SELECTIVE ACTION OF MYCOBACILLIN ON THE CELLULAR PERMEABILITY OF *ASPERGILLUS NIGER*

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Cells of *Aspergillus niger* normally release not all but of some specific cell constituents *viz.*, lysine, proline, ATP, P_i, Na^+, K^+ and Ca^{2+} in the absence of mycobacillin. Mycobacillin enhances the release of these materials without causing lysis. The time and concentration of mycobacillin for optimum release depends on the nature of the materials involved.

It has been reported that polyene antibiotics can interact with and cause permeability changes in a variety of model and biological membranes[1,2]. Filipin which reacts with membrane cholesterol of *Acholeplasma laidlawii* disrupts membrane structure causing the release of both small ions and large protein molecules in a non-specific manner[3]. Other polyene antibiotics *viz.*, amphotericin B, nystatin and etruscomycin react with cholesterol and specifically alter the membrane permeability by causing aqueous pores of specific size in the membrane[3,4]. Polymyxin, a bactericidial antibiotic which lyses the bacterial membrane much like cationic detergents, releases soluble constituents from the sensitive cells[5,6]. Other polypeptide antibiotics *viz.*, tyrocidines, gramicidins and bacitracin also lyse protoplasts and affect membrane permeability[7~9]. Mycobacillin[10], an antifungal antibiotic, causes release of UV-absorbing materials from sensitive cells[11,12], although it does not cause lysis being fungi-static in action[12]. This effect on permeability was antagonized not only by commercial lipids and sterols[13,14], but also by those isolated from the very sensitive cells due to interaction between the antibiotic and the antagonists[14~17]. The present paper aims at the characterization of released materials and the study of the nature of the releasing process in presence and absence of the antibiotic.

Materials and Methods

Microorganism

A sensitive strain of *Aspergillus niger* (G_3Br) was used throughout the experiments. Mycelial growth (as spherules) of log phase cells (2 days old) grown in Czapeck broth at 32 ± 1°C with shaking was used. Average size of spherules was measured microscopically and was found to be 2.1 mm in diameter.

Preparation of Cell Supernatant

Log phase cells were harvested and washed twice by resuspension in an equal volume of cold 50 mM Tris-maleate buffer (pH 7.0), filtered and uniformly soaked with blotting paper and a definite weighed amount of soaked cells was taken in Erlenmeyer flasks (50-ml), containing 9.0 ml 50 mM Tris-maleate buffer (pH 7.0) and 1.0 ml alcohol or mycobacillin solution in varying concentrations and 100 mg wet weight (corresponding to 20 mg dry weight) of mycelia. The mixture was shaken at 37°C for 24 hours and then filtered to obtain the cell supernatant for future study.

Identification and Estimation of 260 nm Absorbing Materials (Bases and Nucleotides) by Paper Chromatography

The cell supernatant was dried under reduced pressure and the dried mass was then dissolved in a minimum volume of water and chromatographed in the solvent systems (i) butanol saturated with water and (ii) 5% formic acid in water. Spot(s) were then located under an ultraviolet lamp (Hanovia chromatolite with fluorescence filter), according to the method of MARKHAM and SMITH[18]. Each of the spots was eluted with water and then absorption was taken at 260 nm. The standard curve of absorption vs. amount of reference material prepared in the same manner gave the amount of the material present in the cell supernatant. Paper blanks were obtained by cutting out adjacent areas of equal size as recommended by HOTCHKISS[19].

Identification and Quantitative Estimation of Amino Acids by Paper Chromatography

The dried mass (obtained under reduced pressure) of cell supernatant was desalted by extracting in minimum volume of ethanol (containing 10% of 10 M HCl) and was analyzed by two dimensional chromatography in the solvent systems (i) 70% ethanol in water and (ii) butanol - acetic acid - water (4: 1: 1). The amino acids appearing in the chromatogram were then identified and estimated by the ninhydrin method[20].

Inorganic Phosphate Estimation

The cell supernatants were directly used for the estimation of inorganic phosphate according to the method of AMES[21].

