PHYSICO-CHEMICAL INTERACTION OF MYCOBACILLIN WITH *ASPERGILLUS NIGER* PROTOPLAST MEMBRANE, THE SITE OF ITS ACTION

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Mycobacillin partially quenched the strong fluorescence when 1-anilino naphthalene 8-sulfonate (ANS) was added to protoplast or plasma membrane but is without any effect on weak fluorescence when added to cell-free extract. There are two classes of ANS binding sites on protoplast or plasma membrane of which one class is sensitive to mycobacillin, being competitively abolished by it. Mycobacillin also non-competitively inhibits the binding of pyrene, a lipid specific probe. Thus it follows from the inhibition by mycobacillin of ANS or pyrene binding to protoplast or plasma membrane that the site of action of the antibiotic is located in the plasma membrane. Interaction between mycobacillin and the plasma membrane is physico-chemical in nature.

Mycobacillin[1] causes release of UV absorbing materials from sensitive fungal cells, which is antagonized not only by commercial lipids and sterols but also by those isolated from cells (lipids *e.g.* lecithin with its oleic acid component having unsaturation at the 9 : 10 position with *cis*-configuration or simply oleic acid and sterols *e.g.* cholesterol having 3β-OH group) due to physico-chemical interaction between mycobacillin and the antagonist[2~5]. Recently it has been observed that mycobacillin in its action on a sensitive strain of *Aspergillus niger* G_3Br causes enhanced release of some normally releasable (releasable in small concentration in absence of the antibiotic) specific cell constituents (viz. lysine, proline, ATP, Pi, Na^+, K^+ and Ca^{2+}) leaving undisturbed the intracellular pool concentration of other cell constituents (viz. aspartic acid, alanine, Mg^{2+}, UTP *etc.*)[6]. Interestingly it has also been observed that the antibiotic selectively enhanced the uptake not of non-releasable but of normally releasable cell constituents[7]. The permeability (release and uptake) is controlled by the membrane whose functional activities (viz. study of squid axon membrane[8], D-lactic acid dehydrogenase coupled membrane transport[9], *etc.*) have been investigated with fluorescence probes. The present work has been undertaken to study the binding of mycobacillin to protoplast or plasma membrane using fluorescence probes so as to identify the mycobacillin binding site, characterize membrane structure in the region of binding site and determine the nature of interaction involved in the binding process.

Materials and Methods

Chemicals

Mycobacillin was prepared from the culture filtrate of *Bacillus subtilis* B_3 according to the method of MAJUMDAR and BOSE[1]. *Helix pomatia* extract was purchased from L'Industrie Biologique Francaise Gennevilliers (Seine), France. Both 1-anilino naphthalene 8-sulfonate (ANS, NH_4-salt) and β-glucuronidase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Pyrene was purchased from Fluka, West Germany. All other chemicals were of reagent grade.

Preparation of Protoplast

Protoplasts were prepared from the mycobacillin-sensitive organism *Aspergillus niger* G_3Br by the method of BACHMANN and BONNER[10]. For this purpose a dense spore suspension was prepared by washing the slant with sterile distilled water followed by removal of hyphal filaments by filtration through glass wool. Ten ml of the spore suspension was used as inoculum for each of the conical flasks containing 100 ml of CZAPECK broth. After 24 hours incubation at 37°C under stationary condition the fragile mycelial mat was harvested by filtration through Buchner funnel, care being taken during filtration so as to keep the mycelium moist all the time. It was washed thrice with sterile water. It was then transferred with the aid of the loop handle to a 50-ml Erlenmeyer flask containing 25 ml of 20% sucrose in 0.05 M potassium phosphate buffer, pH 6.8. The flask was slowly shaken for a few minutes. It was repeated twice and then the wet mat was added to a mixture of sucrose - phosphate buffer (25 ml), 0.1 M glutathione (0.2 ml) and 2.0 ml of a mixture of β-glucuronidase and *Helix pomatia* extract (1 : 3). After overnight incubation at 37°C, the contents of the flask were mixed by gentle shaking and the suspension was filtered through glass wool to remove the major portion of hyphal filaments. The debris left could be removed completely by centrifugation ($600 \times g$). Microscopic observation revealed that this suspension contained high concentration of *A. niger* protoplasts. Protoplasts were harvested by centrifugation at $2,000 \times g$ for 10 minutes.

For each experiment a fresh preparation of protoplasts was made as the survival of protoplasts was found to be short.

