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STUDIES ON UPTAKE OF FATTY ACIDS INTO MYCOBACTERIAL CELLS (I) EFFECTS OF p-CHLOROPHENOXYISOBUTYRATE ON THE UPTAKE OF ACETATE INTO THE CELLS

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Abstract

It was demonstrated that p-chlorophenoxyisobutyrate (CPIB), a potent hypolipidemic agent, has several powerful anti-biological activities against *Mycobacterium smegmatis*, such as growth inhibition, inhibition against uptaking of acetate and decreasing of ATP's content in the cells. On the other hand, 4-pentenoate, an effective reagent for reducing free CoA's concentration in living organisms, was found to inhibit also the acetate-uptake of the cells. From these results obtained, it could be imagined that acetyl CoA synthetase reaction is involved in the uptake of acetate into the cells. Occurence of at least two inhibitory steps of CPIB against biosynthesis of lipids in this micro-organisms *in vivo* was postulated, also.

INTRODUCTION

Comparing the recent progress in studies on transport mechanisms of cations (Na^+,K^+etc.), saccharides and amino acids through biomembranes, mechanism of fatty acid transport is still the subject of some debates. For instance, certaine studies on the uptake of fatty acids by some animal cells are compatible with a non-enzymatic diffusion process[1,2], whereas other studies using bacterial cells suggest a translocation mechanism such as vectorial acylation involving acyl CoA synthetase[3,4,5,6]. Our work has been started to elucidate the discrepancy as described using mycobacterial cells. As the first report, effects of p-chlorophenoxyisobutyrate (CPIB), one of a potent hypolipidemic drug, on the uptake of acetate into *Mycobacterium smegmatis* are presented in this paper.

MATERIALS AND METHODS

Cultivation. M. smegmatis, strain ATCC 14468, was supplied by the Department of Tuberculosis, Research Institute for Microbial Diseases,

Osaka University. This strain was grown with continuous shaking, at 37°C for 3 days (until the begining of stationary phase) in Sauton's medium containing 0.06% Tween 80, then harvested by filtration *in vacuo* and succeeding washing with enough water.

System to measure acetate-uptake. Assay to measure acetate-uptaking activity of *M. smegmatis* was carried out as follows; 1.25 ml of 0.1 M phosphate buffer (pH 7.2), 0.25 ml of 5% beef serum albumin (fraction V) solution, 1 ml of cell suspension (25 mg wet weight of cells/ml) with or without 0.25 ml of CPIB-ethanol solution (variable concentration) were succesively taken into the main room of Warburg's vessel (an accesory of Warburg's manometric apparatus) and this mixture was preincubated at 37°C for 1 hr with continuous shaking in order to make CPIB penetrate into the cells, then incubated at 37°C for another 1 hr with shaking after addition of 1 micromol of Sodium 2-^{14}C-acetate (0.5 Ci/mol) to the mixture. $^{14}CO_2$ released during the incubation was measured by a slight modification of Anderson & Snyder's method[7]. Isolation of cells from the mixture was carried out by filtration of the mixture *in vacuo* through filter paper, Whatman No. 1, and succesive washing with enough cold water. The paper attached with the cell paste was dipped into chloroform: methanol mixture (2 : 1, v/v), then lipids were completely extracted from the cells by continuous shaking at 37°C overnight. After the extraction of lipids, the defatted cell residue was at first removed by filtration *in vacuo*, then the lipid extract was fractionated furthermore into phospholipids, glycerides, free fatty acids etc. by thin layer chromatography (TLC) on a plate of silica gel G using a solvent system of pertoleum ether : ether : acetic acid (70 : 30 : 1, v/v/v). Radioactivities in the total lipid fraction as well as in the individual lipid fraction obtained by extraction of the silica gel scrapped from the corresponding area on TLC plate with toluene base's solution for liquid scintillation counting were counted by Packard's Liquid Scintillation Spectrometer, Model 3385 or 2450. The radioactivity in the defatted cells (non-lipid fraction) was also measured by the liquid Scintillation Spectrometer after oxidation of the sample with Packard's Sample oxidizer, Model 306. The amount of taken acetate in cells was calculated by summing up the radioactivities from released $^{14}CO_2$ and incorporated Sod. 2-^{14}C-acetate in the lipid and non-lipid fractions.

