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STUDIES ON UPTAKE OF FATTY ACIDS INTO MYCOBACTERIAL CELLS (2) FURTHER STUDIES ON THE UPTAKE OF ACETATE INTO *MYCOBACTERIUM SMEGMATIS*

Manabu SATO, Teruaki AKAO and Takashi KUSAKA

Department of Biochemistry, Kawasaki Medical School
Kurashiki, 701-01 Japan

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Abstract

Based upon further investigations on the uptake of acetate into *Mycobacterium smegmatis*, especially using its membrane preparation, ghosts, a non-passive transport of acetate involving adenosine triphosphate (ATP) as a stimulator was elucidated. Uridine triphosphate (UTP) was found to be also stimulative as ATP to the uptake of acetate in the ghosts. The mechanism of these stimulations of ATP as well as UTP to the uptake of acetate is still equivocal at present.

INTRODUCTION

Although studies on the transport of fatty acids are being cumulated, interpretations of the mechanism for transport of fatty acids are still diverse; for example a passive diffusion[1,2], an active transport[3,4], a translocation involving a vectorial enzyme[5,6,7], etc., depending on the experimental materials and the authors. On the other hand, studies on the transport of acetate, which could be chosen as a model for fatty acid, are presented not yet abundantly, though studies concerning incorporations of extra-cellular acetate are being augmented[8,9]. Wagner et al.[10] reported an active transport of acetate in *Escherichia coli* which is assumed to occur in two different fashions depending on the concentration of acetate. On the other hand, it was found in our previous study[11] that a transport of acetate in *M. smegmatis* is inhibited by p-chlorophenoxy isobutyrate (CPIB), 2, 4-dinitrophenol (DNP), potassium cyanide, etc. and an involvement of ATP in the transport of acetate was suggested. In addition, an involvement of coenzyme A (CoA) in that transport was also supposed from an inhibition of 4-pentenoate, a special reagent for reducing the content of free CoA in animal cells[12]. Further studies on the uptake of acetate in *M. smegmatis*, especially using its membrane preparation, ghosts, are presented in this paper, in which

an involvement of ATP in the uptake of acetate in *M. smegmatis* is elucidated.

MATERIALS AND METHODS

1) *Cultivation of M. smegmatis.* The strain of *M. smegmatis* used and methods for its cultivation and harvest were the same as reported previously[11].

2) *Measurements of the uptake of acetate in the intact cells and the ghost preparation.* Activity of the uptake of acetate in the intact cells or the ghosts was measured by summing radioactivities remaining in these cells or the ghosts and in CO_2 released after the following incubations. In the case of intact cells, 3 ml of the incubation medium containing 0.2 mmol phosphate buffer (pH 7.2), 10 μmol 2-^{14}C-sodium acetate (0.5 Ci/mol) and 25 mg wet weight of the intact cell of *M. smegmatis* was pipetted in the main room of Warburg's vessel, then was incubated at 37°C for 1 hr with a continuous shaking of the vessel. In the case of the ghosts, 2 ml of the incubation medium containing 0.2 mmol trihydroxymethylaminomethane (Tris)-HCl buffer (pH 7.2), 20 μmol magnesium chloride, 10 μmol dithiothreitol (DTT), 20 nmol 2-^{14}C-sod. acetate (50 Ci/mol) and the ghosts (protein content, 0.6-0.7 mg) was pipetted in the main room of Warburg's vessel, then was incubated at 30°C for 20 min with the continuous shaking. $^{14}CO_2$ released during the above two kinds of incubations was trapped in 0.3 ml 10 % potassium hydroxide solution put in the center well of vessel, then its radioactivity was measured by the method of Anderson & Snyder[13]. The radioactivity remaining in the intact cells was measured with Packard's Liquid Scintillation Counter, Model 3385 after the collection of cells by filtration through the paper (Whatman, No. 1), the washing of cells with water, then the combustion of cells with Packard's Sample Oxidizer, Model 306. Measurement of the radioactivity remaining in the ghosts was performed in the following way. After the collection of ghosts on the membrane filter (Toyo Roshi Co. TM-2, 0.45 μm), the ghosts were washed 5 times with 10 mM Tris-HCl buffer (pH 7.2) containing 0.1 M calcium chloride and 10 mM magnesium chloride, then solubilized with 1 N sod. hydroxide. To the solubilized sample was added Insta Gel (a liquid scintillation cocktail for aqueous solutions, Packard) to form gel, whose radioactivity was counted with the scintillation counter.

