), modified as follows to minimize cell aggregation: PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)]-NaOH buffer (pH 6.5) was used at the lowest concentration (2 mM) sufficient for keeping the suspension pH in the range 6 to 6.5 during the period of the assay (the β -lactamase activity was shown not to vary within this pH range); MgCl_2 was omitted; and cephalosporin was added at a final concentration of 0.5 mM, except in some experiments (see Results). The cell suspension was diluted to obtain a final turbidity of 100 Klett units. The optical density was recorded over a period of 40 min at 25°C with a lambda 4B spectrophotometer (The Perkin-Elmer Corp.). However, even under these conditions, a slight aggregation of the cells occurred, contributing to the decrease in the optical density at 260 nm, as assessed by controls run without cephalosporin. In order to correct for the optical density decrease due to aggregation, we used a correction factor calculated as follows. At different stages of the hydrolysis of 1 mM solutions of the different cephalosporins, we recorded the spectrum of absorption between 200 and 300 nm and determined the wavelength for which no modification of absorption occurred, i.e., the isobestic point. The isobestic point was 240 nm for cephapirin and cephaloram; 241 nm for cephaloridine; 242 nm for cephacetrile, cephalothin, and benzothienylcephalosporin; and 244 nm for cefazolin. For each batch of cell suspension, we measured simultaneously, by assays performed without cephalosporin, the decreases of optical density at 260 nm and at the different isobestic points and calculated the ratios between the two. In the assays with cephalosporin, the optical density was also simultaneously recorded at both 260 nm ($\text{slope}_{260 \text{ nm observed}}$) and at the isobestic wavelength ($\text{slope}_{\text{isobestic}}$). The ratio was used as a factor to correct the $\text{slope}_{260 \text{ nm observed}}$ for the decrease of optical density related to aggregation ($\text{slope}_{\text{isobestic}} \times \text{correction factor}$), finally yielding the actual decrease related to

the cephalosporin hydrolysis by the cells ($\text{slope}_{260 \text{ nm calc}}$), using the equation $\text{slope}_{260 \text{ nm calc}} = \text{slope}_{260 \text{ nm observed}} - (\text{slope}_{\text{isobestic}} \times \text{correction factor})$. For nitrocefin, a chromogenic cephalosporin, the hydrolysis was recorded at 482 nm, a wavelength at which aggregation produced negligible change in optical density, making correction unnecessary.

One portion of the initial cell suspension was also sonicated and then centrifuged (15 min at $13,000 \times g$), and another portion was only centrifuged. The sonic extract and the cell suspension supernatant thus obtained were assayed for β -lactamase activity under the same conditions as used for the intact cells, except that for the former the duration of the assay was only 10 min. The rate of hydrolysis obtained with the sonic extract was used to determine the V_{max} of the β -lactamase contained in the sonicated cells, and that obtained with the cell suspension supernatant was used to correct the hydrolysis activity of intact cells for the hydrolysis by enzyme which had leaked out. Finally, a portion of the initial cell suspension was dried and weighed to measure the exact concentration of cells used in the assay (mean dry weight, 0.38 ± 0.06 mg/ml).

The permeability coefficient P was calculated by the method of Zimmermann and Rossetlet (46) by combining the Fick first law of diffusion through permeable membranes with the Michaelis-Menten equation governing β -lactamase hydrolysis. Because there are no precise data on the surface area of mycobacteria, we used as an approximation the surface area/weight ratio of *Salmonella typhimurium*, i.e., $132 \text{ cm}^2/\text{mg}$ (dry weight), considering that the sizes of both organisms are in the same range (22, 43) and that even the maximal threefold difference in width (0.3 instead of $0.9 \mu\text{m}$) would affect the surface area/weight ratio by a factor of less than three, for example, 2.76 when the cells are assumed to have a cylindrical middle $1 \mu\text{m}$ long and hemispherical ends.

Determination of the octanol-water partition coefficient. The octanol-water partition coefficient of the unionized form (P_u) was determined for nitrocefin by the method previously used for other monoanionic cephalosporins (30).

Measurement of permeability to small nutrient molecules.