Monovalent and Divalent Cation Estimation

Na^+ and K^+ in the cell supernatant were estimated flame photometrically and Ca^{2+} atomic absorption spectroscopically (Perkin Elmer model 303).

Free Amino Acid Estimation in the Cell Pool

Amino acids in the cell pool were extracted according to the method of PILLAI and SRINIVASAN[22]. The cells (500 mg wet weight corresponding to 100 mg dry weight) previously soaked in filter paper were ground with 10 ml of 70% ethanol in water in a glass mortar for about 30 minutes. The suspension was centrifuged and the clear supernatant collected in a conical flask. The solid residue was re-extracted twice in the same manner. All the clear supernatants after centrifugation were pooled and evaporated under reduced pressure to dryness and made up to 1.0 ml with 10% 2-propanol in water. Amino acids were separated and then estimated according to the paper chromatographic technique[20].

Nucleotide and Phosphate Estimation in the Cell Pool

Nucleotides in the cell pool were extracted according to the method of HURLBERT *et al.*[23]. Cells (0.5 g wet weight) were ground with 2 volume of cold 0.6 N perchloric acid (PCA) in a glass mortar for 20 minutes. The suspension was centrifuged and the residue was reextracted twice with 2 volume of 0.2 N PCA in the same manner. All the supernatants after centrifugation were pooled for nucleotide estimation. The pooled supernatant was neutralized with 5.0 N KOH to pH 6.0~7.0 and the mixture was chilled and centrifuged. Nucleotides in the supernatant were estimated according to MARKHAM and SMITH[18]. Another aliquot of PCA extract was used for estimation of phosphate according to the method of AMES[21].

Cation Estimation in the Cell Pool

Cells (0.5 g wet weight) were ashed in a closed muffle furnace. For estimation of Na^+ and K^+, ashed samples were extracted with double distilled water (free from any contaminating ions) and measured directly using a flame photometer. For estimation of Ca^{2+} and Mg^{2+}, ashed samples were extracted with 2.5 ml concentrated HCl, then mixed with 2.5 ml 1 M tartaric acid and 2.5 ml NaCl (1%) and finally the volume was made up to 25 ml with double distilled water, and the cation estimation was made by atomic absorption spectroscopy.

Preparation of Preloaded Cells

In order to perform release experiments of different permeable substrates from preloaded cells,

cells were depleted first and then preloaded with different substrates. Depletion was carried out by suspending the log phase cells in 50 mM Tris-maleate buffer (pH 7.0) at a concentration of 10 mg wet weight cells per ml and incubated with gentle shaking at 37°C for 2 hours. Depleted cells thus formed were collected by filtration and finally preloaded in 100 mM Tris-maleate buffer (pH 7.0) containing in all a concentration of 10 mM non-labeled and labeled substrates, the labeled substrates [0.5 μCi/ml for L-[^{14}C]proline (specific activity 100 mCi/mmol) and L-[^{14}C]lysine (specific activity 240 mCi/mmol), 5.0 μCi/ml for $H_3{}^{32}PO_4$ (specific activity 10 mCi/mmol) and [^{3}H]ATP (specific activity 2,500 mCi/mmol)] were used. In the case of non-labeled substrates cells were preloaded against the 10 mM NaCl or KCl or $CaCl_2 \cdot 2H_2O$ without use of any label. Release against different extracellular substrate concentrations were determined by the difference of radioactivity for lysine, proline, ATP and P_i before and after the incubation of preloaded cells. In the case of other non-labeled ionic substrates the preloaded cells were ashed before and after incubation to determine their ionic concentrations by atomic absorption spectroscopy.

Regarding the preloading experiment with ATP, "a double isotope" method[24] was employed to follow the nucleotide radioactivity. Cells were incubated in 50 mM Tris-maleate buffer (pH 7.0) containing ATP labeled with 8-^{14}C in the adenine ring and ^{32}P in the α phosphorous position. Thereafter the incubation buffer was chromatographically checked to show the absence of ATP degradation products and the ratio of ^{32}P to ^{14}C in the whole cell and that in the initial incubation buffer was 1.40 and 1.42 respectively. These values, being similar, might be taken to mean thant ATP did not undergo cleavage during preloading.