The *de novo* biosynthetic activity of fatty acid from Sod. 2-^{14}C-malonate, *in vitro*, was assayed by the method of Piérrard & Goldman[8]. Radiogas-chromatography was carried out with Packard's Proportional Counter, Model 894 connected to Packard's gas-chromatography, Model

8067. Acyl CoA synthetase activity was assayed by the method of Samuel & Ailhaud[9]. Determination of ATP's content with luciferase system was carried out by the method of Stanley & Williams[10], but isolation of ATP from the bacillary cells was achieved by the method of Davidson & Fynn[11].

RESULTS

Effects of CPIB on the growth of M. smegmatis. At first, effect of CPIB on the growth of *M. smegmatis* was examined. In consequence, CPIB was found to have a potent inhibitory effect against the growth of this micro-organism. The effective dosis of CPIB seemed to depend on its administration time, for example, 0.4 mM was sufficient for the growth inhibition when added at the inoculation time, whereas 1-2 mM was necessary when added at the beginning of logarithmic phase (Fig. 1).

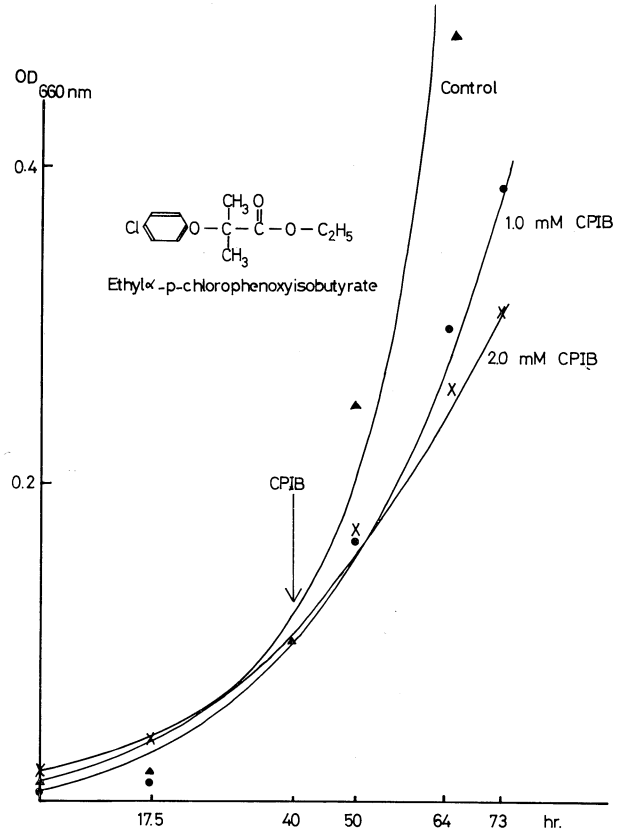

Fig. 1. Effects of CPIB on the growth of *M. smegmatis*. Optical density at 660 nm ($OD_{660\ nm}$) of the cell suspension in culturing glass tube was measured occasoinally as indicated on the abscissa. The arrow indicates adding time of CPIB.

It seems probable, therefore, that penetration of CPIB into the cells should become more difficult according to increasing of cell age.

Effects of CPIB on acetate-uptake of M. smegmatis in vivo. As shown in Table 1, CPIB was found to decrease every radioactivities in lipid and non-lipid fraction as well as in released CO_2 after the incubation of cells with 2-^{14}C-acetate. Among these results, the decreased percent of radioactivity in lipid fraction is the most prominent. According to these results, it could be assumed that CPIB inhibit not only the uptake of acetate into cells but also further biosynthesis of lipids from the taken acetate. The result shown in Table 2 indicates effects of CPIB on synthesis of individual lipids. As shown in the Table, decreased percents of radioactivity in phospholipid and triglyceride due to presence of the described concentration of CPIB are very close each other. It could be implied, therefore, that CPIB might inhibit synthesis of the common precursor of phospholipid and triglyceride such as fatty acids.