Preparation of Plasma Membrane

The protoplasts of *A. niger* G_3Br cells were osmotically lyzed in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.1 M $MgCl_2$ and the lysate centrifuged at $5,000 \times g$ for 10 minutes. The pellet so obtained was finally fractionated using a discontinuous sucrose density gradient centrifugation[11]. The gradient was prepared by layering in succession 5 ml of each 40%, 35%, 30%, 25% and 20% sucrose (w/v) in 100 mM Tris-HCl buffer (pH 7.2) containing 0.1 M $MgCl_2$ over a 50% sucrose (w/v) as the cushion. The centrifugation was carried out in a SW 27-rotor at $100,000 \times g$ for 15 hours at 4°C in Beckman ultracentrifuge. The membrane fraction was sedimented at 30% (w/v). The purity of the membrane fraction was checked by chitin synthetase[12] and succinate dehydrogenase[13] activity.

Fluorescence Measurements

Fluorescence studies were carried out in Carl-Zeiss spectrofluorometer (Model No. ZFM/4C). Measurement of ANS fluorescence was carried out with the use of the excitation wavelength at 380 nm and the emission wavelength at 480 nm. Measurement of pyrene fluorescence was done with the use of the excitation wavelength at 335 nm and the emission wavelength at 385 nm.

ANS Binding Studies

ANS binding on protoplast and plasma membrane preparation and the resulting fluorescence were measured according to the method of AZZI[14]. The reaction mixtures whose composition is given in the legends to the Fig. 2 was incubated for 10 minutes until the steady state level of fluorescence was reached. After cooling the incubation mixtures around 0°C and subsequently centrifuging them at 0°C (protoplast at $2,000 \times g$ for 10 minutes and membrane fraction at $30,000 \times g$ for 30 minutes), free ANS in the supernatant and bound ANS in the pellet were measured fluorometrically in the presence of 3% Triton X-100.

Pyrene Binding Studies

Pyrene binding on protoplast or on a plasma membrane fraction was carried out by incubating the reaction mixtures whose composition is given in the legends to the Fig. 3 in various concentrations of pyrene solution at 32°C for 30 minutes. In practice a saturated pyrene solution was prepared in 0.05 M sodium phosphate buffer containing 20% sucrose (pH 7.0) by shaking at 37°C for 4 hours. Based on fluorescence intensity measurements, the pyrene concentration of this preparation was 10 μM. Pyrene solutions of various concentrations were made therefrom by diluting with the same sucrose - phosphate buffer. After incubation, mixtures were cooled down to around 0°C and centrifuged. Free pyrene in the supernatant and bound pyrene in the pellet were measured fluorometrically using the

techniques as described by ARCHIBALD and COCHRANE[15].

Estimation of Protoplast or Plasma Membrane Concentration

The amounts of protoplast or plasma membrane that came up in the mathematical calculations were measured in terms of their protein content. The amount of protein was measured according to the method of GORNALL *et al.*[16].

Quantum Yield of ANS

Quantum yield (ϕ) of ANS was obtained with the help of the equation $\phi=(0.4\times \mathrm{Fmax})/F_{ethanol}$ as developed by AZZI[17], where 0.4, the quantum yield of ANS in absolute ethanol[18]; $F_{ethanol}$, the fluorescence emitted by ANS in absolute ethanol and Fmax, the limiting fluorescence of ANS at infinite protoplast or plasma membrane concentration. Fmax was obtained from the double reciprocal plot of fluorescence vs. protoplast or plasma membrane concentrations both in presence and in absence of mycobacillin (figures not shown). A biphasic curve was obtained both in presence (10 μM) and in absence of mycobacillin. Quantum yields of ANS were found to be 0.42 and 0.43 for the stronger binding phase with protoplast and plasma membrane respectively; 0.29 and 0.28 for the weaker binding phase with protoplast and plasma membrane respectively. Mycobacillin has no effect on the quantum yields of ANS for the two phases of binding (data not shown).

Results

Effect of Mycobacillin on the ANS Binding to Protoplast (or Plasma Membrane)

ANS gives strong fluorescence upon binding with protoplast and plasma membranes (Fig. 1) but very weak one with the cell-free extract (data not shown). The strong fluorescence with protoplast and plasma membrane increased with the increasing concentrations of the probe till it attained the

Fig. 1. Effect of mycobacillin on the interaction of ANS with protoplast (○) and plasma membrane (●).

