

Dithiocarbamate系殺菌剤の作用機作に関する研究 (2)

イネ白葉枯病菌の脂質合成に及ぼす sodiumdimethyldithiocarbamateの作用

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Studies on the Fungicidal Action of Dithiocarbamates

2. Effect of sodium dimethyldithiocarbamate on the lipid synthesis of *Xanthomonas oryzae*

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米山勝美・見里朝正 : Dithiocarbamate 系殺菌剤の作用機作に関する研究 2. イネ白
葉枯病菌の脂質合成に及ぼす Sodium dimethyldithiocarbamate の作用

Abstract

The mechanism of action of sodium dimethyldithiocarbamate (NaDMDC) was examined by using *Xanthomonas oryzae*, the causal pathogen of bacterial leaf blight on rice plant. The result showed that NaDMDC inhibited selectively the incorporation of ^{14}C -acetate into the lipids of *X. oryzae*, and this inhibition was exerted more strongly when the cells were active in metabolism. Also, NaDMDC inhibited nonspecifically the incorporation of radioactive precursors into all lipid components of cells. In the phosphatidylethanolamine from ^{14}C -glucose labeled cells, labeling of the fatty acid constituent, rather than the glycerolphosphorylethanolamine constituent, was markedly inhibited by NaDMDC. However, NaDMDC did not inhibit the incorporation of radioactive long-chain fatty acids into the phospholipids of cells. These results suggested that the process from long-chain fatty acids to phospholipids was not affected by NaDMDC. Thus, the primary action of NaDMDC may be involved in the fatty acid synthesis of *X. oryzae*.

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Introduction

Since the discovery of antifungal effect of dithiocarbamates made by Tisdale and Williams in the latter half of 1920's, this group of organic compounds exerted a very important role for the protection of plant diseases in agriculture. A large number of different derivatives have been in practical use as fungicides or bactericides. They are principally divided into three types in chemical structure, such as monoalkylamine, dialkylamine and alkyldiamine. Our concern here was focused on the dialkylamine type, because it includes nickel dimethyldithiocarbamate (SANKEL®) which was developed in Japan as an antibacterial agent by showing the high protective effect against bacterial leaf blight of rice plant. With the mechanism of action for this type of compounds, particularly, an interesting matter is to know much about the biochemical effect on the metabolism of bacterial cells.

On the mode of action of sodium dimethyldithiocarbamate (NaDMDC), it has been already speculated that the toxic mechanism was involved in the inactivation of various enzymes, such as enzymes concerned with organic acid synthesis of fungal cells¹⁰⁾, SH-containing enzymes¹³⁾ or coenzymes¹¹⁾, and metal-containing enzymes^{5, 8, 9)}. However, none of these studies were enough to offer a satisfactory explanation for

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the antimicrobial effects of NaDMDC, and the problem still remains to be studied, especially on effect against the macromolecular biosynthesis of cells.

To elucidate this point, the authors tried to examine in detail about the inhibitory action of NaDMDC on the cellular metabolism of *X. oryzae*. It was found that NaDMDC inhibited remarkably the incorporation of radioactive precursors into the lipids of *X. oryzae*, whereas this compound had little inhibitory effects on the respiration and the protein, nucleic acid and cell wall synthesis of the cells.¹⁵⁾

This paper is a report of an advanced investigation about the inhibitory effects of NaDMDC on the lipid metabolism of *X. oryzae*.

Materials and Methods

Test organism and chemicals. The culture of *X. oryzae* (strain: H-5809) was provided from the Division of Plant Pathology, National Institute of Agricultural Sciences, Tokyo. NaDMDC was purchased from the Tokyo Kasei Kogyo Co., Ltd. All the radioactive compounds used in the experiments were purchased from the Radiochemical Center, Amersham, England. Silicic acid was obtained from the Mallinckrodt Organic Works and prepared by the method of Bullen *et al*³⁾. Phosphatidylethanolamine (PE) was obtained from the Wako Chemical Co., Ltd.

Incorporation of radioactive precursors into the lipids of cells. Cell suspension of *X. oryzae* was prepared with 1/15 M phosphate buffer pH 7.0 unless otherwise described. All labeling experiments were performed by the same procedure as described previously¹⁵⁾, except that the reaction mixtures of radioactive palmitic acid and oleic acid incorporations contained 10 μ M of respective unlabeled compounds. The final radioactivities of precursors used, namely acetate-U-¹⁴C (45.2 mCi/mmol), glucose-U-¹⁴C (5.0 mCi/mmol), ³²P-H₃PO₄ (52.0 Ci/mmol), palmitic acid-l-¹⁴C (57.9 mCi/mmol) and oleic acid-l-¹⁴C (59.7 mCi/mmol) were 0.1 μ Ci/ml, 0.5 μ Ci/ml, 2.0 μ Ci/ml, 0.5 μ Ci/ml and 0.5 μ Ci/ml, respectively. Lipid extraction was carried out by the method of Bligh and Dyer²⁾.

