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Selective action of mycobacillin on the uptake of releasable cell materials by *Aspergillus niger*

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The uptake of normally releasable (i.e. releasable in the absence of the antibiotic) cell constituents (namely lysine, proline, ATP, P_i, Na^+, K^+ and Ca^{2+}) by sensitive cells of *Aspergillus niger* that occurs in the absence of mycobacillin is gradually enhanced with increase in concentration of the antibiotic until the uptake attains the maximum. With still higher concentrations the uptake decreases until it becomes the same as in the control without mycobacillin. Uptake follows saturation kinetics both in the absence and in the presence of the antibiotic. Mycobacillin significantly increases $V_{max.}$ for uptake with any effect on K_m. Mycobacillin has no action on the uptake of non-releasable materials.

INTRODUCTION

Mycobacillin (Majumdar & Bose, 1958) causes enhanced release of some normally releasable (i.e. releasable when cells were exposed to buffer in the absence of the antibiotic) specific cell constituents (namely lysine, proline, ATP, P_i, Na^+, K^+ and Ca^{2+}) from the sensitive strain, leaving undisturbed the intracellular pool concentrations of other cell constituents (namely alanine, aspartic acid, Mg^{2+}, UTP etc.). In other words mycobacillin did not disorganize or even affect permeability barriers except at certain particular sites, causing release of specific cell materials. In this respect mycobacillin differs firstly from filipin, tyrocidin, polymyxin etc., which cause major disorganization of the cytoplasmic membrane, releasing non-specifically both large and small molecules (Cerny & Teuber, 1971; De Kruijff *et al.*, 1974; Gale *et al.*, 1981), secondly from gramicidin A, nystatin, amphotericin etc., which cause non-specific release of small soluble materials and ions through transmembrane channels (Kinsky, 1961; Chappell & Crofts, 1965; Goodall, 1970; De Kruijff *et al.*, 1974), and thirdly from valinomycin, enniatins etc., which cause the uptake of specific univalent cations, the antibiotic itself acting as a carrier (Moore & Pressman, 1964; Bhattacharya *et al.*, 1971; Ovchinnikov, 1979). In view of these diversified actions of membrane-active antibiotics, it was decided to examine whether mycobacillin enhances specific uptake as it does specific release.

MATERIALS AND METHODS

Chemicals and radiochemicals

Mycobacillin was prepared from the culture filtrate of *Bacillus subtilis* B_3 by the method of Majumdar & Bose (1960). Carbonyl cyanide *m*-chlorophenylhydrazone, actinomycin D, cycloheximide, ATP, ADP, AMP, adenosine, IMP, inosine and hypoxanthine were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [α-^{32}P]ATP (triethylammonium salt) and [8-^{14}C]ATP (tetrasodium salt) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. $H_3{}^{32}PO_4$, [^{3}H]ATP, L-[^{14}C]lysine, L-[^{14}C]proline, L-[^{14}C]aspartic acid and L-[^{14}C]alanine were all purchased from Bhabha Atomic Research Centre, Trombay, India. All other chemicals used were reagent grade from commercial sources.

Organism

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

Preparation of depleted cells of *A. niger*

Exponential-phase cells were harvested and washed by resuspension in an equal volume of ice-cold 50 mM-Tris/maleate buffer, pH 7.0, filtered and uniformly soaked with blotting paper, and then suspended in 50 mM-Tris/maleate buffer, pH 7.0, at a concentration of 10 mg wet wt. (≡ 2 mg dry wt.) of cells/ml of buffer and incubated for 2 h with gentle shaking at 37 °C. Depleted cells thus formed were collected by filtration.