(A) A given amount of protoplasts (3.4 mg protein/ml) or plasma membranes (1.25 mg protein/ml) suspension in sucrose-phosphate buffer (pH 7.0) was titrated with various concentrations of ANS (0~60 μM) and the fluorescence was measured at each concentration of the probe.

(B) Maximum fluorescence attained in (A) by the given amount of protoplast or plasma membrane in presence of the saturated concentration of ANS was again titrated with various concentrations of mycobacillin (0~20 μM).

Data are the mean of four different sets of experiment.

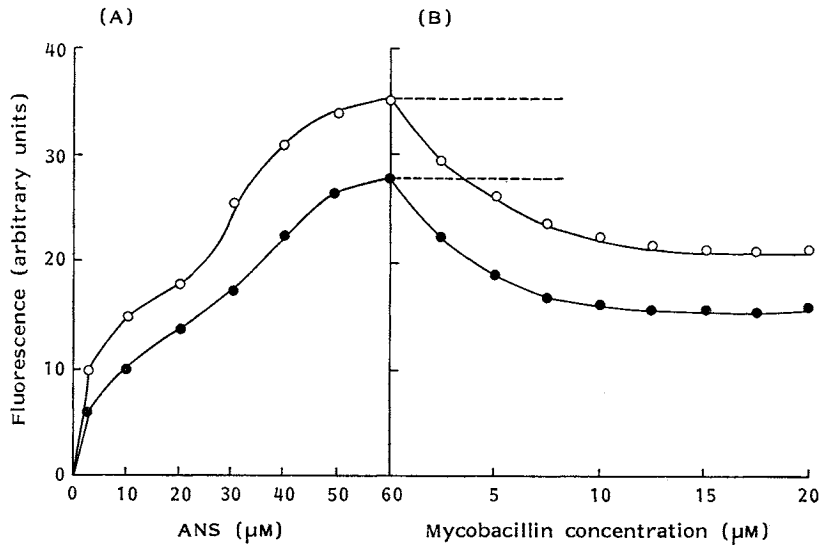
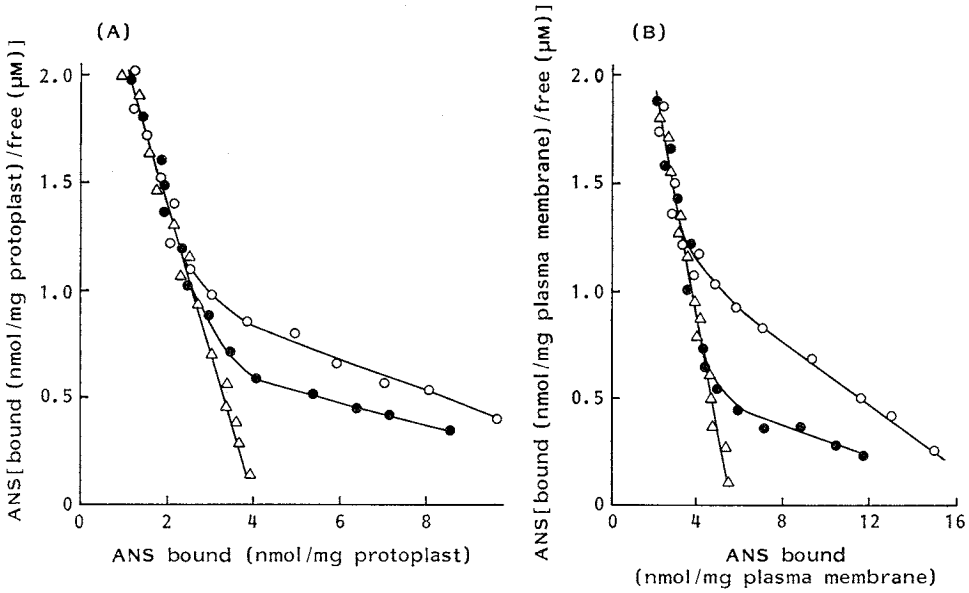


Fig. 2. Scatchard plot of ANS binding to protoplasts (A) and plasma membranes (B) in presence and absence of mycobacillin.

The reaction mixtures (3.0 ml) containing 2.2 mg protoplast/ml or 0.9 mg plasma membrane/ml, 0.05 M sodium phosphate buffer (pH 7.0), 20% sucrose and ANS (in varied concentrations 1.0~50 μM) were incubated as detailed in the text. The concentrations of ANS were determined in supernatants and pellets. Data are the means of three different sets of experiment.