The permeability to small hydrophilic molecules acting as carbon and nitrogen sources was estimated from their uptake kinetics. Cell suspensions were diluted in 2 mM PIPES-NaOH (pH 6.5) and 0.05 mM MgCl_2 to obtain a final turbidity of 1,000 Klett units. [^{14}C]glucose (final specific activity, 2 mCi/mM), [^3H]glycerol (2 mCi/mmol), [^{14}C]glycine (1.6 mCi/mmol), or L-[^3H]leucine (59 mCi/mmol; all from Amersham Corp.) substrate was added to obtain a range of final concentrations of 0.05 to 2 mM (glucose), 0.1 to 10 mM (glycerol), or 0.01 to 1 mM (glycine and leucine). The mixture was kept at room temperature, and a 100- μl sample was removed at 0.5, 2, 5, 10, and 15 min (for the sugars) or at 0.5, 1, 2, 3, and 5 min (for the amino acids), filtered through a 0.45- μm -pore-size HA membrane filter (Millipore Corp.) which was washed then twice with 5 ml of cold distilled water, dried, and counted in a liquid scintillation counter. A portion of the initial cell suspension was dried and weighed to measure the exact concentration of cells used in the assay (mean dry weight, 4 ± 0.8 mg/ml). The rate of uptake by the cells was expressed as nanomoles per milligram (dry weight) of cells per minute.

The V_{max} and K_m values of the overall transport process were calculated graphically from the rates of uptake found for the different concentrations of substrates, by using the double-reciprocal plotting of Lineweaver-Burk.

The permeability coefficient was estimated by assuming that the substrates used penetrate into the cell by first

TABLE 1. Permeability coefficient (P) of *M. chelonae* PS4770 for cephalosporins

Substrate	No. of expt ^a	P (10^{-8} cm/s)	SD
Cephaloridine	11	10	3.3
Cephapirin	1	4.1	
Cephacetrile	3	1.8	0.4
Cefazolin	5	2.3	0.7
Cephalothin	6	2.7	1.3
Cephloram	3	4.2	3.5
Benzothienylcephalosporin	3	0.9	0.3
Nitrocefin	5	7.9	4.1

^a Independent experiments.

diffusing passively through the cell wall and then being actively transported through the cytoplasmic membrane. Therefore, at the steady state, the passive diffusion through the cell wall, which follows the Fick first law of diffusion

$$J = P \cdot A \cdot (C_o - C_p) \quad (1)$$

(where A is the area of cell wall per unit of cell weight) and the active transport through the cytoplasmic membrane, which is governed by the Michaelis-Menten equation, are functioning at the same velocity. By definition, the overall transport system is functioning at half its maximal capacity, i.e., $V_{\max}/2$, when the external concentration (C_o) is equal to the overall K_m (28, 45). Under that condition, the Fick first law of diffusion can be written as

$$V_{\max}/2 = P \cdot A \cdot (K_m - C_p) \quad (2)$$

or

$$P = (V_{\max}/2)/A \cdot (K_m - C_p) \quad (3)$$

C_p , which under the same condition is equal to the true K_m of the active transport system (Michaelis-Menten equation), is unknown, but because of the active and efficient transport system through the cytoplasmic membrane, it can be assumed to be much smaller than C_o (28, 45). Therefore, if C_p is neglected, the value of the overall K_m gives us a minimal estimate of the coefficient of permeability, P . It should be noted that high values of overall transport K_m indicate a low permeability.

RESULTS

Permeability to cephalosporins. The method first described by Zimmermann and Rosselet (46) and already successfully used to measure the permeability of several species of gram-negative bacteria (29, 45) was applied to *M. chelonae*, although significant modifications were necessary to circumvent the technical problems due to the slow aggregation of the cells. Because of the relatively low β -lactamase activity and the low permeability of the strain, the hydrolysis with intact cells was measurable only for eight readily hydrolyzable compounds. The permeability coefficient, P , ranged from $(0.9 \pm 0.3) \times 10^{-8}$ cm/s for benzothienylcephalosporin to $(10 \pm 3.3) \times 10^{-8}$ cm/s for cephaloridine, demonstrating the very low permeability of *M. chelonae* to the tested drugs (Table 1). Even when the uncertainty of the area/weight ratio used for the calculations is considered, these values are markedly lower than those reported for gram-negative bacteria. The permeability of the *M. chelonae* envelope to cephaloridine, cephacetrile, and nitrocefin is 1,000 times lower than that of the *Escherichia coli* envelope and even 10

FIG. 1. Comparative permeability of *M. chelonae* PS4770, *E. coli*, and *P. aeruginosa* to some cephalosporins and sugars. Permeability is expressed as coefficient P , in centimeters per second. The data for *E. coli* are from references 28, 29, and 30 and from J. Hellman and H. Nikaido (unpublished observations), and those for *P. aeruginosa* are from references 25 and 45. In the case of *P. aeruginosa*, glucose has been omitted because it penetrates the cell via a specific channel (13, 41), resulting in a high permeation rate, and has been replaced by fructose, which has the same molecular weight.

times lower than that of the very impermeable envelope of *Pseudomonas aeruginosa* (Fig. 1) (25, 29, 45).