Uptake of ATP by depleted cells

This was studied by using the method of Chaudry & Baue (1980). Washed depleted cells were incubated for 1 h with shaking at 37 °C in incubation medium (10 ml), harvested (with retention of cell-free supernatant for further study) and washed with ice-cold incubation buffer until the washings were free of radioactivity. Cells were homogenized (Hurlbert *et al.*, 1954) and extracted first once with 2 vol. of ice-cold 0.6 M-$HClO_4$ and then twice with 2 vol. of 0.2 M-$HClO_4$ in a glass mortar, and the extract was centrifuged, neutralized with 5.0 M-KOH to pH 6.0–7.0 and finally the mixture was chilled and centrifuged. Samples (50 μl) of the cell pool and cell-free supernatant of the incubation medium were separately applied to a Whatman 3MM paper strip, as also were 10 μl volumes of marker solutions (5 mM) of ATP, ADP, AMP, adenosine, IMP, inosine and hypoxanthine. After electrophoretic separation by the method described by Wadkins & Lehninger (1963), spots were located under a u.v. lamp (Hanovia Chromatolite with fluorescence filter), cut from the paper and placed in a counting vial together with 5 ml of liquid-scintillation fluid. Radioactivity was then counted in a Packard scintillation counter.

Table 1. Uptake of ATP by depleted cells of *A. niger*

Depleted *A. niger* cells (0.25 g wet wt. ≡ 0.05 g dry wt.) were incubated for 1 h at 37 °C in 10 ml of the incubation mixture containing 50 mM-Tris/maleate buffer, pH 7.0, and 5 mM-ATP {including 2 μCi of [α-^{32}P]ATP (sp. radioactivity 12.0 Ci/mmol) and 2 μCi of [8-^{14}C]ATP (sp. radioactivity 2.8 Ci/mmol)}. After the incubation cells were harvested and processed and radioactivities of samples were determined as described in the text.

	Radioactivity (c.p.m./50 μl)		^{32}P/^{14}C ratio
	^{32}P	^{14}C	
Extracellular-medium nucleotides*			
ATP	2201	1572	1.40
Intracellular nucleotides*			
ATP	2075	1461	1.42
ADP	1125	803	1.40

* No radioactive spots other than those indicated.

The cell-free supernatant of the incubation system contained only ATP as initially added and none of its degradation products (Table 1), which shows that ATP was not extracellularly degraded. However, the intracellular pool of the harvested cells from the incubation system contained both ATP and ADP. Interestingly, the ^{32}P/^{14}C ratios in ATP both from the cell-free supernatant and from the cell pool were almost identical, which shows that intact molecules of ATP were taken up by the cells. The ^{32}P/^{14}C ratio in ADP in the cell pool was the same as that in ATP both inside and outside the cells, which indicates that ATP after penetration as intact molecules was partly degraded intracellularly to ADP. These preliminary experiments were done to prove that intact ATP molecules were taken up by the cells, even though they are highly charged molecules that are not easily released or taken up. Uptake of intact ATP has also been reported in other biological systems (Gajdos *et al.*, 1968; Chaudry & Gould, 1970; Winkler, 1976; Pant *et al.*, 1979).

Uptake of different substrates by depleted cells under conditions of non-incorporation and of incorporation into cell polymers

Uptake was performed in an incubation mixture (2.0 ml) containing 20 mM-Tris/maleate buffer, pH 7.0, 10 mM of appropriate substrate with tracer amount of radioactive label {5.0 μCi/ml for $H_3{}^{32}PO_4$ (sp. radioactivity 10 Ci/mol) or [^{3}H]ATP (sp. radioactivity 2500 Ci/mol); 0.5 μCi/ml for L-[^{14}C]lysine (sp. radioactivity 240 Ci/mol) or L-[^{14}C]proline (sp. radioactivity 100 Ci/mol) or L-[^{14}C]aspartic acid (sp. radioactivity 152 Ci/mol) or L-[^{14}C]alanine (sp. radioactivity 60 Ci/mol)}, 10 mg wet wt. (≡ 2 mg dry wt.) of depleted cells, 10 μl of mycobacillin solution of various concentrations or 10 μl of ethanol and 10 μl of the respective inhibitor solution [cycloheximide (200 μg/ml) for amino acids, actinomycin D (100 μg/ml) for ATP and carbonyl cyanide *m*-chlorophenylhydrazone (0.1 mM) for P_i] or 10 μl of the appropriate solvent for the uptake under conditions of non-incorporation or of incorporation. Preincubation of the reaction system without the appropriate substrate was for 10 min with shaking at 37 °C. Thereafter the reaction was initiated by addition of appropriate substrates. After the required intervals incubation was stopped by rapid separation of the cells from the medium with filtration under vacuum suction, and cells were thoroughly washed three times with 5 ml of ice-cold incubation buffer. Then the filter pads were secured to counting vials, dried at 60 °C and radioactivity was counted by using a liquid-scintillation fluid consisting of toluene/Triton X-100 (2:1, v/v) containing 4 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) per litre of toluene. Uptake was expressed as the amount of substrate incorporated in nmol/mg cell dry wt., being calculated from the observed radioactivity (c.p.m.) per mg cell dry wt. and the specific radioactivity, i.e. c.p.m. per nmol of substrate.