Determination of leakage of lipid components from the cells. Thirty ml of bacterial suspension prepared with Suwa liquid medium¹⁴⁾ containing 10% peptone was incubated in the presence of ¹⁴C-acetate (final radioactivity: 0.5 μ Ci/ml) with shaking for 30 min at 28°C. The cells were collected by centrifuging at a low speed, washed twice with cold distilled water, and resuspended in the same fresh medium of original volume. Then, 5 ml aliquots was reincubated with shaking after added with a given concentration of NaDMDC, and 1 ml of the sample was removed at the given time intervals and was subjected to the extraction of lipids by the procedure described above. The extracted lipids were analyzed for radioactivity by a windowless gas flow counter.

Silicic acid column chromatography of lipids. Incorporation tests of radioactive precursors were executed in the volume of 70 ml of bacterial suspension. The extraction of lipids from the cells was carried out by the method of Folch and Stanley⁵⁾, the lipid extract was evaporated to dryness under reduced pressure and redissolved in 5 ml of chloroform. The lipids were applied on a silicic column according to the method of Sakagami *et al*¹²⁾, and eluting solution was fractionated in the volume of 5 ml. One ml of each fraction was placed on a counting plate and dried. Radioactivity was determined by a gas flow counter.

Identification of phospholipids and their alkaline hydrolyzates. Main lipid component obtained by silicic acid chromatography was spotted on a silica gel TLC plate. The plate was developed with chloroform-methanol-water (65:25:4, v/v), dried at a room temperature, then sprayed with ninhydrin reagent. PE was identified

as one ninhydrin-positive spot whose position coincided with authentic PE. An additional detection for PE was made with the water-soluble deacylated phospholipid, which was obtained from the mild alkaline hydrolysis of phospholipid by the method of Dawson⁴⁾. The deacylated phospholipid was spotted on a Whatman No.1 paper, and ascending chromatogram was developed using the phenol solvent saturated with water-acetic acid-ethanol (100:10:12, v/v). For color development, the chromatogram was sprayed with ninhydrin reagent or ammonium molybdate-perchloric acid solution modified by Dawson⁴⁾. The deacylated product of PE, glycerylphosphorylethanolamine (GPE), was identified by the location on the chromatogram which coincided with the GPE prepared from authentic PE.

Analysis of radioactivity for hydrolytic products of PE.

The cells were labeled with ^{14}C -glucose in the presence or absence of NaDMDC for 30 min, then were subjected to the extraction of lipids. The extracted lipids were chromatographed on a silica gel plate with the solvent system described above. PE region was detected by radio-TLC scanning, and scraped from the plate. Then, the PE was eluted with methanol and followed by the evaporation of solvent under nitrogen. The radioactive PE was submitted to preferential fatty ester hydrolysis, and the hydrolytic products were partitioned between GPE and fatty acids according to the procedure of Dawson⁴⁾. The GPE and fatty acid fractions were separately analyzed for radioactivity.

