Kitazin P, Inhibitor of Phosphatidylcholine Biosynthesis in *Pyricularia oryzae**

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The effect of organophosphorus fungicide, Kitazin P (IBP, *S*-benzyl diisopropyl phosphorothiolate), on lipid biosynthesis of *Pyricularia oryzae* was investigated. Addition of IBP to the mycelial cells suspension of *P. oryzae* induced a striking decrease in incorporation of methionine-methyl-^{14}C, *S*-adenosylmethionine-methyl-^{14}C, and glycerol-1-^{14}C into phosphatidylcholine, which is the most abundant phospholipid in *P. oryzae*, but incorporation of choline-methyl-^{14}C into phosphatidylcholine and that of methionine-methyl-^{14}C into simple lipids were not affected. Incorporation of methionine-methyl-^{14}C into phosphatidylcholine is found to be directly proportional to mycelial cells growth of *P. oryzae*. Enzymes responsible for the biosynthesis from glycerol to phosphatidylcholine through Greenberg's pathway, except phospholipid *N*-methyltransferase, were not inhibited by IBP. IBP concentration required for 50% inhibition of phospholipid *N*-methyltransferase was 40 ppm. IBP had no effect on activities of glycerokinase, glycerophosphate acyltransferase, phosphatidic acid cytidyltransferase, phosphatidylserine synthetase, and phosphatidylserine decarboxylase, respectively. Therefore, the specific inhibition of conversion from phosphatidylethanolamine to phosphatidylcholine by the transmethylation of *S*-adenosylmethionine might be regarded as one of the modes of action of IBP.

IBP has been widely used as an organophosphorus fungicide to control rice blast caused by *P. oryzae*. Kakiki *et al.*[1] reported that EBP (Kitazin) inhibited the incorporation of glucosamine-1-^{14}C into the cell wall of *P. oryzae* without affecting respiration, protein biosynthesis, and nucleic acid biosynthesis. Since they suggested that a mode of action of EBP might be the inhibition of the cell wall biosynthesis especially the chitin biosynthesis, several famous scientists[2~4] quote the results of an experiment by Kakiki *et al.*

Maeda *et al.*[5] found that mycelia of *P. oryzae* treated with IBP accumulated UDP-*N*-acetylglucosamine. They considered that the accumulation of UDP-*N*-acetylglucosamine results from the inhibition of the cell wall synthesis system by IBP. They also discussed that IBP seems to affect the permeation of chitin precursors through cell membrane and presented some speculations from several points of view.

De Waard[6] reported that the effect of EDDP (edifenphos, Hinosan) and IBP on cell membrane permeability is probably primarily responsible for their fungitoxicity and that the interference with chitin synthesis has to be regarded as a secondary effect.

However, the authors[7] reported earlier in the short communication that IBP markedly inhibited the incorporation of methionine-methyl-^{14}C into phospholipid, especially phosphatidylcholine, at the concentration of 50 ppm. Therefore, the inhibition of phosphatidylcholine biosynthesis by IBP might be regarded as one of the modes of action of IBP.

Katagiri and Uesugi[8] reported that cross resistance was found between organophosphorus thiolate fungicides (IBP, EDDP) and isoprothiolane (Fuji-One). This finding suggested a similarity in the mode of action of organophosphorus thiolate fungicides and isoprothiolane.

Shimosako and joint researcher, Kakiki,[9~11]

* Mechanisms of Action of Organophosphorus Fungicides. Part I.

have presented several times the mode of action of isoprothiolane at the Annual Meeting of Pesticide Science Society of Japan. Isoprothiolane inhibited lipid biosynthesis without affecting protein and nucleic acid biosynthesis in mycelia of *P. oryzae*, and this fungicide inhibited especially the incorporation of methionine-methyl-^{14}C into phospholipid and that of acetate-2-^{14}C into fatty acid and triglyceride, whereas the incorporation of *S*-adenosylmethionine-methyl-^{14}C into phosphatidylcholine was not inhibited by isoprothiolane at 50 ppm in the cell-free system. Kakiki and Misato[12] showed that isoprothiolane strongly inhibited the incorporation of suger-^{14}C such as ribose, galactose, mannose and glucose into the cell wall, whereas it did not inhibit the incorporation of glucosamine-1-^{14}C.