○ Control, ● mycobacillin 10 μM, △ mycobacillin 20 μM.



maximum. If added to the reaction system under condition of maximum fluorescence mycobacillin decreased the fluorescence. This decreased value attained a minimum constant independent of the concentrations of mycobacillin both in the cases of a protoplast and plasma membrane. However, the feeble fluorescence as obtained with the cell-free extract remained unaffected by mycobacillin (data not shown).

Analysis of Mycobacillin Sensitive and Insensitive ANS Binding to Protoplast or Plasma Membrane

ANS bound by a given amount of protoplast or plasma membrane and ANS remaining free in the presence of increasing concentrations of ANS with or without mycobacillin were determined (Fig. 2). The Scatchard plot of the data in absence of mycobacillin indicates that ANS has at least two types of binding both with protoplast or plasma membrane arbitrarily named as A-type (stronger binding) and B-type (weaker binding) whose respective dissociation constants (K_A and K_B) and maximum capacity for bound ANS (n_A and n_B) are presented in the Table 1. In the presence of mycobacillin (10 μM) the A-type of binding of ANS to protoplast or plasma membrane as characterized by K_A and n_A remained unaltered while the B-type by K_B and n_B was decreased (Table 1). Interestingly the value of K_B was increased by mycobacillin which had no effect on the value of n_B. However, the picture was radically altered when ANS binding was studied with a still higher concentration of mycobacillin (20 μM) when ANS showed only A-type of binding (Fig. 2) whose respective dissociation constant and maximum capacity for bound molecules of ANS were the same as before (Table 1), while the B-type of binding was abolished.

Analysis of Mycobacillin-sensitive Pyrene Binding to Protoplast or Plasma Membrane

Pyrene bound by a given amount of protoplast or plasma membrane and pyrene remaining free in the presence of increasing concentrations of pyrene with or without mycobacillin were determined (Fig. 3). The Scatchard plot of the data in absence of mycobacillin indicates that pyrene followed only one type of binding both with protoplast or plasma membrane, whose dissociation constant (K_d) and maximum capacity for bound pyrene (Bmax) were calculated as shown in the Table 2. The Scatchard plot of the data in presence of mycobacillin at 10 μM which decreased pyrene binding to protoplast or plasma membrane also showed one type of binding whose dissociation constant (K_d) and maximum capacity of bound pyrene (Bmax) are also shown in the Table 2. Similar results showing one type of binding were also obtained with a still higher concentration of mycobacillin (20 μM) whose binding parameters (K_d and Bmax) are also shown in the Table 2. Interestingly the value of K_d remained unaltered in the presence and in the absence of mycobacillin whereas Bmax decreased with increase in the concentration of the antibiotic.

Table 1. ANS binding parameters to protoplast or plasma membrane in presence and in absence of mycobacillin.

ANS binding	Concentration of mycobacillin (μM)	ANS binding parameters			
		A-type		B-type	
		Dissociation constant, K_A (μM)	Maximum capacity of bound molecules, n_A (nmol/mg protoplast or plasma membrane)	Dissociation constant, K_B (μM)	Maximum capacity of bound molecules, n_B (nmol/mg protoplast or plasma membrane)
Protoplast	0	1.4	4.1	13.4	15.3
	10	1.5	4.1	20.5	15.2
	20	1.4	4.1	—	—
Plasma membrane	0	1.7	5.6	13.8	18.5
	10	1.7	5.6	28.5	18.5
	20	1.8	5.6	—	—

Data were calculated from the Scatchard plot of ANS binding as indicated in Fig. 2.
—: Binding was not observed.

Table 2. Pyrene binding parameters to protoplast or plasma membrane in presence and in absence of mycobacillin.