Results

Effect of some nutrient media on the inhibitory action of NaDMDC

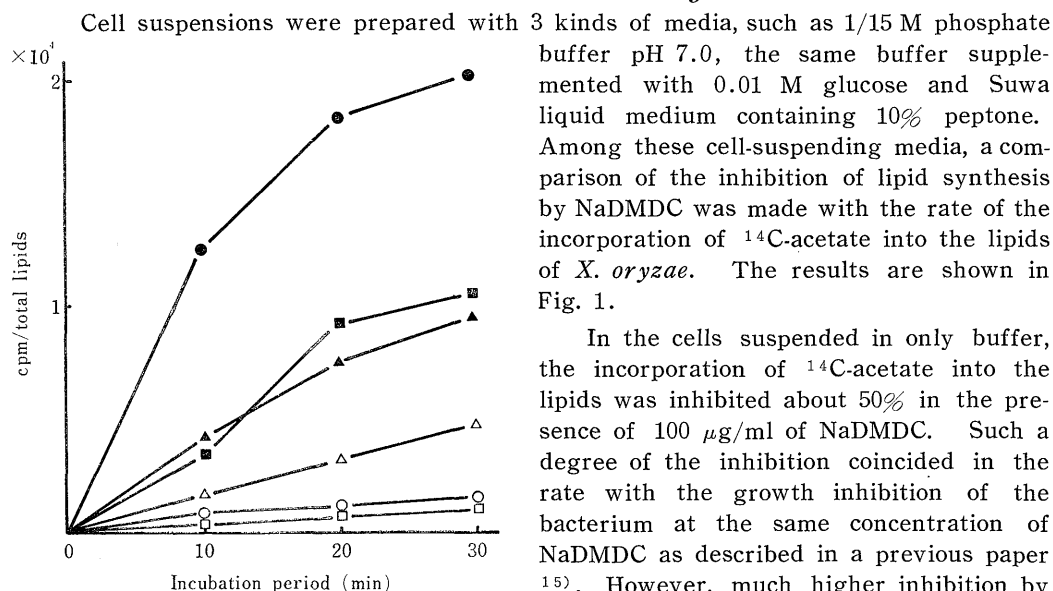


Fig 1. Effect of some nutrient media on the inhibition of lipid synthesis by NaDMDC. Bacterial suspensions were prepared from phosphate buffer pH 7.0, with (—△—) or without (—▲—) NaDMDC, phosphate buffer pH 7.0 containing 0.01M glucose, with (—○—) or without (—●—) NaDMDC, and Suwa liquid medium containing 10% peptone, with (—□—) or without (—■—) NaDMDC.

In the cells suspended in only buffer, the incorporation of ^{14}C -acetate into the lipids was inhibited about 50% in the presence of 100 $\mu\text{g}/\text{ml}$ of NaDMDC. Such a degree of the inhibition coincided in the rate with the growth inhibition of the bacterium at the same concentration of NaDMDC as described in a previous paper¹⁵⁾. However, much higher inhibition by NaDMDC was given with both the cells suspended in Suwa medium and the cells suspended in the glucose-supplemented buffer, and in either case the rates of inhibition were approximately 90%. On the other hand, the incorporation of ^{14}C -acetate into the lipids was markedly increased in the cells suspended in the glucose-supple-

mented buffer as compared with the cells suspended in the buffer solution, but this phenomenon was not observed in the cells suspended in Suwa medium. In the latter case, low labeling of the lipids is perhaps due to competition with the nutrient contents of Suwa medium in the incorporation of ^{14}C -acetate precursor into the lipids, because the medium is very rich in nutrient source. These facts suggest that NaDMDC may be more effective on the inhibition of lipid synthesis under the condition of activating cellular metabolism.

Effect of NaDMDC on the leakage of lipids from cells

Table 1. Effect of NaDMDC on the leakage of radioactive lipids from ^{14}C -acetate labeled cells

Concentrations of NaDMDC	Incubation time (min)			
	0	10	20	40
Control	10479	14469	10217	10637
10 $\mu\text{g/ml}$	10513	11229	10610	10832
100	10408	11267	9678	9896

Data are expressed as cpm per total lipids.

The loss of radioactively labeled lipids from the cells was determined in the presence or absence of NaDMDC. As shown in Table 1, the residual radioactivity in the lipids of NaDMDC-treated cells was almost the same as in the lipids of untreated cells, even though the cells were incubated under the presence of 100 $\mu\text{g/ml}$ of NaDMDC for 60 min. Therefore, it is unlikely that NaDMDC brings about the leakage of lipid components from the cells.

Comparative chromatography of lipids between NaDMDC-treated and untreated cells

The analysis of lipids was made by a silicic acid column chromatography in order to determine the inhibitory site of NaDMDC in the course of lipid synthesis. The lipids were extracted from the cells labeled with ^{14}C -acetate in the presence or

absence of 50 $\mu\text{g/ml}$ NaDMDC for 30 min. The chromatographic patterns of the lipids are illustrated in Fig. 2. In the lipids of untreated cells serving as control, the lipid components were fractionated as several small radioactive peaks and one extremely large peak, the large peak occupying an overwhelming major portion of the total radioactivity. The lipid revealed by the large peak therefore was considered as a main component of cellular lipids in *X. oryzae*. Also, a similar chromatographic pattern was obtained with the lipids of NaDMDC-treated cells, but all of the radioactive peaks were remarkably lowered at a nearly equivalent rate in their height or size as compared with those of un-