In this paper, we will describe the effect of IBP on lipid biosynthesis in detail and discuss the mechanism of action of IBP.

MATERIALS AND METHODS

Chemicals. L-Methionine-methyl-^{14}C, *S*-adenosylmethionine-methyl-^{14}C, glycerol-1-^{14}C, choline-methyl-^{14}C, L-glycerol 3-phosphate-U-^{14}C, CTP-U-^{14}C, and L-serine-U-^{14}C were obtained from The Radiochemical Centre, Amersham. Phosphatidylserine-U-^{14}C was synthesized from CDP-dioleoyl and L-serine-U-^{14}C using a particulate fraction of *P. oryzae* as enzyme source.[13] CDP-dioleoyl was obtained from Miles Laboratories Ltd. L-α-Phosphatidic acid, *S*-palmitoyl-CoA, and α-glycerophosphate dehydrogenase were purchased from Sigma Chemical Company. IBP was the gift of Kumiai Chemical Industry Co., Ltd.

Organism and culture condition. Wild type strain of *P. oryzae* designated as P-2 was used. Spores of *P. oryzae* were inoculated into 100 ml of liquid medium containing 20 g glucose, 5 g yeast extract, and decoction of 10 g rice straw in 1 liter (pH 7.0). After cultivation for 3~4 days at 27°C with reciprocal shaking, 10 ml of the medium was transfered to a shaking flask containing 100 ml of the fresh medium and cultured for 14 hr. The mycelial cells were harvested by filtration and washed with distilled water.

Radioisotope labeling. The washed mycelia (120 mg fresh weight) of *P. oryzae* were incubated in 5 ml of 0.05 M phosphate buffer (pH 7.0) at 27°C containing methionine-methyl-^{14}C (2 μCi) with or without IBP. After incubation, the mixture was filtered and washed with distilled water. The lipids of *P. oryzae* were extracted according to the method of Bligh and Dyer,[14] and the lipids were separated by thin-layer chromatography on silica gel G plates using the solvent systems of chloroform–methanol–ammonia water (70: 35: 5, v/v) and heptane–isopropyl ether–acetic acid (60: 40: 4, v/v). Spots were detected with iodine vapor, Dittmer reagent,[15] Dragendorff reagent,[16] and ninhydrin reagent.[17] The radioactive lipids were detected with an autoradiographic technique (X-ray film). The ^{14}C-labeled lipid fraction was scraped out into a scintillation vial containing 0.5 ml of ethyl acetate, after 30 min the 5 ml of scintillator (4 g PPO, 1 liter toluene) was added and then radioactivity was measured with a liquid scintillation counter (Beckman, LS–100C).

Determination of phospholipid composition. Relative phospholipid composition was determined with quantitative thin-layer chromatography (silica gel H) using the solvent system of chloroform–methanol–ammonia water (70: 35: 5, v/v) combined with phosphorus estimation. Phospholipids were extracted from silica gel H by the neutral Bligh and Dyer procedure.[18] Lipid phosphorus was determined by the method of Bartlett.[19]

Preparation of cell-free extracts for enzyme assay. Glycerokinase. Mycelia of *P. oryzae* were suspended in 4 volumes of 0.25 M sucrose containing 0.1 mM EDTA. The suspension was blended for 1 min in a Waring Blender microcup and homogenized in a Potter's homogenizer, the homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant fraction was centrifuged at 105,000 × *g* for 1 hr. The 105,000 × *g* supernatant solution was brought to 70% saturation with solid $(NH_4)_2SO_4$ and allowed 1 hr. The precipitate was collected by centrifugation at 20,000 × *g* for 15 min and dissolved in 0.01 M phosphate buffer (pH6.7) containing 2.2 M $(NH_4)_2SO_4$, 0.01 M glycerol, and 1 mM EDTA.