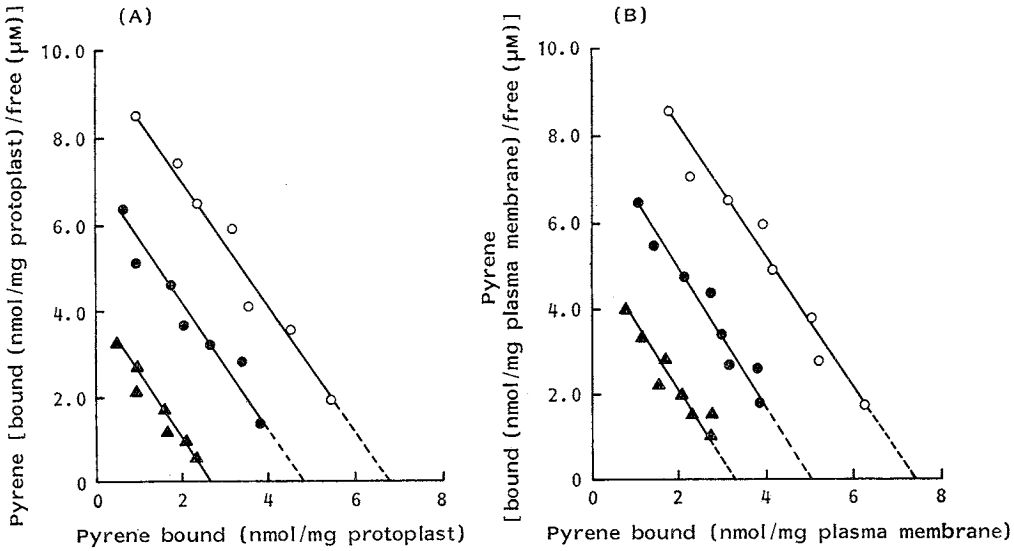
Pyrene binding	Concentration of mycobacillin (μM)	Pyrene binding parameters	
		Dissociation constant, K_d (μM)	Maximum capacity of bound molecules, Bmax (nmol/mg protoplast or plasma membrane)
Protoplast	0	0.68	6.74
	10	0.66	4.71
	20	0.66	2.64
Plasma membrane	0	0.65	7.35
	10	0.63	4.97
	20	0.64	3.24

Data were calculated from the Scatchard plot of pyrene binding as indicated in Fig. 3.

Fig. 3. Scatchard plot of pyrene binding to protoplast (A) and plasma membrane (B) in presence and absence of mycobacillin.

The reaction mixtures (3.0 ml) containing protoplast (2.2 mg/ml) or plasma membrane (0.9 mg/ml), 0.05 M sodium phosphate buffer (pH 7.0), 20% sucrose and pyrene (in varied concentrations 0.1~10 μM) were incubated for 30 minutes at 32°C and centrifuged. Pyrene in the supernatant and in the pellet was measured fluorometrically as described in the text. Data are the mean of three different sets of experiment.

○ Control, ● mycobacillin 10 μM, ▲ mycobacillin 20 μM.



Mycobacillin Effect as a Function of Temperature on the ANS Binding to Plasma Membrane in Presence of the Saturated Concentration of the Probe

The effect of mycobacillin added to a given concentration of plasma membrane suspension containing the saturated concentration of the probe was studied at four different temperatures. This showed that the fluorescence at a given concentration of mycobacillin decreased with increase in temperature. From the double reciprocal plot (change of fluorescence decrement vs. mycobacillin concentration) the values of K_{app} at four different temperatures were graphically determined (Fig. 4) to calculate the thermodynamic parameters *i.e.*, ΔG^0, ΔH^0 and ΔS^0 (Table 3), which showed that the value of ΔG^0, apparent Gibbs potentials were negative. It indicates spontaneity of the reaction between mycobacillin and plasma membrane. It appears from a comparative study of these data that

Fig. 4. Reciprocal plot of fluorescence decrement vs. mycobacillin concentration at four different temperatures.

Fluorescence decrement is the difference between the maximum ANS fluorescence with a given amount of plasma membrane (1.25 mg protein/ml) in absence of mycobacillin and that in presence of each of the different concentrations of mycobacillin. Mycobacillin concentrations were varied from 3 μM to 18 μM.

△ At 22°C, ○ at 31°C, ● at 42°C, □ at 50°C.

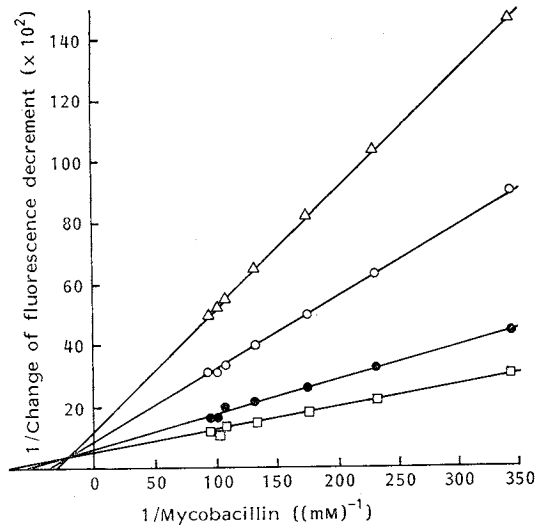


Table 3. Thermodynamic parameters of mycobacillin-protoplast interaction.