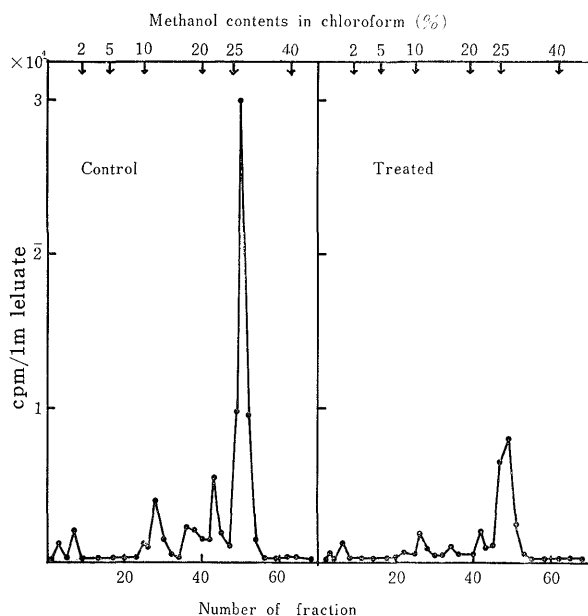


Fig. 2. Silicic acid column chromatography of the labeled lipids from the cells exposed to ^{14}C -acetate

untreated cells.

In the other experiment, the lipids were extracted from the cells labeled with $^{32}\text{P}\text{-H}_3\text{PO}_4$, and chromatographed for the detection of phospholipids and for determi-

nation of the inhibitory site of NaDMDC on phospholipid synthesis. As shown in Fig. 3, some radioactive peaks were revealed at the late stage of elution, the peaks

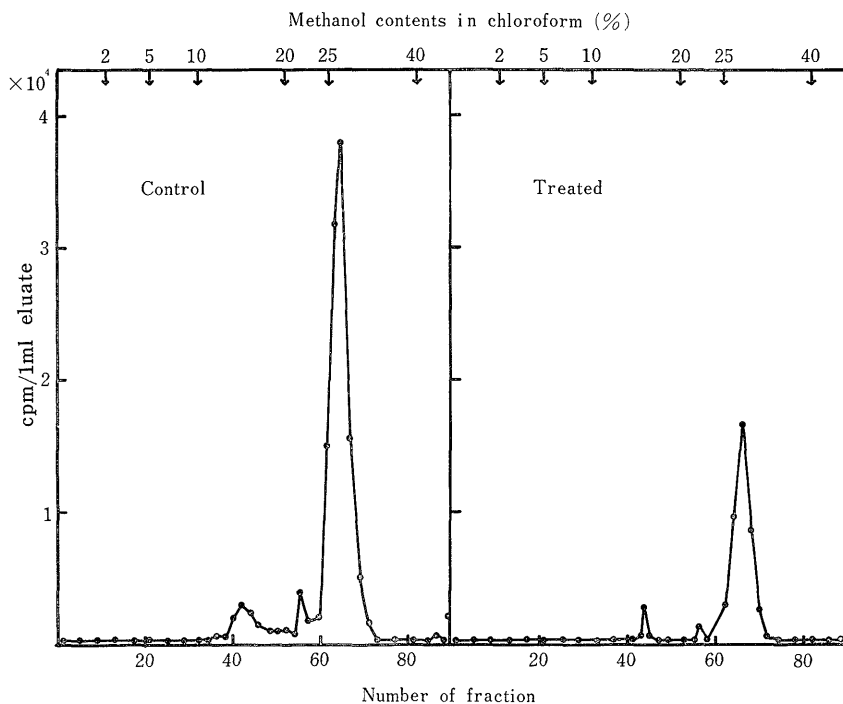


Fig. 3. Silicic acid column chromatography of the labeled lipids from the cells exposed to $^{32}\text{P}\text{-H}_3\text{PO}_4$

consisting of one outstanding large peak and two small peaks. These peaks show to be those of the lipids coupled with H_3PO_4 , phospholipids. A comparative chromatography between the ^{32}P -labeled lipids of NaDMDC-treated cells and those of untreated cells, showed that the incorporation of $^{32}\text{P}\text{-H}_3\text{PO}_4$ into all components of lipids was inhibited at a constant rate in the presence of NaDMDC. These results are in full accord with the chromatographic result from incorporation tests for ^{14}C -acetate. Thus, it is clear that NaDMDC inhibits nonspecifically the synthesis of all phospholipids in *X. oryzae*.