Glycerophosphate acyltransferase and phosphatidic acid cytidyltransferase. The pellet obtained from the 105,000 × *g* centrifugation was suspended in 0.32 M phosphate buffer (pH 7.5).

Phosphatidylserine synthetase. Mycelia of *P. oryzae* were suspended in 4 volumes of 0.01 M Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 5 mM 2-mercaptoethanol. The suspension was homogenized by the above method. The homogenate solution was centrifuged at 10,000 × *g* for 45 min. The 10,000 × *g* supernatant solution was brought to 80% saturation with solid $(NH_4)_2SO_4$ and allowed 1 hr. The precipitate was collected by centrifugation at 24,000 × *g* for 30 min and dissolved in the buffer. The preparation was dialyzed for 10 hr against the buffer. $MnCl_2$ was added to the dialyzed solution to a final concentration of 0.05 M and allowed 30 min. The resulting precipitate

was collected by centrifugation at 24,000×*g* for 30 min. The pellet was dissolved in 0.28 M Tris-phosphate buffer (pH 7.9).

Phosphatidylserine decarboxylase. Mycelia of *P. oryzae* were suspended in 3 volumes of 0.1 M phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$ and 10 mM 2-mercaptoethanol. The suspension was homogenized by the above method and the homogenate was centrifuged at 1,000×*g* for 10 min and the supernatant fraction was centrifuged at 105,000×*g* for 1 hr. The pellet was suspended in 0.2 M phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol.

Phospholipid N-methyltransferase. Mycelia of *P. oryzae* were suspended in 4 volumes of 0.25 M sucrose and the suspension was homogenized by the above method. The homogenate was centrifuged at 1,000×*g* for 10 min and the supernatant fraction was centrifuged at 10,000×*g* for 10 min. The 10,000×*g* supernatant fraction was centrifuged at 25,000×*g* for 20 min and the 25,000×*g* supernatant fraction was centrifuged at 105,000×*g* for 1 hr to obtain the microsomal fraction. The microsomal fraction was washed by resuspension in 0.25 M sucrose followed by sedimentation again at 105,000×*g* for 1 hr. Washed microsomal pellet was resuspended in 0.05 M Tris-HCl buffer (pH 8.2).

Enzyme assay. Each enzyme reaction mixture contains one to five mg of protein. Results were obtained under conditions of linearity with respect to both time and protein concentration.

Glycerokinase activity was determined by means of a spectrophotometric method according to the procedure of Kennedy.[20]

Glycerophosphate acyltransferase activity was assayed by measuring incorporation of L-glycerol 3-phosphate-U-^{14}C into lipid fraction in the presence of palmitoyl-CoA. The reaction mixture consisted of L-glycerol 3-phosphate-U-^{14}C (0.5 μCi), 2 mg fatty acid-poor bovine albumin, 1.5 mM $MgCl_2$, 75 mM Tris-HCl buffer (pH 7.5), 0.085 mM *S*-palmitoyl CoA, and the enzyme preparation in a final volume of 0.5 ml. Incubation was carried out for 10 min at 37°C, after which the lipid was extracted from the reaction mixture according to the method of Bligh and Dyer, and the incorporated radioactivity was measured.

Phosphatidic acid cytidyltransferase activity was measured by the incorporation of CTP-U-^{14}C into lipid fraction in the presence of phosphatidic acid. The reaction mixture consisted of CTP-U-^{14}C (0.4 μCi), 2 mM phosphatidic acid, 0.25% Triton X-100, the enzyme preparation, and 4 mM $MgCl_2$ in a final volume of 0.5 ml. The reaction mixture was incubated at 37°C for 5 min and the lipid was extracted.