Results

Partial Characterization of Intracellular Pool in Absence of Mycobacillin and Complete Characterization of Materials Released from Cell Pool in Presence and Absence of Mycobacillin

The partial characterization of the cell pool of *A. niger* (G_3Br) indicates the presence of lysine, proline, leucine, aspartic acid, alanine and tyrosine amongst amino acids, ATP, GTP, UTP amongst nucleotides and Na^+, K^+, Ca^{2+}, Mg^{2+}, and P_i amongst cations and anions (Table 1) while the complete characterization of materials released from the cell pool in absence of mycobacillin indicated the presence only of lysine and proline amongst amino acids, only ATP among nucleotides and Na^+, K^+, Ca^{2+} and P_i amongst cations and anions. However the release of ATP needs a comment. ATP, being a highly charged molecule, is rigidly excluded by the membrane[25], although there are indications in the literature that such charged molecule as ATP might be able to cross the cell membrane[26,27].

Interestingly, not all but some of the specific constituents of the cell pool were released. Now considering the fact that some materials like aspartic acid, alanine, tyrosine, leucine, UTP, GTP and Mg^{2+} which were present in fairly good amounts in the cell pool were not released, this might indicate that the presence of so-called released materials in the supernatant were caused by actual release and not by autolysis which was further supported by viability of spherules determined with dye exclusion test[28]. Then again the complete characterization of materials relaesed from the cell pool in the presence of mycobacillin indicates that no materials other than those normally permeable in the absence of mycobacillin were found in the supernatant. The action of mycobacillin was therefore limited only to an enhancing effect on the release of normally permeable materials and this action was specific not in the restricted sense of the term. The fact that the pool constituents like aspartic acid, alanine, tyrosine, leucine, UTP, GTP and Mg^{2+} as referred to above were not released by mycobacillin might be an indication that mycobacillin did not cause extensive non-specific damage to the cell membrane. This was further confirmed by microscopic observation of protoplast made from vegetative cells in presence

Table 1. Effect of various concentrations of mycobacillin on the release of different cellular materials.

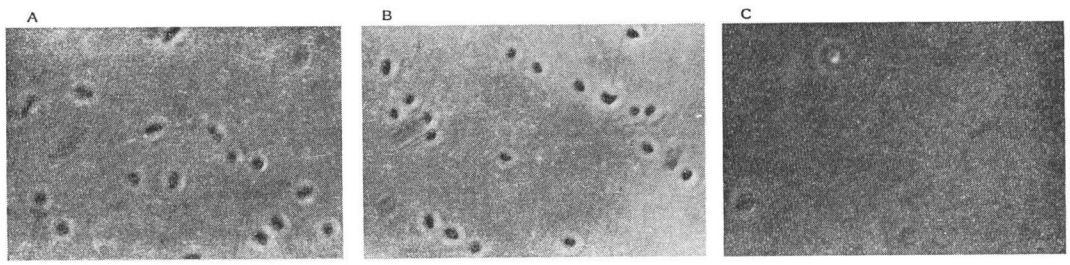
Materials	Amount of materials in the cell pool	Amount of materials released to the cell supernatant in presence of mycobacillin (μg/ml)							
		0	15	20	50	100	150	200	250
Lysine	75.8	23.9 (1.0)	38.2 (1.6)	43.0 (1.8)	55.3 (2.3)	55.6 (2.3)	55.4 (2.3)	55.0 (2.3)	55.4 (2.3)
Proline	823.6	373.8 (1.0)	486.0 (1.3)	521.0 (1.4)	725.0 (1.9)	760.0 (2.0)	762.0 (2.0)	761.0 (2.0)	762.0 (2.0)
Leucine	65.2	—	—	—	—	—	—	—	—
Aspartic acid	291.5	—	—	—	—	—	—	—	—
Tyrosine	155.5	—	—	—	—	—	—	—	—
Alanine	494.5	—	—	—	—	—	—	—	—
ATP	43.5	2.7 (1.0)	3.0 (1.1)	3.5 (1.3)	6.5 (2.4)	20.5 (7.6)	35.1 (13.0)	39.4 (14.6)	39.9 (14.7)
GTP	20.0	—	—	—	—	—	—	—	—
UTP	28.5	—	—	—	—	—	—	—	—
P_i	35.3	12.7 (1.0)	15.2 (1.2)	16.0 (1.3)	19.0 (1.5)	19.2 (1.5)	19.2 (1.5)	19.3 (1.5)	19.2 (1.5)
Na^+	180.2	43.2 (1.0)	69.1 (1.6)	78.2 (1.8)	130.4 (3.0)	156.2 (3.6)	156.4 (3.6)	156.5 (5.6)	156.4 (3.6)
K^+	194.2	86.6 (1.0)	95.3 (1.1)	99.6 (1.2)	109.8 (1.3)	109.8 (1.3)	109.7 (1.3)	109.9 (1.3)	109.7 (1.3)
Ca^{2+}	7.5	2.2 (1.0)	4.2 (1.9)	4.4 (2.0)	4.5 (2.0)	4.6 (2.1)	4.7 (2.1)	4.6 (2.1)	4.6 (2.1)
Mg^{2+}	12.5	—	—	—	—	—	—	—	—