3) *Other procedures.* The assays for fumarase [E. C. 4. 2. 1. 2], acetanilide hydrolase, reduced nicotinamide adenine dinucleotide (NADH) -3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT)

oxidoreductase in the ghosts were carried out by the methods of Racker[14], Akao[15] and Cesari[16], respectively. Proteins were determined by the method of Lowry et al.[17].

4) *Chemicals.* Sod. 2-^{14}C-acetate was purchased from the Radiochemical Centre, Amersham. Lysozyme, deoxyribonuclease I (DNase I) and 4-pentenoic acid were purchased from B. D. H. Biochemicals, Sigma Chemical Co. and K and K Fine Chemicals, respectively. All other chemicals were reagent grade from commercial sources.

RESULTS

1) *Dependency of the uptake of acetate in the intact cells on the initial concentration of acetate.* Dependency of the uptake of acetate in the intact cells on the initial concentration of acetate in the medium was found to be expressed as a typical hyperbolic curve as shown in Fig. 1.

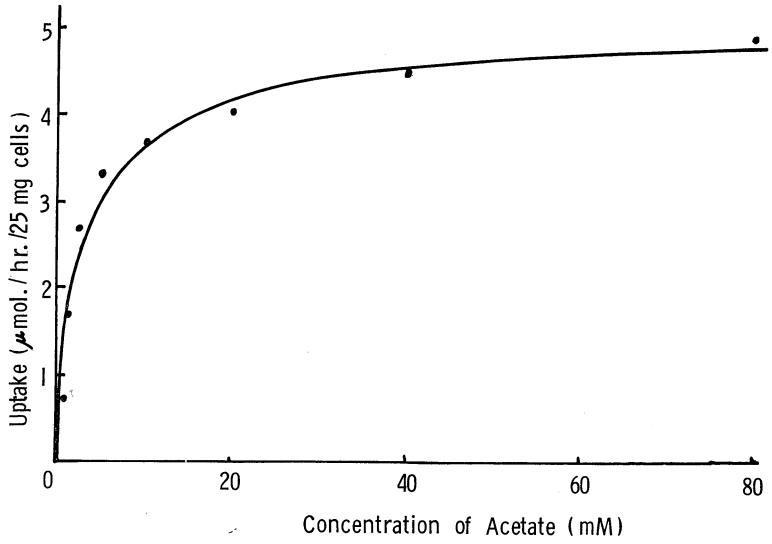

Fig. 1. Dependency of the uptake of acetate in the intact cells on the initial concentration of acetate. Incubation systems were as described in MATERIALS & METHODS except that the initial concentration of acetate was varied as indicated.

In addition to the previous finding that the uptake of acetate in *M. smegmatis* is inhibited by various reagent[11], this result is also in accord with the understanding that acetate should not be taken up in *M. smegmatis* by a simple diffusion.

2) *Preparation of the ghosts from M. smegmatis and assays of some enzyme activity in the ghosts.* It seemed of interest to use a membrane preparation, ghosts, from *M. smegmatis* for studying the transport of acetate because it was reported that[18] the membrane of ghosts prepared from *Mycobacterium phlei* was supposed to be oriented as in the intact cell and ghosts seemed evidently more simple than the intact cells for approaching to the study on the transport of acetate.