Uptake of different cations by depleted cells

Depleted cells [0.05 g wet wt. (≡ 0.01 g dry wt.)] were suspended in 10 ml of incubation medium containing 50 mM-Tris/maleate buffer, pH 7.0, 10 mM-$CaCl_2$ or 5 mM-$MgSO_4$ or 10 mM-KCl or 10 mM-NaCl for uptake of their respective cations and 10 μl of mycobacillin solution of various concentrations. After incubation at 37 °C with gentle shaking, cells were rapidly filtered and washed as indicated above. After the washing cells were dried and ashed in a closed muffle furnace. For determination of Na^+ and K^+, ashed samples were extracted with double-distilled water (free from any contaminating ions) and measured directly by using a flame photometer. For the determination of Ca^{2+} and Mg^{2+} ashed samples were extracted with 2.5 ml of conc. HCl, then mixed with 2.5 ml of 1 M-tartaric acid and 2.5 ml of 1% (w/v) NaCl, and finally the volume was made up to 25 ml with double-distilled water and measurement was done by atomic absorption spectroscopy.

RESULTS

Effect of mycobacillin on the uptake of releasable cell materials

Table 2 shows that in the absence of mycobacillin there was uptake of all normally releasable cell materials (namely lysine, proline, ATP, P_i, Na^+, K^+ and Ca^{2+}). On addition of mycobacillin to the reaction system the uptake of different materials was enhanced and further continued to increase with mycobacillin concentrations until it attained a peak value. The peak value was found to be different for different cell materials. However, on further increase in the concentrations of mycobacillin beyond the peak values, the uptake slowly decreased until it attained the same value as in the control without mycobacillin. This decrease in uptake with increase in mycobacillin concentration almost to the level of no uptake might be caused by a simultaneous releasing action of mycobacillin. The optimum concentrations of mycobacillin for uptake and release for a given material were different, e.g. 20 μg/ml (Table 2) and 100 μg/ml respectively for lysine. Therefore at a concentration of 20 μg of mycobacillin/ml, when the uptake for lysine attained the maximum value, release still persisted, causing an apparent decrease in the peak value of uptake at higher concentration of the antibiotic. The pattern of uptake was found to be the same under conditions of

Table 2. Effect of various concentrations of mycobacillin on the uptake of different releasable cell materials

Cell suspension (0.005 g wet wt./ml) was incubated for 10 min with mycobacillin (0–200 μg/ml) with (*a*) or without (*b*) respective inhibitor of incorporation of substrates into cell polymers. Then the uptake was initiated with addition of appropriate substrates (for details see the Materials and methods section) with incubation for 1 h at 37 °C. Values in parentheses indicated the fold increment of uptake with respect to control sample.