Phosphatidylserine synthetase activity was measured with the incorporation of L-serine-U-^{14}C into chloroform phase in the presence of CDP-diglyceride. The incubation mixture contained 1 mM CDP-dioleoyl, L-serine-U-^{14}C (0.2 μCi), 0.1% Triton X-100, 2 mM EDTA, 80 mM Na_2SO_4, 4 mM 2-mercaptoethanol, and the enzyme preparation in a final volume of 0.5 ml. After 60 min at 37°C, the lipid was extracted from the reaction mixture and the radioactivity of chloroform phase was measured with a liquid scintillation counter.

Phosphatidylserine decarboxylase activity was measured by release of $^{14}CO_2$ from phosphatidylserine-U-^{14}C as described by Dowhan *et al.*[13]

The enzyme assay for phospholipid *N*-methyltransferase was based on the reaction condition outlined by Rehbinder and Greenberg.[21] The reaction mixture consisted of the enzyme preparation, *S*-adenosylmethionine-methyl-^{14}C (0.5 μCi), and 0.5 ml of 0.2 M Tris-HCl buffer (pH 8.2) in a total volume of 1.2 ml. Incubation was carried out for 60 min at 30°C and the lipid of the reaction mixture was extracted by the method of Bligh and Dyer. The formation of radioactive phosphatidylcholine, which was shown by thin-layer chromatography, was measured with a liquid scintillation counter.

Protein concentration was determined by the method of Lowry *et al.*[22] using bovine serum albumin as a standard.

RESULTS

IBP inhibited cell growth without affecting the shape of mycelial cells. By the addition of 50 ppm IBP to the medium, dry weight increase of mycelial cells was suppressed strongly

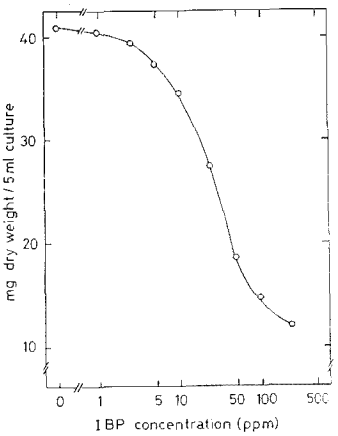

FIG. 1. Effect of IBP on the Growth of *P. oryzae*. Mycelia of *P. oryzae* (12 mg dry weight) were cultivated in 5 ml of the liquid medium containing various concentrations of IBP. After cultivation for 10 hr, each dry weight of mycelial cells was determined.

TABLE I. EFFECT OF IBP ON THE INCORPORATION OF METHIONINE-METHYL-^{14}C INTO LIPID FRACTIONS OF *P. oryzae*

The mycelia of *P. oryzae* (120 mg fresh weight) were incubated in 5 ml of 0.05 M phosphate buffer (pH 7.0) containing methionine-methyl-^{14}C (2 μCi) for 2 hr at 27°C. The lipids of *P. oryzae* were extracted by the method of Bligh and Dyer. Simple and compound lipids were separated by thin-layer chromatography.

Lipid fraction	Methionine incorporated (nmol/5 ml reaction mixture)		Inhibition (%)
	Untreated control	IBP treated at 50 ppm	
Total lipids	5.52	2.49	55
Simple lipids	1.22	1.16	5
Compound lipids	3.81	1.13	70

(Fig. 1). Therefore, the following experiments were conducted at a final concentration of 50 ppm in the reaction mixture.

Concerning the effect of IBP on the incorporation of methionine-methyl-^{14}C into lipid fractions, it can be seen from Table I that IBP markedly reduced the incorporation of methionine-methyl-^{14}C into compound lipids. Therefore, by the use of autoradiographic technique, effect of IBP on the incorporation of methionine-methyl-^{14}C was examined (Fig. 2). The most remarkable incorporation of methionine-methyl-^{14}C was observed in fraction I, whereas the incorporation into fraction I is markedly inhibited by treatment with 50 ppm IBP. Fraction I reacted positively to the addition of