Effects of CPIB on de novo synthesis of fatty acids, in vitro. In order to search for step of CPIB's inhibition on the pathways of *de novo* synthesis of fatty acids, effect of CPIB on an incorporation of radio-

TABLE 1.

Effects of CPIB on 2-^{14}C-acetate uptake in *M. smegmatis*.

final conc. of CPIB added	$^{14}CO_2$ released dpm (%)*	radioactivity in the cells		total uptake of 2-^{14}C-acetate dpm (%)*
		in lipid fract. dpm (%)*	in non-lipid fract. dpm (%)*	
0 mM	345,600 (100)	31,000 (100)	185,800 (100)	562,400 (100)
2 mM	211,100 (61)	9,800 (31)	108,800 (59)	329,700 (59)
4 mM	198,500 (57)	7,500 (24)	89,800 (48)	295,800 (53)

* Average values from 4 experiments, whose conditions are described in text.

TABLE 2.

Effects of CPIB on biosynthesis, *in vivo*, of various lipids in *M. smegmatis* from 2-^{14}C-acetate.

final conc. of CPIB	phospholipid dpm (%)*	triglyceride dpm (%)*	free fatty acid dpm (%)*	sum dpm (%)*
0 mM	11,670 (100)	3,584 (100)	170 (100)	15,424 (100)
2 mM	1,740 (15)	606 (17)	95 (56)	2,441 (16)
4 mM	1,250 (11)	489 (14)	53 (31)	1,792 (12)

* Average values from 4 experiments, whose conditions are described in text.

activity from 2-^{14}C-malonate into long chain fatty acids was investigated. As shown in Table 3, CPIB seemed to have no effect on total incorporation of malonate into long chain fatty acids. Furthermore, as shown in Fig. 2, the incorporation of the radioactivity from 2-^{14}C-malonate into synthesized fatty acids was not changed by addition of CPIB, *in vitro*. These results indicate that CPIB might inhibit biosynthesis of fatty acids, if any, at step earlier than the malonyl CoA's formation.

Experiments for elucidation of inhibitory mechanism of CPIB against the acetate-uptake of M. smegmatis. According to recent reports[3,4,5,6], several

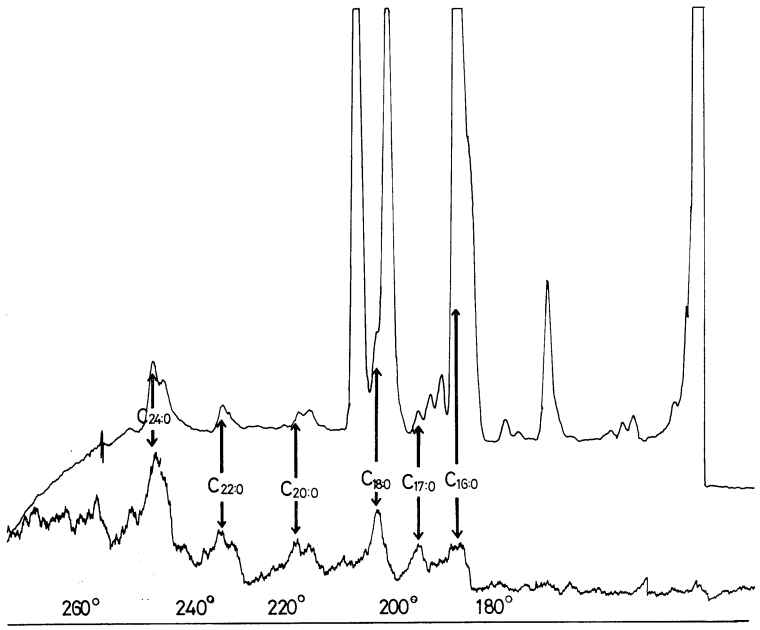

Fig. 2(a). Incorporation of the radioactivity from 2-^{14}C-malonate into synthesized fatty acids after incubation without CPIB.