Cell material taken up	Concn. of mycobacillin (μg/ml) . . .	Uptake (nmol/mg cell dry wt.)								
		0	10	15	20	30	50	100	150	200
Lysine	(*a*)	32.0 (1.0)	41.4 (1.29)	47.0 (1.47)	58.4 (1.82)	58.5 (1.82)	50.1 (1.56)	39.1 (1.22)	34.3 (1.07)	32.1 (1.00)
	(*b*)	37.2 (1.0)	56.2 (1.51)	62.5 (1.68)	75.9 (2.04)	76.2 (2.04)	62.5 (1.68)	44.0 (1.18)	38.0 (1.02)	37.3 (1.00)
Proline	(*a*)	155.0 (1.0)	367.2 (2.37)	530.0 (3.41)	560.3 (3.61)	560.5 (3.61)	415.0 (2.68)	225.0 (1.45)	160.7 (1.03)	159.3 (1.02)
	(*b*)	260.0 (1.0)	411.3 (1.58)	552.4 (2.12)	575.0 (2.21)	576.1 (2.21)	475.2 (1.82)	275.3 (1.06)	260.9 (1.00)	260.4 (1.00)
ATP	(*a*)	12.0 (1.0)	17.3 (1.44)	20.4 (1.70)	24.8 (2.07)	24.8 (2.06)	20.2 (1.68)	14.5 (1.20)	12.4 (1.03)	12.5 (1.04)
	(*b*)	16.9 (1.0)	20.1 (1.19)	24.0 (1.42)	28.5 (1.68)	29.0 (1.72)	24.5 (1.45)	17.9 (1.06)	17.5 (1.03)	17.2 (1.0)
P_i	(*a*)	6.5 (1.0)	9.2 (1.42)	12.8 (1.96)	14.0 (2.15)	13.9 (2.13)	13.0 (2.00)	7.8 (1.20)	6.9 (1.06)	6.5 (1.0)
	(*b*)	7.0 (1.0)	10.9 (1.56)	14.5 (2.07)	18.5 (2.64)	18.7 (2.67)	16.5 (2.36)	8.2 (1.17)	7.2 (1.02)	7.3 (1.04)
Na^+	(*b*)	110.0 (1.0)	132.1 (1.20)	138.5 (1.25)	136.4 (1.24)	128.4 (1.16)	125.0 (1.13)	115.5 (1.05)	110.6 (1.00)	110.2 (1.00)
K^+	(*b*)	102.5 (1.0)	125.4 (1.22)	143.2 (1.40)	142.5 (1.39)	138.4 (1.35)	135.0 (1.31)	110.7 (1.08)	104.6 (1.02)	103.2 (1.00)
Ca^{2+}	(*b*)	2.5 (1.0)	2.9 (1.16)	3.5 (1.40)	3.7 (1.48)	3.7 (1.48)	3.5 (1.40)	3.2 (1.28)	2.6 (1.04)	2.5 (1.00)

both incorporation and non-incorporation (where studied).

Time course of uptake for different releasable cell materials in the presence of peak and beyond-the-peak concentrations of mycobacillin

Figs. 1(*a*)–1(*g*) show that the uptake, which occurred almost instantaneously for all of the releasable cell materials, was linear with time until they all attained the peak values in the absence and in the presence of the peak and beyond-the-peak concentrations of mycobacillin, but the time taken to reach the peak value was found to be different for different materials. However, these peak values remained constant for the observed period (60 min) for some materials (namely Na^+, K^+ and P_i) but showed gradual decrease for others (namely lysine, proline, ATP and Ca^{2+}).

Effect of a given concentration of mycobacillin on the rate of uptake in the presence of various extracellular concentrations of different normally releasable cell materials