Identification of a main phospholipid of X. oryzae

To identify the phospholipid revealed as the largest peak on the above chromatograms, the phospholipid component was chromatographed on a silica gel TLC plate. When the plate was sprayed with phospho-molybdate solution or ninhydrin reagent, one spot was detected on the chromatogram, which was positive against both sprayed reagents. The R_f value was 0.75. This lipid seems to be a phospholipid containing amino groups. For definite identification, this phospholipid was hydrolyzed under the condition of mild alkali, and the water-soluble hydrolytic product, a deacylated phospholipid was chromatographed on a paper. Detection for spot was made by the color development of the same two reagents as used above. The positive spot was observed at the position of R_f value 0.61, where coincided with the R_f value for the hydrolytic product of authentic PE, and this value also has a close resemblance to the R_f value for the hydrolytic product of PE reported by Dawson⁴⁾. Consequently, this lipid was identified as PE.

Distribution of radioactivity in the fatty acid and GPE parts of PE

In the hydrolytic products of radioactively labeled PE as described in Method, the radioactive ratios of fatty acid fraction to GPE fraction are shown in Table 2.

Table 2. Effect of NaDMDC on the incorporation of ^{14}C -glucose into the fatty acid (FA) and glycerylphosphorylethanolamine (GPE) parts of phosphatidylethanolamine molecules

Concentrations of NaDMDC	Incubation time (min)					
	15			30		
	FA	GPE	FA/GPE	FA	GPE	FA/GPE
Control	7344cpm	579cpm	12.7	8573cpm	673cpm	12.7
10 $\mu\text{g/ml}$	3146	562	5.6	4820	749	6.4
100	934	198	4.7	1652	418	4.0

The radioactive ratios for the PE of untreated cells were approximately 12 at any incubation time. In the PE of NaDMDC-treated cells, however, the ratios were remarkably decreased with increasing concentration of NaDMDC: the ratios were about 4 at a concentration of 100 $\mu\text{g/ml}$ of NaDMDC regardless of incubation time. These decreased ratios are presumably resulted from a low radioactivity of the fatty acid fractions of PE, because the incorporation of ^{14}C -glucose into GPE fractions was not decreased under the presence of 10 $\mu\text{g/ml}$ of NaDMDC, in spite of the decrease of the incorporation into fatty acid fractions. The results suggest that NaDMDC acts on either the synthesis of fatty acids or the transfer of fatty acids to glycerol-3-phosphate.

Incorporation of radioactive fatty acids into the phospholipids

The lipids were extracted from the cells labeled with radioactive long-chain fatty acids for given times, and the extracted lipids were chromatographed on a silica gel plate in order to separate phospholipid components. After the phospholipid-containing region was scrapped from the plate, the phospholipid sample was analyzed for radioactivity. As shown in Fig. 4, the incorporation of ^{14}C -palmitic acid into the phospholipids had a rapidly increasing tendency with the elapsed time

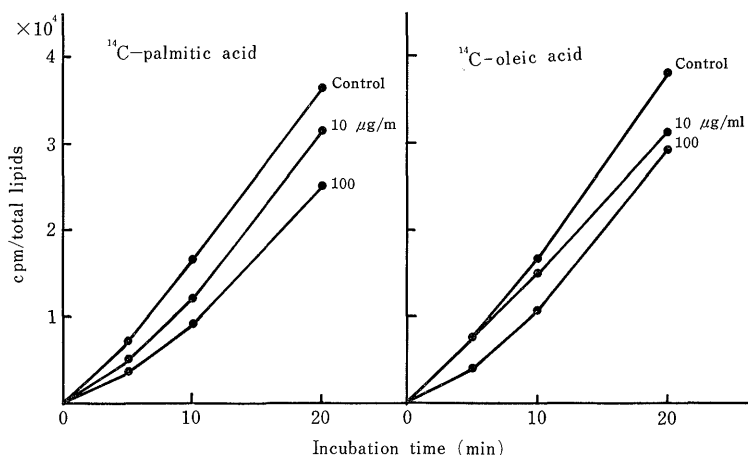


Fig. 4. Effect of NaDMDC on the incorporation of labeled fatty acids into the phospholipid fraction of *X. oryzae*

of incubation. A similar pattern of incorporation was also observed in the case of ^{14}C -oleic acid. In the NaDMDC-treated cells, the incorporation of two radioactive compounds into the phospholipids was not inhibited even at a concentration of 100 $\mu\text{g}/\text{ml}$ NaDMDC, in contrast with the incorporation of ^{14}C -acetate or ^{14}C -glucose into the lipid fraction. Therefore, it is concluded that NaDMDC has no influence upon the transfer enzymes of long-chain fatty acids to glycerol-3-phosphate.