Although the rate of hydrolysis by β -lactamase which had leaked out was always very low (less than 1% of that by cell extracts), this rate nevertheless was significant in comparison with the rate of hydrolysis measured with intact cells (for example, from 6 to 37% [mean, 24%] for cephaloridine), somewhat limiting the precision and the reproducibility of the permeability determinations. But when the assays were performed at different concentrations of cephaloridine (in the range of 0.125 to 1 mM), there were only limited variations in the calculated permeability values (less than 50% variation for a fourfold decrease in concentration), indicating that the methodology followed was correct, without too much interference from enzyme adhering to the cell surface or from damaged cells (14).

In view of the fluctuations observed (Table 1), and to allow more accurate comparisons between the compounds, the permeability coefficient was also expressed relative to that of cephaloridine, which was used as a control in each set of experiments. Figure 2 shows such a set of values as a function of the hydrophobicity of the molecules, expressed as an octanol-water partition coefficient. The range of the relative permeation rates was rather narrow, i.e., an order of magnitude between the highest and lowest values. Cephaloridine, a dipolar ionic compound, penetrated three to nine times faster than the monoanionic compounds. Cephapirin, also a dipolar ion, penetrated barely faster than the monoanionic compounds, with a rate corresponding to 40% of that for cephaloridine. Among monoanionic compounds, the permeation rate changed very little although the hydrophobicity of the molecules increased almost 100-fold from cephacetrile to benzothienylcephalosporin. It has to be pointed out that the latter compound, which is very hydrophobic, penetrated rather slowly. Unexpectedly, nitrocefin, an even more hydrophobic monoanion ($\log P_u = 2.1$), penetrated

FIG. 2. Permeability of *M. chelonae* PS4770 to monoanionic cephalosporins and cephaloridine, expressed as a percentage of the permeation rate of cephaloridine. Hydrophobicity values, expressed as an octanol-water partition coefficient, are from reference 30.

markedly faster than the other compounds of the same group. A similar finding was made with *E. coli*, in which nitrocefin was found to penetrate more than 10 times faster than expected from its hydrophobicity value (30; J. Hellman and H. Nikaido, unpublished results). This particular behavior can be due to the presence of an extended conjugated double-bond system, which could produce dynamic polarization of the molecule.

Since the degree of temperature dependence gives valuable information on the penetration pathways, assays were performed comparatively at 25 and 35°C for cephaloridine, cephalothin, cefazolin, and cephaloram. The permeability coefficients measured at 35°C were only 1.6 to 3 times higher than those measured at 25°C, indicating that the permeation of cephalosporins across the envelope of *M. chelonae* is not strongly temperature dependent.

Permeability to small nutrient molecules. Although the Zimmermann and Rosselet method based on the hydrolysis rate of β -lactams is one of the few methods allowing accurate measurements of the permeability barrier in intact cells (27), the results provided are indeed limited to one class of molecules. Therefore, in order to confirm the very low permeability of *M. chelonae*, the permeability coefficient was also calculated for small nutrient molecules, using the results of uptake kinetic studies. In order to calculate the permeability from the kinetic data, we assumed that the nutrients are actively transported across the cytoplasmic membrane (see Materials and Methods). Such an assumption is justified, since energy-coupled systems of amino acid transport have been found in *M. phlei* (21, 33, 34) and since the uptake kinetics of amino acids and sugars suggested the existence of active transport systems in *M. smegmatis*, *M. phlei*, and *M. tuberculosis* (1, 23, 40, 44), as well as in *Nocardia asteroides*, a species related to the mycobacteria (3).