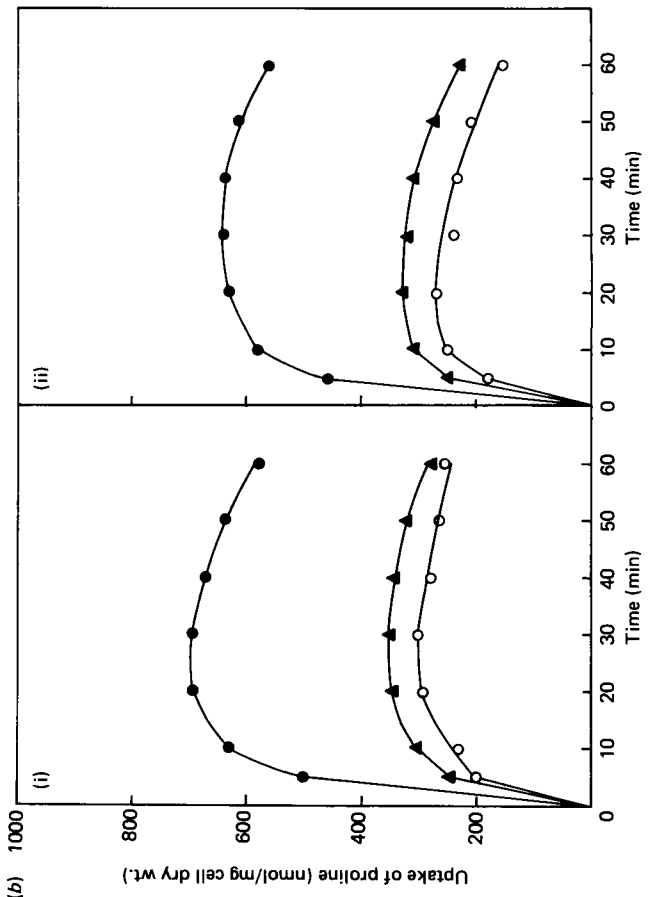
Uptake of all the releasable cell materials over the range 10–15 mM was non-linear and followed Michaelis–Menten kinetics both in the presence and in the absence of mycobacillin {results not shown except that for lysine under conditions of incorporation and non-incorporation [Figs. 2(*a*)(i) and 2(*a*)(ii)]}. Kinetic constants (Table 3) were calculated from Lineweaver–Burk plots {not shown except that for lysine [Figs. 2(*b*)(i) and 2(*b*)(ii)]}, which suggests that the apparent K_m values for uptake of different cell materials in the presence and in the absence of mycobacillin were very much comparable, whereas $V_{max.}$ values increased significantly in the presence of mycobacillin. Thus mycobacillin did not appear to compete with the substrates transported for their respective transport systems. Interestingly, the values of K_m and $V_{max.}$ were somewhat lower under conditions of non-incorporation than under conditions of incorporation.

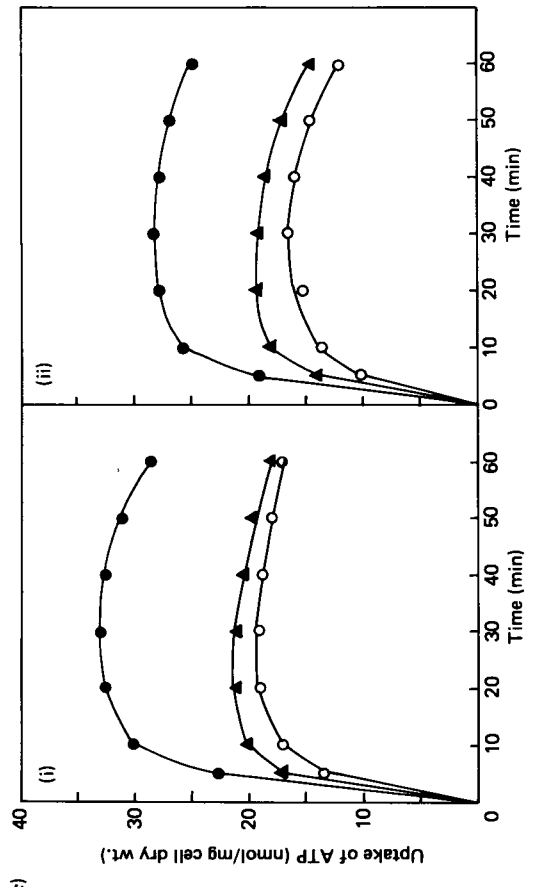
Effect of mycobacillin on the uptake of cell materials not normally releasable by mycobacillin

Uptake of non-releasable cell materials (namely alanine, aspartic acid and Mg^{2+}) at a given extracellular substrate concentration was studied in the absence and in the presence of various concentrations of mycobacillin; the results showed that mycobacillin did not possess any effect on their uptake (Table 4).

DISCUSSION

In continuation of previous studies showing that mycobacillin enhanced the release of some normally releasable specific cell constituents from a sensitive strain of *Aspergillus niger* without causing lysis or extensive damage of the cell, we have now reported here the enhancing effect of mycobacillin on the uptake of those very cell materials whose release was enhanced by mycobacillin. Hence the possibility of both the processes being controlled by the same mechanism could not be ruled out. These sensitive sites for uptake might be


(a)
Uptake of lysine (nmol/mg cell dry wt.)
(i)
(ii)
Time (min)
(b)
Uptake of proline (nmol/mg cell dry wt.)
(i)
(ii)
Time (min)