Cell suspension (0.002 g dry weight/ml) was incubated with 9.0 ml 50 mM Tris-maleate buffer (pH 7.0) and in the presence or absence of the antibiotic (1.0 ml). Release of different cellular materials to the suspending fluid was measured after 24 hours incubation. Amount of materials in the cell pool were measured as described in the text. Amounts were expressed in terms of nmol/mg cell dry weight. Figures in the parentheses indicated the number of fold increment of release in respect of the control sample.

— Not released.

Fig. 1. Phase contrast microgrphs of untreated and mycobacillin or Triton X100 treated *A. niger* protoplasts.

A: Untreated, B: mycobacillin (50 μg/ml)-treated, C: Triton X100 (2%)-treated.



and absence of mycobacillin and also in presence of a lytic detergent Triton X100 which clearly shows that the cells of *A. niger* did not undergo lysis in presence of the antibiotic (Fig. 1).

Metabolite-ion Release as a Function of Mycobacillin Concentration

Table 1 shows the effect of various concentrations of mycobacillin on the release of different celluar materials. Release appears to be a function of mycobacillin concentration till it attained a constant value irrespective of its concentration. The concentration of mycobacillin required for maximal release of a given material varies with the material, approximately 50 μg/ml for lysine, P_i and K^+; 100 μg/ml for proline, Ca^{2+} and Na^+; 200 μg/ml for ATP. The maximum enhancing effect of mycobacillin on release varied from 1.3~3.6 fold depending on the nature of the material except ATP, in whose case the enhancing effect was 14.6 fold.

Kinetics of Metabolite-ion Release in Presence of Different Concentrations of Mycobacillin