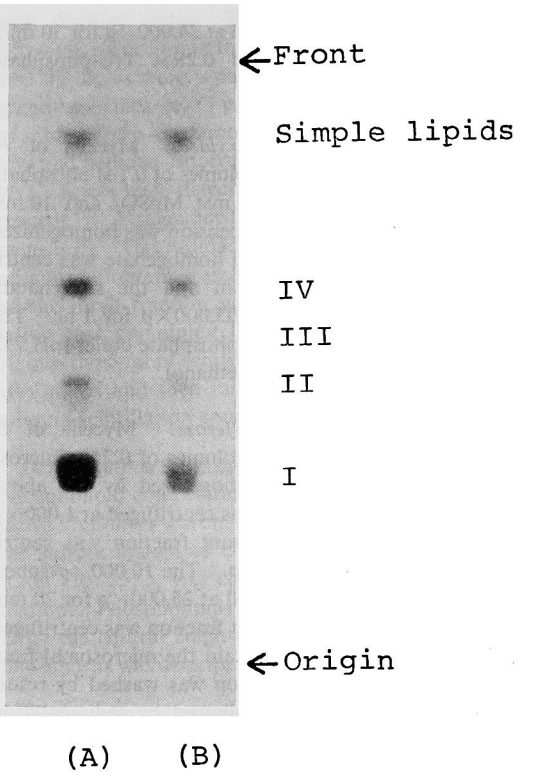

FIG. 2. Autoradiogram Showing Thin-layer Chromatographic Separation of Labeled Lipids in *P.oryzae*.
The mycelia of *P. oryzae* (120 mg fresh weight) were incubated in 5 ml of 0.05 M phosphate buffer (pH 7.0) containing methionine-methyl-^{14}C (2 μCi) for 4 hr at 27°C. The lipids of *P. oryzae* were separated by a one-dimensional thin-layer chromatography on a silica gel G plate with the following system: chloroform–methanol–ammonia water (70: 35: 5, v/v).
(A) untreated control, (B) IBP treated at 50 ppm.

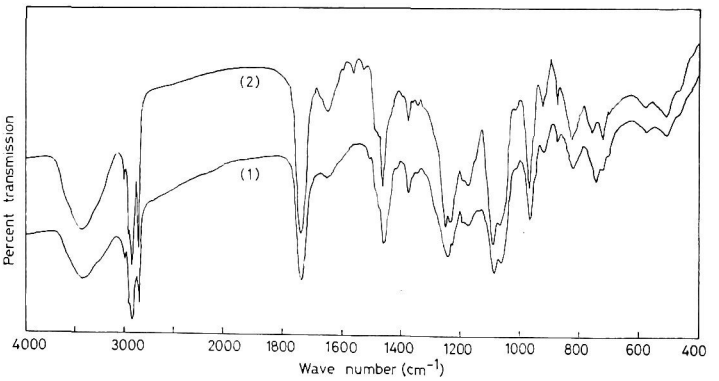

Fig. 3. Infrared Absorption Spectra of Fraction I (1) and Phosphatidylcholine Isolated from Egg Yolk (2) (KBr pellets).

TABLE II. PHOSPHOLIPID COMPOSITION OF *P. oryzae*

The phospholipids of *P. oryzae* were separated by a one-dimensional thin-layer chromatography on a silica gel H plate with the following system: chloroform–methanol–ammonia water (70: 35: 5, v/v). Each phospholipid was extracted from silica gel H and lipid phosphorus was determined.