(c)
Uptake of ATP (nmol/mg cell dry wt.)
(i)
(ii)
Time (min)

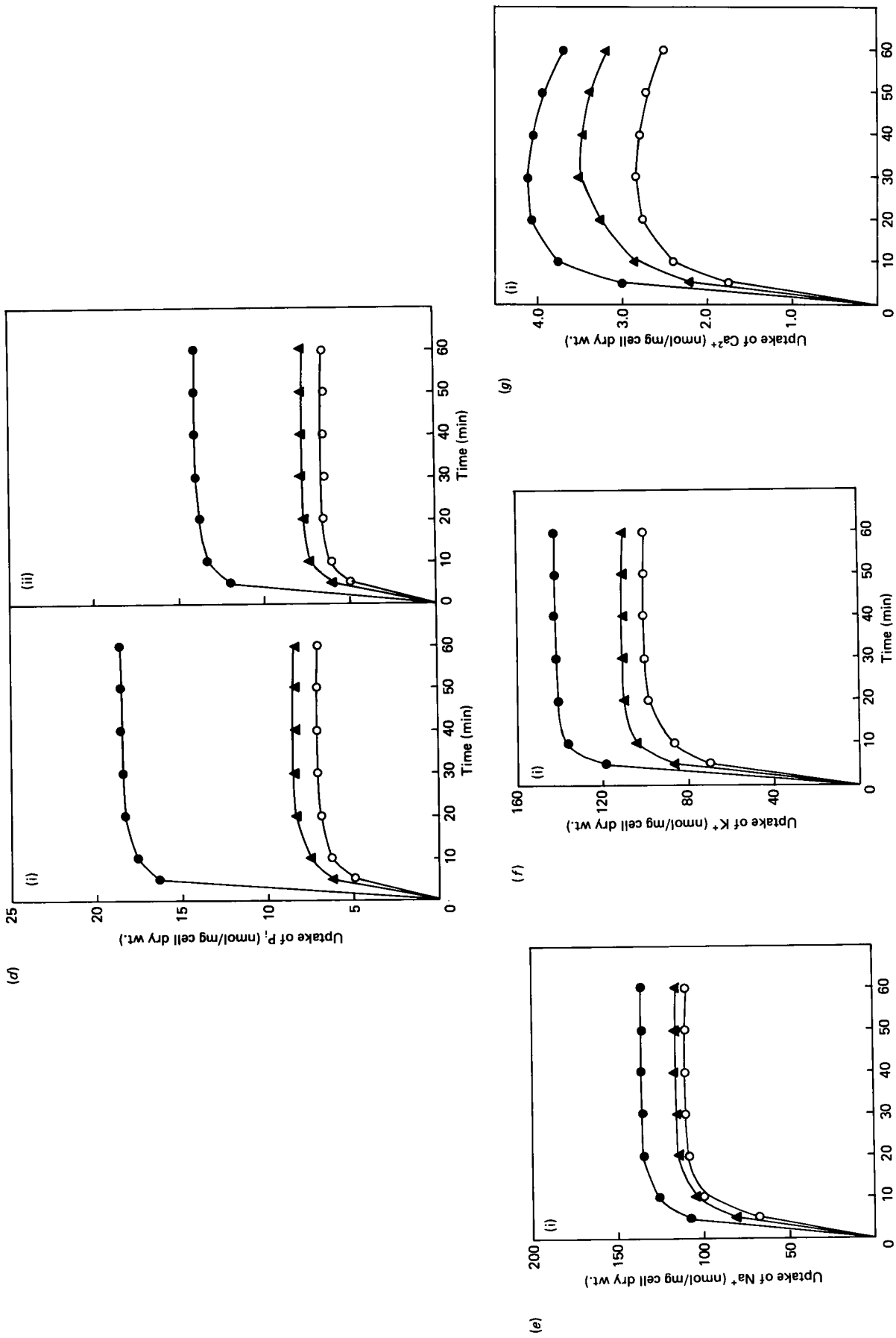


Fig. 1. Effect of concentrations of mycobacillin on the kinetics of uptake under condition of incorporation (i) for (*a*) lysine, (*b*) proline, (*c*) ATP, (*d*) P_i, (*e*) Na^+, (*f*) K^+ and (*g*) Ca^{2+} and under conditions of non-incorporation (ii) for (*a*) lysine, (*b*) proline, (*c*) ATP and (*d*) P_i

Experimental conditions are indicated in the text. For uptake of each of the cell materials, concentration in the incubation medium was 10 mM. Mycobacillin was added at zero time. ○, Control; ●, 20 μg of mycobacillin/ml; ▲, 100 μg of mycobacillin/ml.