The values of thermodynamic parameters were calculated from the following equations applying the method adapted by CHATTERJEE and CHATTORAJ[20].

$\Delta G^0_{av} = RT \text{ in } K_{app}$, where T is the average temperature in between two sets of temperature.

$$\Delta H^0_{av} = \frac{G_1{}^0/T_1 - G_2{}^0/T_2}{1/T_1 - 1/T_2}$$

$$\Delta G^0_{av} = \Delta H^0_{av} - T\Delta S^0_{av}$$

Results are the mean±SD of five determinants.

	Temperature (Å)	K_{app} (μM)	ΔG^0_{av} (kcal/mol)	ΔH^0_{av} (kcal/mol)	$T\Delta S^0_{av}$ (kcal/mol)
1.	295.0	29.0±1.2	—	—	—
	299.5 (av)	—	−6.3±0.2	3.0±1.0	9.3±0.8
2.	304.0	25.0±1.9	—	—	—
	309.5 (av)	—	−6.7±0.3	7.5±0.5	14.2±0.2
3.	315.0	16.0±2.4	—	—	—
	319.0 (av)	—	−7.1±0.1	7.3±0.4	14.4±0.3
4.	323.0	13.0±1.5	—	—	—

although ΔH^0 has got some positive value, that of $T\Delta S^0$ is high enough to make ΔG^0 appreciably negative. The value of ΔG^0 in the process was predominantly affected by $T\Delta S^0$, in other words, the binding process was accompanied by increase of entropy of the system. Thus mycobacillin binding by plasma membrane might be considered more a physico-chemical phenomenon rather than a covalent one.

Discussion

The earlier studies showed that mycobacillin causes not only specific metabolite release as antagonized by cell lipids and sterols[2,6] but also specific metabolite uptake which follows saturation kinetics implicating a carrier-mediated process[7]. The present work has therefore been undertaken to locate the site of mycobacillin action, to chemically characterize the target site, and to determine the physico-chemical nature of the reaction occurring at the target using ANS and pyrene as membrane reacting fluorescence probes. Mycobacillin, although it did not have any effect on the weak ANS fluorescence in presence of cell-free extract of *A. niger* caused a definite decrease in the strong ANS fluorescence in the presence of protoplast or plasma membrane containing the saturated concentration of the probe. Hence the mycobacillin target site might be located on the membrane. The kinetic parameters as obtained by the Scatchard plot of the data on equilibrium binding indicates that one of the ANS binding sites (B-type) was competitively inhibited by mycobacillin (K_B being variable, n_B remaining constant). This might be interpreted to mean that mycobacillin interacts with one class of binding site located on the membrane thereby exerting its antifungal action.

Since the quantum yield of ANS fluorescence (ϕ 0.29) at the weaker binding phase (B-type) which is antagonized by mycobacillin was very much similar to the reported quantum yield of ANS fluorescence obtained when the probe reacted with the lipid moiety of the membrane (ϕ 0.30[19]), the mycobacillin target site might be in its lipid region. In support of our contention a lipid specific probe pyrene which reacts specifically in the hydrophobic lipid region of the membrane was also used in fluorescence studies. The kinetic parameters as obtained by the Scatchard plot of the data on equilibrium binding with pyrene was interpreted to indicate that mycobacillin interacts with only one class of non-competitive binding site (K_d being constant and Bmax being variable) in the hydrophobic lipid region of membrane. Since mycobacillin action has been reported to be antagonized[4] not only by lecithin but also by oleic acid, a constituent of lecithin, the mycobacillin binding site in the lipid region might be further pinpointed at the 9 : 10 unsaturated position with the *cis*-configuration of oleic acid of membrane lipid.

The nature of the reaction occurring at the mycobacillin binding site was then studied by noting

the temperature dependence of the antibiotic effect on the ANS binding to the membrane (Fig. 4). In Table 3, it is shown that the value of ΔG^0 decreased with the increase of temperature, indicating that the reaction between mycobacillin and plasma membrane was favored at higher temperature; in other words structural disorderliness favored the reaction. Therefore the binding reaction between mycobacillin and lipid region of plasma membrane might be physico-chemical in nature.

Thus the site of action of mycobacillin is located in the plasma membrane whose interaction with the antibiotic is physico-chemical in nature.

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