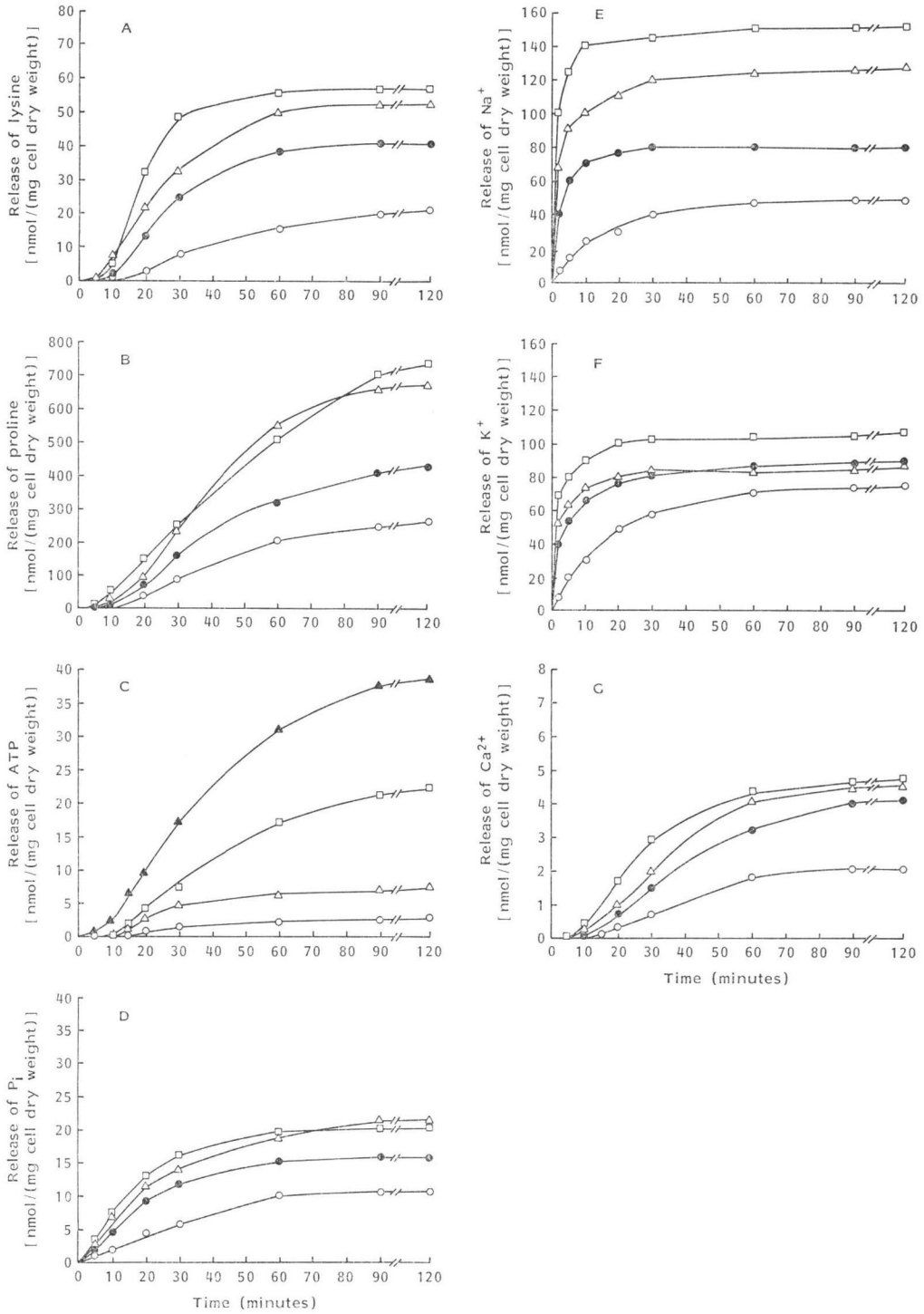
Studies on kinetics of release (Fig. 2A~G) in absence and presence of various concentrations of mycobacillin indicate that Na^+, K^+ and P_i did not show any time lag whereas other permeable materials like lysine, proline, ATP and Ca^{2+} showed lag which was partly reduced in presence of mycobacillin. The time lag for highest concentration of mycobacillin approximated to 5~7 minutes for lysine and 12~15 minutes for proline and Ca^{2+} and 5 minutes for ATP. The lag was followed by linear release till it became constant at a given time which varied from one material to another. In case of Na^+, K^+ and P_i which did not show any lag the pattern of release was the same as the above.