Table 3. Comparison of apparent K_m and $V_{max.}$ values for the uptake of different releasable cell materials (from Lineweaver–Burk plots)

(*a*) Under conditions of incorporation; (*b*) under conditions of non-incorporation.

Cell material taken up	K_m (mM)				$V_{max.}$ (nmol/min per mg cell dry wt.)			
	Without mycobacillin		With mycobacillin (20 μg/ml)		Without mycobacillin		With mycobacillin (20μg/ml)	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Lysine	5.3	3.1	5.3	3.1	13.3	7.6	18.2	13.1
Proline	10.3	3.3	10.3	3.3	80.0	78.4	100.0	121.2
ATP	4.2	3.9	4.3	3.9	2.9	1.8	4.8	2.8
P_i	14.3	5.9	14.5	5.9	5.9	2.8	12.5	4.3
Na^+	5.8		5.8		25.6		30.3	
K^+	6.3		6.3		21.7		32.3	
Ca^{2+}	6.0		6.0		0.5		0.8	

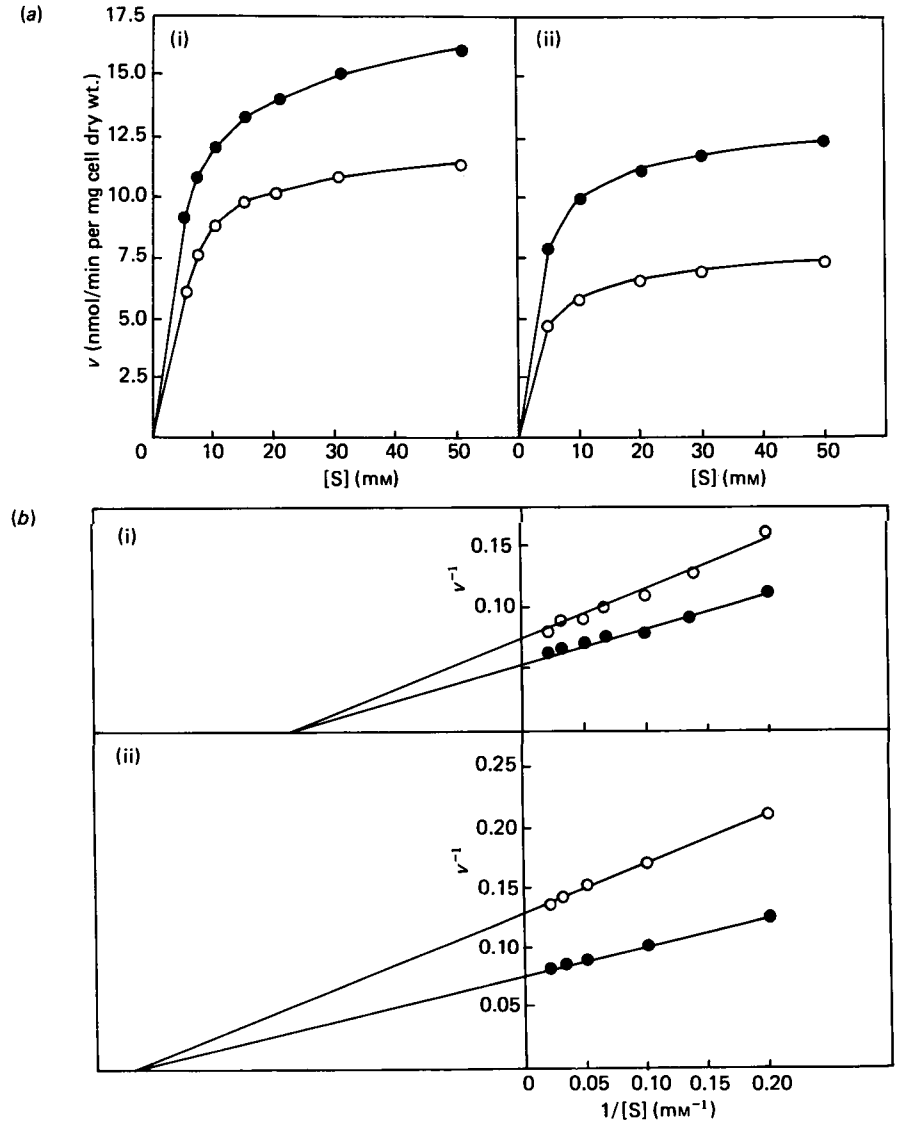


Fig. 2. Effect of mycobacillin on the rate of uptake under conditions of incorporation (i) and of non-incorporation (ii) of lysine in the presence of its increasing extracellular concentrations

Extracellular concentrations of lysine were varied in the range 0–50 mM. The data were obtained with a 1 min incubation period. Experimental conditions are the same as indicated in Fig. 1 legend. ○, Control; ●, 20 μg of mycobacillin/ml.

Table 4. Effect of mycobacillin on the uptake of non-releasable cell materials

The incubation time was 1 h, and 5.0 mM extracellular concentration was used in each case.

Cell material taken up	Concn. of mycobacillin (μg/ml)...	Uptake (nmol/mg cell dry wt.)			
		0	15	20	50
Aspartic acid*		35.0	34.5	34.5	34.7
Alanine*		41.8	42.0	42.1	42.0
Mg^{2+}		8.4	8.5	8.5	8.5

* Results given were obtained under condition of non-incorporation.

different for different cell materials because of the difference in the nature of the materials taken up and also that in the enhancing effect produced by mycobacillin.

Saturation kinetics [Figs. 2(*a*)(i) and 2(*a*)(ii)] showed the uptake to be a carrier-mediated process, and the non-competitive action of mycobacillin on the uptake might be taken to mean that the antibiotic might bind to the carrier at a site other than the substrate-binding site.