The K_m of the overall transport of glycerol and glucose ranged from 200 to 1,000 μ M, very high values which suggest a rather low permeability of the cell wall. These values are higher than those published for *P. aeruginosa* (45) and are in the range of those published for highly impermeable, porin-deficient mutants of *E. coli* (29). The K_m values of the overall transport of glycine and L-leucine were lower than those of the sugars, a result consistent with the notion that the affinity of the membrane systems transporting growth factors is

TABLE 2. Permeability coefficient (P) of *M. chelonae* PS4770 for small nutrient molecules

Substrate	Expt ^a	V_{max} (nmol/mg per min)	K_m (μ M)	P (10^{-8} cm/s)
Glucose	1	4.76	1,070	28
	2	2.34	550	27
Glycerol	1	0.57	254	14
	2	0.59	214	17
Glycine	1	2.04	20	620
	2	1.85	31	360
L-Leucine	1	0.28	103	17

^a Independent experiments.

higher than the affinity of those transporting carbon sources (19, 31). The permeability coefficients calculated from the kinetic data ranged from 15×10^{-8} cm/s for glycerol to 490×10^{-8} cm/s for glycine (Table 2). These values confirmed the very low permeability of *M. chelonae* as measured with cephalosporins and are similarly lower than the values reported for *E. coli* and *P. aeruginosa* (28, 29, 45). Not surprisingly, the small solutes tested ($M_w, \leq 200$) penetrated more rapidly than the cephalosporins, which are larger ($M_w, 400$ to 500), a fact previously observed in *E. coli* and *P. aeruginosa* (29, 30, 45).

DISCUSSION

The primary purpose of the present study was to confirm, by direct and quantitative methods, the role of the mycobacterial cell wall as an efficient permeability barrier. Although earlier studies indicated that β -lactamase activity was cryptic in mycobacterial cells (17), the degree of crypticity is not related, in any simple manner, to the strength of the permeability barrier (27). We have shown, by the Zimmermann-Rosselet assay, that the barrier to β -lactam antibiotics in *M. chelonae* is about 3 orders of magnitude less permeable than the outer membrane of *E. coli* and significantly less permeable even than that of *P. aeruginosa*. Since the β -lactam antibiotics act on penicillin-binding proteins that are located on the external surface of the cytoplasmic membrane, the inactivating enzyme (β -lactamase) must be present outside the cytoplasmic membrane, as was indeed found with gram-negative bacteria. Thus the barrier that exists between the external medium and the β -lactamase molecules undoubtedly corresponds to the mycobacterial cell wall.

In view of the data obtained, it is clear that the low cell wall permeability plays an essential role in making *M. chelonae* resistant to many antibiotics, and in this respect the case of the β -lactams is particularly relevant. Because the very effective permeability barrier reduces drastically the stream of β -lactam molecules entering the cell, even the low level of β -lactamase produced (10 to 100 times lower than that of enteric bacteria or pseudomonads carrying R factors or overproducing their chromosomal cephalosporinase) is sufficient to decrease radically the concentration of the drug at the target, explaining the low in vitro activity of the β -lactams against *M. chelonae* (4, 5, 38, 39). For example, our data showed that, when the concentration of cephalothin or cephaloridine was 500 μ M outside the cells, its concentration was only 0.1 to 1 μ M inside the envelope. In contrast, in *E. coli*, the periplasmic concentrations reached by the same cephalosporins were found to be 73 and 787 μ M,

respectively, when their external concentration was 1 mM, even in the presence of the TEM-type β -lactamase at a very high level (28, 30). It is also likely that the low permeability contributes to the resistance of *M. chelonae* to aminoglycosides, since this species probably produces aminoglycoside-modifying enzymes, as does *M. fortuitum*, a closely related species (42). But other relatively hydrophilic drugs such as tetracyclines or chloramphenicol, for which inactivating mechanisms in mycobacteria have not been described, should penetrate the cell envelope, though at low rates, and reach their target. The mechanism of resistance of *M. chelonae* to these drugs (38, 39) remains to be clarified.

The overall transport kinetics for several hydrophilic nutrients showed apparent K_m values mostly in the range of 100 to 1,000 μ M, with the exception of glycine (Table 2). These values are much higher than those found in the similar nutrient transport processes in *E. coli*, for which K_m values of 1 to 10 μ M or less are commonly observed (19, 29). The high apparent K_m values mean low minimal estimates of cell wall permeability, as described in Materials and Methods (Table 2). Although these are only minimal estimates, the true values of the permeability coefficients may not be much higher. The true K_m of the active transport systems, which was measured by Brodie and co-workers with cytoplasmic membrane vesicles from *M. phlei*, is in the range of 3 to 6 μ M for several amino acids (21, 33). Thus C_p in equation 3 is likely to be much smaller than the K_m for overall transport, and our estimated values of P are probably rather close to the correct values.