$C_{16:0}$, $C_{17:0}$, $C_{18:0}$, $C_{20:0}$, $C_{22:0}$. $C_{24:0}$ indicate n-hexadecanoic-, n-heptadecanoic-, n-octadecanoic-. n-eicosadecanoic-, n-docosadecanoic-, n-tetracosadecanoic-acids, respectively. The upper curve indicates usual gas-chromatographic recording, the lower curve indicates radioactivity recorded simultaneously. Column of SE 30 (10%) on Schimalite (60-100 mesh) was used under temperature-programmed condition from 140°C to 180°C as indicated on the lowest line in the Figure (temperature-raising rate: 2.5°C/min). Being passed through the column, gas sample is split into two portions, one is conducted to a flame-ionization detector and the other is to the gas-proportional counter. Methylation of fatty acids synthesized during the incubation as in Table 3 was carried out with 14% BF_3 in methanol solution.

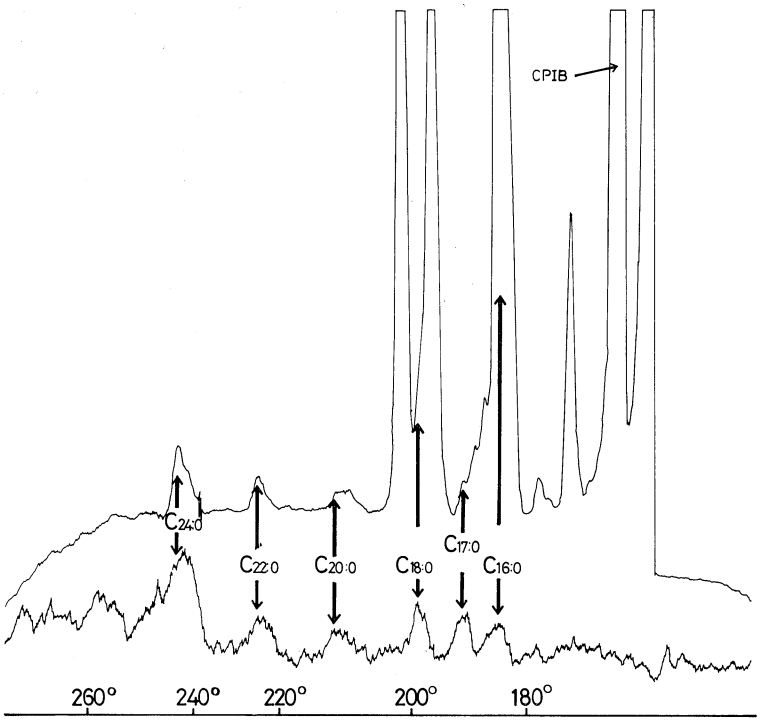

Fig. 2(b). Incorporation of the radioactivity from 2-^{14}C-malonate into synthesized fatty acids after incubation with CPIB.
The experimental condition was the same as Fig. 2(a) except the incubation with CPIB.

TABLE 3.

Effects of CPIB on *de novo* synthesis of Fatty acids, *in vitro*.

conc. of CPIB added to incubat. mixture*	incorporation of radioactivity from 2-^{14}C-malonate into synthesized fatty acids
0 mM	45,990 dpm
1 mM	53,200 dpm
2 mM	40,250 dpm
0 mM	45,900 dpm
2 mM	40,200 dpm
5 mM	41,820 dpm

* Incubation mixture (± CPIB) was as follows; 0.5 μmol CoA, 5 μmol ATP, 5 μmol $MgCl_2$, 2 μmol NADH, 1 μmol NADPH, 0.01 mmol DTT, 0.1 mmol phosphate buffer (pH, 7.2), 0.5 μmol Sod. malonate-2-^{14}C(0.5 Ci/mol) and 10 mg protein of whole cell extract prepared by sonification of cell suspension. The incubation was performed at 37°C for 1 hr.