As no time lag is required for uptake of any of the releasable cell materials, for which there is a definite time lag for mycobacillin-induced release of some (lysine, proline, ATP and Ca^{2+}) but not for others (Na^+, K^+ and P_i), the transport carrier for the latter might be located across the permeability barrier whereas that for the former might be located not across the permeability barrier but embedded in it near the exterior surface. Unlike the peak release, the peak uptake as a function of time showed a gradual fall for some materials (namely lysine, proline, ATP and Ca^{2+}) and not for others (namely Na^+, K^+ and P_i), which might lend further support to the previous assumption that the transport carrier for the former group of cell materials was embedded in the permeability barrier near the exterior surface, in which case the lack of facilitated transport for the inside of the cell might account for the fall in peak uptake. In the same way for the other group of cell materials, comprising Na^+, K^+ and P_i, having facilitated transport across the membrane, the peak uptake remained constant.

Thus uptake studies coupled with those on release might be taken to mean that cells of *Aspergillus niger* inherently possessed some common mechanism for both release and uptake. The action of mycobacillin, which is limited only to the enhancing effect both for release and for uptake, might be due to the interaction of mycobacillin with the non-substrate-binding component of the transport system, which might possibly be lipid or sterol, as mycobacillin action has already been reported to be antagonized by interaction with lipid and sterol (Halder *et al.*, 1967; Mukherjee *et al.*, 1979; Mukherjee & Bose, 1982). It may be recalled that the inhibition by oligomycin on the $Na^+ + K^+$-stimulated ATPase (transport ATPase) occurred as a result of interaction of oligomycin not with the ATPase but with some organic-solvent-removable component of the membrane, possibly lipid (Glynn, 1962; Palatini & Bruni, 1970; Pittoti *et al.*, 1972; Broughall *et al.*, 1973). Thus mycobacillin might interact similarly with lipid or sterol components of the different transport systems to bring about conformational alterations causing selective enhancing effect on release or uptake of specific cell constituents, depending on the extra- or intra-cellular substrate concentration.

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