The ghosts were prepared from *M. smegmatis* following the method of Asano et al.[18], which is diagrammed in Fig. 2. The quality of the

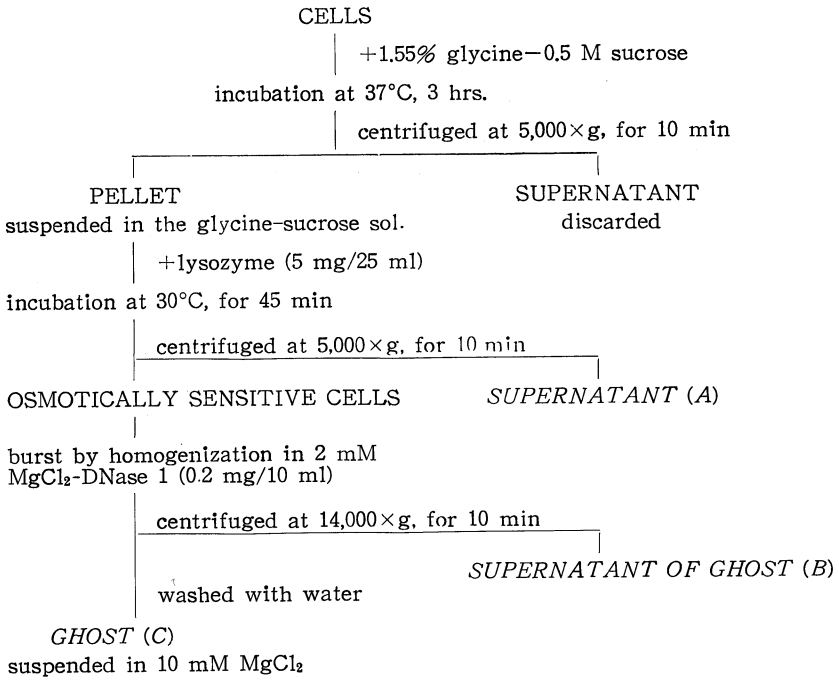

Fig. 2. Preparation of the ghost of *M. smegmatis*.

prepared ghosts was verified by measuring activity of the fumarase, the acetanilide hydrolase, both enzymes are to be localized in the cytosol fraction, and NADH-MTT oxidoreductase, being considered to be generally in the membrane fraction, as well as distribution of the total protein content in the subfractions as indicated in Table 1. According to the results in the Table, the preparation of ghosts appeared to be almost depressed of the cytosolic component while about a half of the membranous enzyme seemed to be residing in the ghosts.

TABLE 1.

Distributions of some enzyme activity and total protein in subfractions of the ghosts

subfraction	A*	B*	C*
protein	28.0%	49.0%	23.0%
fumarase	34.6%	75.4%	0
acetanilide hydrolase	0.6%	92.0%	1.3%
NADH-MTT oxidoreductase	0	59.0%	41.0%

*: Subfractions A, B, C, are as indicated in Fig. 2.

3) *Effects of adding some components to the medium for the uptake of acetate in the ghosts.* For ascertaining participations of ATP and CoA in the uptake of acetate in *M. smegmatis* as postulated previously[11], ATP, CoA as well as ascorbic acid (Asc)-N, N, N′, N′-tetramethyl-p-phenylenediamine dihydrochloride (TPD), a stimulator for the transport of proline in *M. phlei*[19,20], were tried to add to the medium for the uptake of acetate in the ghosts, each separately and in its combinations as in Fig. 3. Among these additions, some stimulations to the uptake of acetate were observed only in the case of adding ATP as shown in Fig. 3.

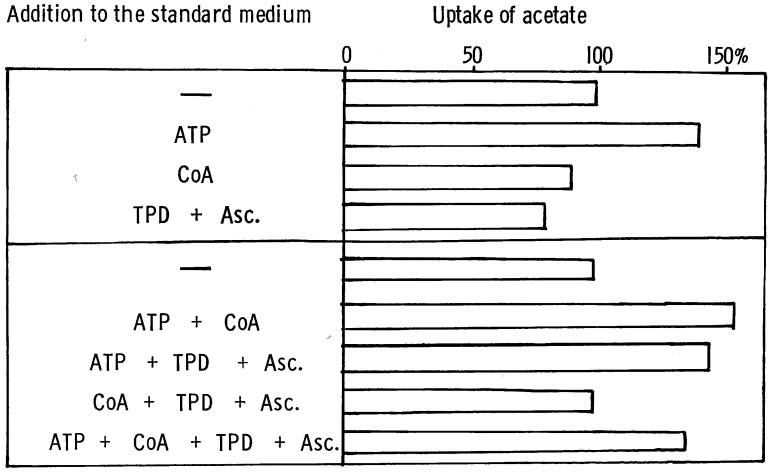

Fig. 3. Effects of adding some components to the medium for the uptake of acetate in the ghosts. Incubation systems were as described in MATERIALS & METHODS except that 20 μmol ATP, 10 μmol CoA, or 34 μmol Asc. -0.75 μmol TPD were added to incubation mixture each separately.