Phospholipid	Composition (%)
Phosphatidylcholine	51
Phosphatidylethanolamine	27
Phosphatidylinositol / Phosphatidylserine	15
Unknown	4

both Dittmer and Dragendorff reagents but negatively to ninhydrin reagent. The *Rf* value of fraction I coincided with the standard phosphatidylcholine from egg yolk. Infrared absorption spectrum of the fraction I also coincided with that of the standard phosphatidylcholine (Fig. 3). Phosphatidylcholine counts approximately 50% of the total phospholipids (Table II) and the radioactivity of phosphatidyl choline accounts for more than 50% of the total radioactivity of lipids in *P. oryzae*. As shown in Table III, the incorporation of choline-methyl-^{14}C into phosphatidylcholine was not inhibited by IBP at 50 ppm, however the incorporation of methionine-methyl-^{14}C, *S*-adenosylmethionine-methyl-^{14}C, and glycerol-1-^{14}C into phosphatidylcholine was markedly inhibited by IBP. In the absence of IBP, incorporation of methionine-methyl-^{14}C into phosphatidylcholine increased in proportion to the growth of *P. oryzae* in medium, while in the presence of IBP at 50 ppm, both incorporation of methionine-methyl-^{14}C into phosphatidylcholine and growth of *P. oryzae* were scarcely observed (Fig. 4).

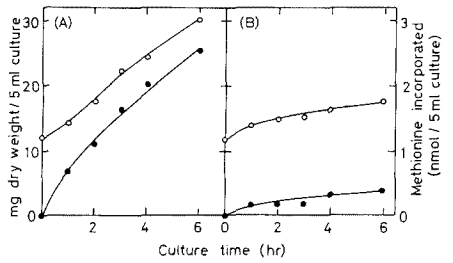

FIG. 4. Effect of IBP on the Growth and the Incorporation of Methionine-methyl-^{14}C into Phosphatidylcholine in *P. oryzae*.

The mycelia of *P. oryzae* (12 mg dry weight) were cultivated in 5 ml of the liquid medium containing 2 μCi methionine-methyl-^{14}C for 6 hr at 27°C with reciprocal shaking. (A) untreated control, (B) IBP reated at 50 ppm.
○—○, dry weight; ●—●, methionine incorporated.

Using microsomal fraction, effect of IBP on

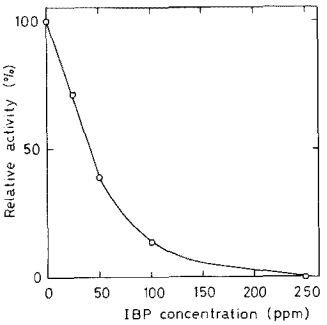

FIG. 5. Effect of IBP on the Activity of Phospholipid *N*-methyltransferase in Microsomal Fraction of *P. oryzae*.

TABLE III. EFFECT OF IBP ON THE INCORPORATION OF CHOLINE-METHYL ^{14}C, METHIONINE-METHYL-^{14}C, *S*-ADENOSYLMETHIONINE-METHYL-^{14}C OR GLYCEROL-1-^{14}C INTO PHOSPHATIDYLCHOLINE IN *P. oryzae*

The mycelia of *P. oryzae* (120 mg fresh weight) were incubated in 5 ml of 0.05 M phosphate buffer (pH 7.0) containing choline-methyl-^{14}C (1 μCi), methionine-methyl-^{14}C (2 μCi), *S*-adenosylmethionine-methyl-^{14}C (0.5 μCi) or glycerol-1-^{14}C (2 μCi) for 4 hr at 27°C. The lipids of *P. oryzae* were separated by a thin-layer chromatography and the radioactivity of phosphatidylcholine was measured with a liquid scintillation counter.

Labeled compound	Labeled compound incorporated (nmol/5 ml reaction mixture)		Inhibition (%)
	Untreated control	IBP treated at 50 ppm	
Choline-methyl-^{14}C	2.34	2.21	6
Methionine-methyl-^{14}C	6.79	1.47	78
S-Adenosylmethionine-methyl-^{14}C	0.85	0.21	75
Glycerol-1-^{14}C	0.32	0.11	67

the incorporation of *S*-adenosylmethionine-methyl-^{14}C into phosphatidylcholine, that is, phospholipid *N*-methyltransferase activity, was examined (Fig. 5). IBP also markedly reduced the incorporation of *S*-adenosylmethionine-methyl-^{14}C into phosphatidylcholine and IBP concentration required for 50% inhibition of phospholipid *N*-methyltransferase was 40 ppm.