It has been frequently assumed that the low rate of growth of mycobacteria is related to a permeability barrier impeding the penetration of nutrients (35, 37). Our data suggest that this assumption may indeed be correct, at least in certain cases. For example, from the data shown in Table 2, we can calculate by using equation 1 that glucose, when present in the external medium at 2 g/liter (11 mM or 11×10^3 nmol/cm³), i.e., the concentration in Middlebrook media supplemented with 10% OADC enrichment medium, will diffuse through the cell wall barrier with the maximal rate of 28×10^{-8} cm/s $\times$ 132 cm²/mg $\times$ 11×10^3 nmol/cm³ $\times$ $60 \times 60 = 1,460$ nmol/mg per h, i.e., 263 μ g/mg per h. If we assume that 80% of the glucose is assimilated, this will result in the increase of 210 μ g/mg of cells, i.e., only 21% of the weight of the cell, per h. Obviously in other cases additional factors, such as low efficiency of the active transport systems or of the metabolic pathways, may make significant contributions to the slow rate of growth.

What component of the cell wall constitutes the permeability barrier in mycobacteria? As stated in the introduction, mycobacteria and their close relatives are unique among gram-positive bacteria in the very high lipid content of their cell wall. The lipidic material was for a long time considered as forming an amorphous, unorganized wax, but it has been hypothesized more recently by several laboratories that it may be organized into a bilayer structure, with the hydrocarbon chains directed more or less perpendicular to the plane of the cell wall (2, 9, 10, 24). Although the supramolecular organization of lipids in the mycobacterial cell wall remains to be elucidated, we note that the proposed bilayer structure bears a striking resemblance to that of the gram-negative cell wall or, more specifically, to that of the outer membrane, which constitutes the permeability barrier in gram-negative bacteria.

Our study does not provide direct information on the pathways of penetration of hydrophilic molecules across the mycobacterial cell envelope. Nevertheless, some indirect

indications can be drawn from the data obtained. The permeation through the lipidic phase is known to be strongly dependent on the hydrophobicity of the molecules. The pioneering work of Collander and Bärilund, using the plant cell *Chara ceratophylla* (7), and studies using liposomes (for example, reference 6) demonstrated that, for molecules of similar size, the rate of penetration through lipidic membranes and the hydrophobicity (or the octanol-water partition coefficient) of the molecule vary together, with a ratio of roughly 1:1. The permeation through the lipidic phase is known to be also strongly dependent on temperature. Diffusion of hydrophobic solutes across erythrocyte membranes has Q_{10} values (multiplication factor for an increase of 10°C) between 3 and 6 (12). The temperature dependence can be even greater for a tightly associated, less permeable lipid barrier, and, for example, an increase of 8°C (from 14 to 22°C) resulted in a more than 20-fold increase in the rate of penetration of nafcillin into a rough mutant of *S. typhimurium* (26). Conversely, the penetration through water-filled channels is (i) relatively independent of the hydrophobicity of the molecule or even, in some cases, is inversely related to the hydrophobicity (27) and (ii) increases very little with the temperature, i.e., by a factor of about 1.3 for an increase of 10°C (29). Considering these differences, we believe that our data are significant in showing that even a 100-fold increase in hydrophobicity produced no striking increase in permeability. Although our data on the effect of temperature are not as clear-cut, the Q_{10} values observed, ranging between 1.6 and 3, are lower than the values usually observed with the lipid bilayer of typical biological membranes and strikingly lower than the Q_{10} values of diffusion through the lipid domains of the bacterial outer membrane. Thus, our data suggest that the diffusion of small, hydrophilic molecules through the mycobacterial cell wall takes place mainly in an aqueous environment, not through the lipid interior. This observation strengthens further the analogy with the gram-negative outer membrane, which contains porins with water-filled diffusion channels (31). We are currently studying the mycobacterial cell wall proteins with regard to their pore-forming functions. Finally, the diffusion of hydrophilic solutes through the presumed aqueous pathway does not mean that this is the only available pathway of diffusion through the cell wall. It is quite possible that some hydrophobic solutes traverse the cell wall layer through dissolution into the lipid phase, and this idea is supported by the generally good antimycobacterial activities of hydrophobic antibiotics and also by some direct measurement (8). Our complete understanding of the mycobacterial cell wall permeation barrier will obviously require also the detailed study of this presumed hydrophobic pathway.

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