NADH, succinic acid, ascorbic acid-phenazinemethosulfate (Asc-PMS), all of which are known to stimulate the transport of proline in *M. phlei*[19,20] were also examined, but no stimulating effect on the uptake of acetate in the ghosts was found from these experiments.

4) *Effects of the times of bursting ghosts on its uptake of acetate in the presence of ATP.* In attempts to amplify the stimulating effect of ATP on the uptake of acetate in the ghosts as shown in Fig. 3, times of bursting the ghosts during their preparation varied to some extent. In consequence, the ratio of the uptake of acetate in the ghosts in the presence of ATP to that in the absence of ATP were found to increase as shown in Table 2, though the total uptake of acetate decreased,

TABLE 2.

Effects of times of bursting the ghosts on its uptake of acetate

	total uptake of acetate (dpm)			
times of burst	1	2	4	6
− ATP*	234,000	118,000	52,000	41,000
+ ATP (final conc., 10 mM)	541,000	331,000	229,000	165,000
ratio (+ATP/−ATP)	2.30	2.81	4.40	4.02

*: Incubation systems were as in MATERIALS & METHODS.

according to the increase in time of bursting ghosts till four times. The reason why the increase in time of the bursting was effective on the stimulation of ATP seemed due either to washing out endogenous ATP from the ghosts, or to facilitation for ATP to enter the ghosts, and or both.

5) *Optimum concentration of ATP in the medium for the uptake of acetate in the ghosts.* As shown in Fig. 4., an optimum concentration (finally 10 mM) of ATP added to the medium for the uptake of acetate in the ghosts could be observed under the fixed condition as in the figure.

6) *Effects of various nucleotides added to the medium for the uptake of acetate in the ghosts.* Among the various nucleotides added to the medium for the uptake of acetate in the ghosts, uridine triphosphate (UTP) was found to be effective next to ATP on the uptake of acetate in the ghosts as shown in Fig. 5. Other nucleotides tested were found to be ineffective.

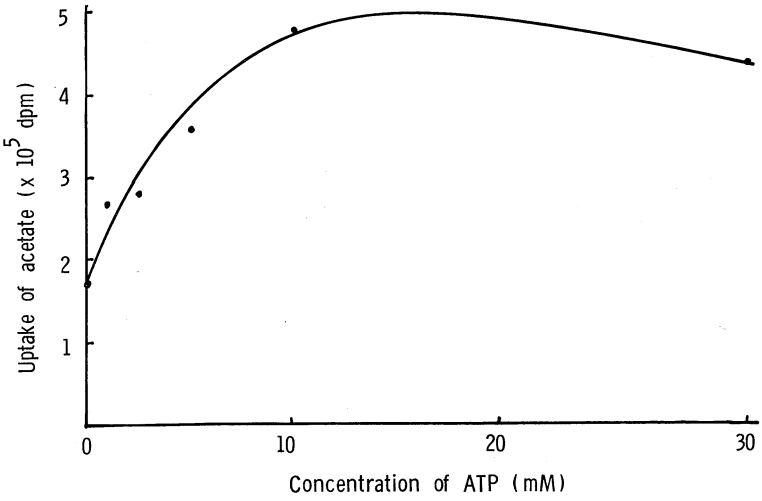

Fig. 4. Optimum concentration of ATP in the medium for the uptake of acetate in the ghosts. Incubation systems were as described in MATERIALS & METHODS except that the concentration of ATP was varied as indicated.