Because incorporation of glycerol-1-^{14}C into phosphatidylcholine was inhibited by IBP (Table III), effects of IBP on enzymatic activities responsible for the biosynthesis from glycerol to phosphatidylethanolamine, that is, glycerokinase, glycerophosphate acyltransferase, phosphatidic acid cytidyltransferase, phosphatidylserine synthetase, and phosphatidylserine decarboxylase were examined respectively, however these enzymes were not inhibited by IBP at 50 ppm (Table IV).

TABLE IV. EFFECT OF IBP ON ENZYMATIC ACTIVITIES RESPONSIBLE FOR PHOSPHOLIPID BIOSYNTHESIS IN *P. oryzae*

Enzyme	Conc. of IBP (ppm)	Relative[a] activity (%)
Glycerokinase	0	100
	25	101
	50	105
Glycerophosphate acyltransferase	0	100
	25	99
	50	118
Phosphatidic acid cytidyltransferase	0	100
	25	101
	50	106
Phosphatidylserine synthetase	0	100
	25	99
	50	101
Phosphatidylserine decarboxylase	0	100
	25	97
	50	115

[a] Relative activity was expressed as percentages to an untreated control.

DISCUSSION

As described in the results, it was noticeable that a large amount of phosphatidylcholine was found *P. oryzae*. As a rule, the biosynthesis of phosphatidylcholine by two distinct pathways has been reported. The first, discovered by Kennedy and Weiss,[23,24] involves the formation from choline to phosphatidylcholine. The second, discovered by Greenberg *et al.*,[25,26] involves the conversion from phosphatidylethanolamine to phosphatidylcholine by transmethylation of *S*-adenosylmethionine. Inhibition of IBP on the incorporation of methionine-methyl-^{14}C, *S*-adenosylmethionine-methyl ^{14}C, and glycerol-1-^{14}C into phosphatidylcholine was found, however no inhibition was found with the incorporation of choline-methyl-^{14}C. This fact suggests that the inhibitory action of IBP is in Greenberg's pathway, but is not Kennedy's pathway. Greenberg's pathway is considered to be the predominant route of phosphatidylcholine biosynthesis in *P. oryzae*, because incorporation of methionine-methyl-^{14}C into phosphatidylcholine is found to be directly proportional to mycelial growth of *P. oryzae*. Enzymatic activities responsible for the biosynthesis from glycerol to phosphatidylcholine by way of Greenberg's pathway, except phospholipid *N*-methyltransferase, were not inhibited by IBP at 50 ppm. Therefore, IBP specifically inhibited the conversion of phosphatidylethanolamine to phosphatidylcholine by methylation utilizing the methyl group of *S*-adenosylmethionine.

Phospholipid is located mainly in the cell membrane and constitutes a substantial component of all membranes. One of the major functions of phosphatidylcholine is thus to constitute the structure and function of cell membranes. Phosphatidylcholine is an essential for the cytochrome P-450—catalyzed reaction,[27] ATPase,[28,29] D-β-hydroxybutyrate apodehydrogenase[30] and other membrane-bound enzymes.[31]

Recently new fungicides, *i.e.*, inhibitors of ergosterol biosynthesis, have been developed. These new fungicides including Triarimol,[32~34] *S*-1358 (Denmert),[35~37] Triforine,[38] Triadimefon,[39] and Imazalil[40] inhibited sterol demethylation in ergosterol biosynthesis without affecting the phospholipid biosynthesis.

Cross-resistance between IBP and EDDP has been reported in *P. oryzae*.[41] Recently the authors discovered that EDDP also inhibited phosphatidylcholine biosynthesis, es-

pecially methylation of phosphatidylethanolamine, without affecting chitin biosynthesis.[42]

These results indicated that the specific inhibition of conversion from phosphatidylethanolamine to phosphatidylcholine by transmethylation of *S*-adenosylmethionine in Greenberg's pathway is one of the modes of action of IBP.

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