Discussion

The incorporation of radioactive precursors into the lipids of *X. oryzae* was markedly inhibited by NaDMDC. To explain the inhibition by NaDMDC, two possible mechanisms were considered: one was the direct inhibition of lipid synthesis, the other was the leakage of lipid components from the cells. The latter mechanism, for example, is shown by the release of lipopolysaccharides from the cell wall of the bacteria treated with ethylenediaminetetraacetic acid⁷⁾. However, our experiments excluded the possibility of the latter mechanism from the fact that NaDMDC did not cause the leakage of radioactive lipids from the labeled cells. Thus, it was concluded that the compound inhibited the process of the lipid synthesis of bacterial cells.

On the other hand, the authors reported in a previous paper¹⁵⁾ that the inhibition of lipid synthesis by NaDMDC was largely increased by the addition of glucose to the cell suspension prepared with a buffer solution. Such a effect of cell-suspending media on the sensitivity of cells to NaDMDC was examined using 3 kinds of media which contained different nutrient sources, and it was found that incorporation of ^{14}C -acetate into the lipids was inhibited more strongly by NaDMDC in the cells suspended in a nutrient-rich medium rather than in the cells suspended in a poor medium. Ballesta and Schaechter¹⁾ reported that the rate of the lipid synthesis in *Escherichia coli* was rapidly decreased by the shift-down of cells from a nutrient-rich medium to a poor one. In contrast, the shift-up cells seem to be greatly increased in the rate of lipid synthesis. Therefore, the inhibition of lipid synthesis by NaDMDC appears to be more effective to the cells being metabolically active, particularly to the cells activating in their lipid metabolism.

A column chromatographic analysis for the labeled lipids of NaDMDC-treated cells, showed that all components of cellular lipids were markedly reduced in their radioactivity at a nearly constant rate as compared with untreated cells. Thus it is unlikely that NaDMDC specifically inhibits the synthesis of particular phospholipids. As an additional gain from lipid analysis, it was shown that a major component of the lipids of *X. oryzae* was PE, supporting the fact that in many bacteria a major portion of cellular lipids is generally occupied with phospholipids, especially glycerophospholipids.

In the PE from ^{14}C -glucose labeled cells, radioactive distribution between fatty acid part and GPE part was determined, and it was found that a decreased radioactivity of PE in NaDMDC-treated cells was resulted from low labeling of fatty acid part, because radioactivity of the GPE part was almost constant in both NaDMDC-treated and untreated cells. Thus, the inhibition of PE production by NaDMDC appears to be involved in either the synthesis of fatty acids or the transfer of fatty acids to glycerol-3-phosphate. However, NaDMDC did not act on the process from fatty acids to phosphatidic acid, since the incorporation of radioactive long-chain fatty acids into the phospholipids was not inhibited by NaDMDC.

These results indicated that the primary action of NaDMDC might be connected with the fatty acid synthesis of *X. oryzae*. *In vitro* action of NaDMDC on fatty acid

synthesis is now in progress using cell-free preparation of *X. oryzae* or *E. coli*, and the results will be reported in the following paper.

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Dithiocarbamate 系殺菌剤の作用機作に関する研究 2. イネ白葉枯病菌の脂質合成に及ぼす Sodium dimethyldithiocarbamate の作用

米山勝美・見里朝正

Dithiocarbamate 系殺菌剤 Sodium dimethyldithiocarbamate (NaDMDC) によるイネ白葉枯病菌の脂質合成阻害作用について検討した。

1. NaDMDC は白葉枯病菌の脂質への ^{14}C -acetate の取り込みを最も顕著に阻害したが、この阻害は本菌の代謝活性が盛んな場合にさらに大きく助長された。

2. NaDMDC 処理菌の脂質をカラムクロマトグラフィーにより分析した結果、全ての脂質成分においてほぼ等しく阻害されることが示された。

3. NaDMDC 処理菌から抽出・単離した Phosphatidylethanolamine を加水分解した結果、 ^{14}C -glucose からの取り込みは glycerylphosphorylethanolamine 部分よりも脂肪酸部分で著しく阻害されていることが認められた。

4. ^{14}C -palmitic acid および ^{14}C -oleic acid のリン脂質分画への取り込みは NaDMDC により殆んど阻害されなかった。

以上の結果から、NaDMDC の作用は脂質合成系における長鎖脂肪酸からリン脂質合成までの過程ではなく、それより以前の脂肪酸合成系に関連していると考えられる。