kinds of fatty acids may be taken into some micro-organisms by a vectorial acylation process catalyzed by acyl CoA synthetase localized on surface of these organisms. On the assumption of the same mechanism in case of acetate-uptake of *M. smegmatis*, effect of CPIB on acetyl CoA synthetase activity in this organism was examined *in vitro*. As shown in Table 4, however, no effect of CPIB was observed. In the next place,

TABLE 4.

Effects of CPIB, *in vitro*, on acetyl CoA synthetase of *M. smegmatis*.

conc. of CPIB added to incubat. mixture*	acetyl CoA formed after incubation
0 mM	17,330 dpm
0.2 mM	20,690 dpm
1.0 mM	14,120 dpm

* Incubation mixture (± CPIB) was as follows; 1.0 μmol CoA, 0.01 mmol ATP, 0.01 mmol $MgCl_2$, 0.1 mmol phosphate buffer (pH, 7.6), 1.0 μmol Sod. acetate-2-^{14}C (0.5 Ci/mol) and 0.7 mg protein of whole cell extract. The incubation was performed at 37°C for 0.5 hr.

effect of 4-pentenoate[12], a special reagent for reducing of free CoA's concentration in living cells, on the acetate-uptake of *M. smegmatis* was investigated. As shown in Table 5, it was found that 4-pentenoate clearly inhibit the uptake of acetate. Inhibitiory effects of potassium cyanide (a respiratory poison), 2, 4-dinitrophenol (an uncoupler) as well as iodoacetamide (a SH-reagent) against the uptake of acetate were also demonstrated as in Table 5. Accordingly, it seemed possible that degree of acetate-uptake in the cells depends upon the contents of both CoA and ATP in cells. Considering these facts as well as another one that radioactivities in released $^{14}CO_2$ showed always much more than those in cell bodies (sum of radioactivities in lipid and non-lipid fractions) after incubations of the cells with 2-^{14}C-acetate as shown in Table 1 & 5, the possibility of participation of acetyl CoA synthetase in the uptake of acetate appeared to increase much more.

From such consideration, any activity of CPIB which may decrease free CoA's or ATP's content in the cells could be postulated. In this connection, uncoupling effects of CPIB on rat liver mitochondria[13,14] have been already reported. Therefore, effect of CPIB on ATP's content of *M. smegmatis* during the incubation was investigated. As shown in Table 6, it was found that CPIB decreased ATP's content very clearly after the incubation and the decreased percent of ATP's content reached

TABLE 5.

Effects of various inhibitors on 2-^{14}C-acetate uptaking activity of *M. smegmatis*.

inhibitor	final conc. of inhibitor added to incubat. system*	$^{14}CO_2$ released dpm (%)	radioactivity in the cells dpm (%)	total uptake of acetate dpm (%)
4-pentenoate	0 mM	360,000 (100)	205,000 (100)	565,000 (100)
	9 mM	309,000 (86)	154,000 (75)	463,000 (82)
	45 mM	95,800 (27)	18,000 (9)	113,800 (20)
potassium cyanide	0 mM	280,000 (100)	190,000 (100)	470,000 (100)
	1 mM	18,800 (7)	5,360 (3)	24,160 (5)
	10 mM	11,600 (4)	2,500 (1)	14,100 (3)
2,4-di nitro phenol	0 mM	385,000 (100)	207,000 (100)	592,000 (100)
	0.5mM	403,000 (105)	102,000 (49)	505,000 (85)
	1 mM	256,000 (66)	40,100 (19)	296,100 (50)
iodoacetamide	0 mM	280,000 (100)	190,000 (100)	470,000 (100)
	1 mM	22,500 (8)	2,400 (1)	24,900 (5)
	10 mM	28,000 (1)	1,100 (0)	3,900 (0)

* Incubation systems were the same as in Table 1.