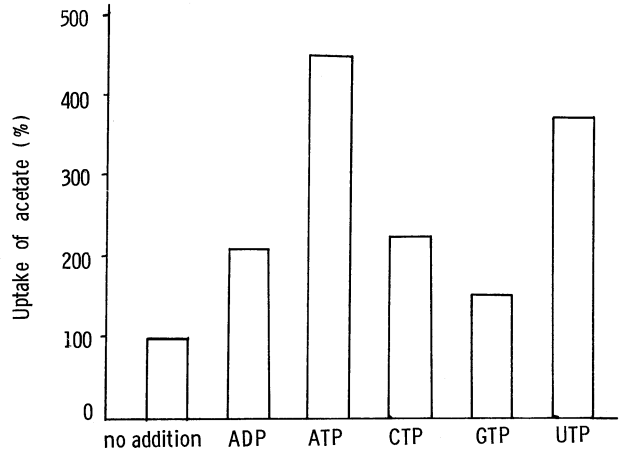

Fig. 5. Effects of various nucleotides added to the medium for the uptake of acetate in the ghosts. Incubation systems were as described in MATERIALS & METHODS except that each nucleotide (10 mM; final conc.) was added to the incubation mixture.

7) *Effects of 4-pentenoate and DNP on the uptake of acetate in the ghosts.* For ascertaining whether the mechanism of the uptake of acetate

in the ghosts, especially stimulated by the exogenous ATP, is similar to or different from that of the uptake of acetate in the intact cells, effects of 4-pentenoate and DNP, both known to be inhibitive against the uptake of acetate in the intact cells[11], on the uptake of acetate in the ghosts in the presence of ATP, were examined. As a result, both inhibitory effects could be definitely observed as shown in Table 3. These results are in favour of an existence of similar mechanism for the transport of acetate in the intact cells and in the ghosts of *M. smegmatis*.

TABLE 3.

Effects of 4-pentenoate and DNP on the uptake of acetate in the ghosts

inhibitor	final conc. of inhibitor mM	uptake of 2-^{14}C-acetate dpm	% of inhibition
4-pentenoate	0	42,900	0
	2	13,800	68
	10	9,100	79
	40	660	98
2, 4-dinitrophenol	0	29,700	0
	0.1	7,900	73
	0.5	6,600	78

Incubation systems were as described in MATERIALS & METHODS except that ATP (10 mM; final conc.) were added to the incubation mixture.

DISCUSSIONS

The ghosts prepared from *M. phlei* is reported[18] to present a permeability barrier to several substances and to require a preincubation of ghosts with the necessary components to achieve the oxidative phosphorylation. By contrast, according to this study, the exogenous ATP was found to be consistently stimulative without preincubation to the uptake of acetate in the ghosts prepared from *M. smegmatis*. Although there is no evidence at present proving whether ATP penetrates the membrane of ghosts, or resides in the membrane itself, or bind to some substances on the surface of membrane, etc., it could be presumed at least from this study that ATP added exogenously to the ghosts could be interacting with some membranous component involved in the transport of acetate during the incubation.

The transport of acetate in the intact cells of *M. smegmatis* could be assumed to be driven by a similar mechanism as the ghosts as discussed already, in RESULTS. Concerning the mechanism of stimulating effect of ATP on the transport of acetate in *M. smegmatis*, the following three possibilities might be postulated. 1) ATP may be utilized as a cofacter of acetyl CoA synthetase catalyzing a vectorial esterification of acetate for the transport, 2) ATP may be utilized directly as an energy source for the transport, 3) ATP may be utilized to energize the membrane for the transport. In the first case, CoA should be also necessary beside ATP to form acetyl CoA during the uptake of acetate. From this point of view, the experimental results showing inhibitory effects of 4-pentenoate against the uptake of acetate in the ghosts (Table 3) as well as in the intact cells[11] appeared to be in accord with the first assumption, provided 4-pentenoate reduced the intracellular concentration of free CoA as in the case of the animal tissue[12]. As a matter of fact, an unexpected result suggesting no function of 4-pentenoate to reduce the concentration of free CoA in *M. smegmatis* was obtained (unshown results). Further studies should be required, therefore, to unravel the mechanism of stimulating effect of ATP on the transport of acetate in *M. smegmatis*. For elucidating the mechanism of stimulating effect of UTP on the uptake of acetate in the ghosts prepared from *M. smegmatis* as shown in Fig. 5, it is necessary at first to elucidate the problem whether UTP may be involved directly in the transport or UTP may be utilized in the transport after transferring its high energy to somewhat to form ATP.

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