Release from Preloaded Cells as a Function of Extracellular Concentration in Absence and in Presence of a Given Concentration of Mycobacillin

It appears from Fig. 3 that with the increase in extracellular cold proline concentration, release of labeled proline from the preloaded cells in absence of mycobacillin gradually decreased and then attained a minimum constant value, independent of extracellular proline concentrations. In presence of mycobacillin (*viz.*, 200 μg/ml and 100 μg/ml) the similar pattern showing constant minimum release independent of extracellular substrate concentrations was observed, though the minimum releasable values were higher for the higher concentrations of the antibiotic. Similar observations were also noted for other permeable materials *viz.*, lysine, ATP, P_i, Na^+, K^+ and Ca^{2+} both in absence and in presence of mycobacillin (Figures not shown).

Fig. 2. Effect of various concentrations of mycobacillin on the kinetics of release.

Cells (10 mg wet weight/ml) were suspended in 50 mM Tris-maleate buffer (pH 7.0) and incubated with shaking at 37°C for different lengths of time. Mycobacillin was added at zero time. ○ Control, ● 20 μg/ml, △ 50 μg/ml, □ 100 μg/ml, ▲ 200 μg/ml.



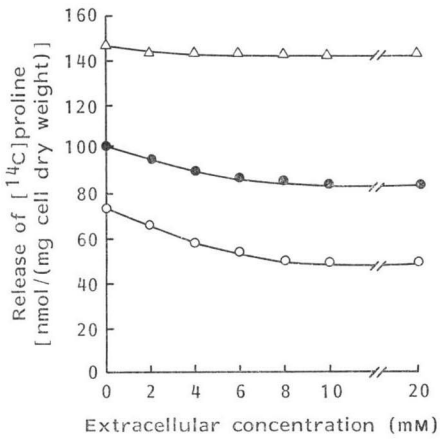
Discussion

While studying the permeability barrier as affected in sensitive cells by mycobacillin, it is observed that the *A. niger* cells, irrespective of the presence of mycobacillin, possessed specific sites for the release only of lysine and proline amongst amino acids, ATP amongst nucleotides and Na^+, K^+, Ca^{2+} and P_i amongst cations and anions. The presence of mycobacillin merely enhanced the release not of any metabolites or ions but of those only which were normally permeable even in absence of mycobacillin. Unlike many other polyene macrolide and polypeptide antibiotics, since mycobacillin did not cause lysis of protoplasts nor even extensive damage, the action being limited to some specific sites, the antibiotic might act as another probe in the study of membrane structure and function.

Release as a function of mycobacillin concentrations indicates the presence of some specific mycobacillin-sensitive sites on the cells which differed in the degree not only of their mycobacillin sensitivity but also of their efficiency in causing release. The fact that these mycobacillin-sensitive sites were all different was also supported by studies on kinetics of release. This showed absence of time lag for release of some cellular constituents or its variation when there was time lag for release of others and also the variation in the time period required for maximum release in presence of optimum concentration of mycobacillin. Interestingly release by preloaded cells appears to be independent of extracellular substrate concentration, the process being sensitive to mycobacillin.

Fig. 3. Effect of mycobacillin on release of [^{14}C]-porline from preloaded cells.

Washed pre-equilibrated cells (5 mg wet weight/ml) were suspended in 0.1 M Tris-maleate buffer (pH 7.0) containing various concentrations (0~20 mM) of non-labeled substrate with or without mycobacillin and incubated for 2 hours at 37°C with gentle shaking. Cells were then filtered and radioactivity was counted on the filter paper. The released amounts of [^{14}C]proline were determined by the difference of radioactivity remaining in the cell (and also in filtrate if necessary) before and after the incubation of preloaded cells. The preloaded amount of [^{14}C]-proline was 250.0 nmol/mg cell dry weight. ○ Control, ● 20 μg/ml, △ 100 μg/ml.



Thus it is suggested that *A. niger* inherently possessed some selective sites causing weak release of some specific cell constituents and these sites happened to be sensitive to mycobacillin action resulting in enhanced release when exposed to it. Furthermore these sites were located on the cell surface probably on membrane whose ubiquitous components lipids or sterols were sensitive to mycobacillin as indicated by the antagonization of mycobacillin action by exogenous addition of commercial lipids or sterols[14,20]. Interestingly mycobacillin appears to serve as interesting probe for membrane studies.

Acknowledgments

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