TABLE 6.

Contents of ATP in *M. smegmatis* after incubation with CPIB.

conc. of CPIB added to incubation system*	incubation time at 37°C	content of ATP in 25 mg wet weight of cells
0	60 min	0.67 n mol (100%)
0.5 mM	60 min	0.59 (88〃)
1.0 mM	60 min	0.40 (60〃)
0	30 min	0.70 (100%)
2.0 mM	30 min	0.53 (75〃)
0	60 min	0.99 (100%)
2.0 mM	60 min	0.48 (49〃)

* Incubation system was the same as the preincubation system described in text.

at approximately the same level of the decreased percent of the acetate-uptake as described in Table 1. According to the results described above, a mechanism of the acetate-uptake of *M. smegmatis* could be infered as shown in Fig. 3. Namely, CPIB could reduce at first ATP's content in the cells required for the activity of acetyl CoA synthetase

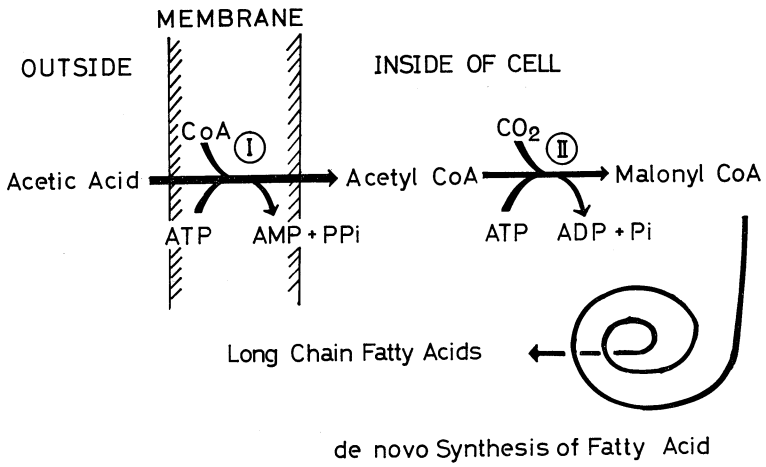

Fig. 3. Postulated mechanism of acetate-uptake into cells of *M. smegmatis*. Two possible steps effected by CPIB (I) acetyl CoA synthetase reaction, (II) acetyl CoA carboxylase reaction.

which may be involved in taking up exogenous acetate. Secondly, CPIB could inhibit *de novo* synthesis of fatty acids by reducing ATP's content which is also required for the activity of acetyl CoA carboxylase. Another possibility of CPIB's effect against acetyl CoA carboxylase itself remaines also because an inhibitory function of CPIB against hepatic acetyl CoA carboxylase has been also reported[15].

DISCUSSION

Many studies on CPIB as a potent inhibitor against biosynthesis of lipids have been reported[15,16,17]. It seemed, therefore, interesting to investigate any function of CPIB on the cellular uptake of acetate, as not only the simplest fatty acid but also a precursor of long chain fatty acids, because activities of nutrients-uptake and further metabolism of those nutrients are often related intimately in microbial cells as the known case of induction of β-galactosidase system in *Escherichia coli*. As reported in Table 1, CPIB was found to inhibit the acetate-uptake itself very clearly, beside another inhibitory function against biosynthesis of lipids. This is a new antibiological function of CPIB ever reported. If the mechanism as postulated in Fig. 3 is true, at least two major inhibitory steps of CPIB should be considered for *de novo* synthesis of fatty acid in microorganisms *in vivo*, the one is taking step of acetate, the other step is an acetyl CoA carboxylase reaction.

Studies to elucidate participation of acetyl CoA synthetase for the

acetate-uptake of this organism should be achieved more directly, for example, by use of membrane vesicle preparation, electron-microscopic observation and so on. It should also be interesting to investigate if a mechanism of acetate-transport through animal's cellular membrane is similar to that in *M. smegmatis* or not.

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