Nutritional Conditions for Microorganisms to Dechlorinate Benthiocarb (Thiobencarb)*

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(Received September 5, 1985)

When dechlorination microbes in the diluted suspension of a soil activated for dechlorination of benthiocarb (thiobencarb, S-4-chlorobenzyl N,N-diethylthiocarbamate) were inoculated to several media, benthiocarb was rapidly dechlorinated after a lag period in an extract solution of reactive soil adjusted to pH 7 with phosphate buffer and in a mineral salt medium added with yeast extract or Bacto Casamino acid. Dechlorination, however, did not occur in usual bacterial media such as bouillon-peptone, V.L. basal and mineral salt medium. The concentration of minerals and organic nutrients in media greatly affected the lag period and the dechlorination rate. The dechlorination activity of soil extracts varied according to their components. The activity of extracts corresponded well to the activity of the original soils. Nitrogen concentration in media greatly affected the activity. Ferrous content in the soil extract was an essential factor for the growth of microbes. The concentration of organic nutrients and mineral salts and the ratio between them in media were also of great importance for the proliferation of dechlorination microbes.

INTRODUCTION

Benthiocarb (thiobencarb, S-4-chlorobenzyl N,N-diethylthiocarbamate) was dechlorinated after a lag period in a certain soil under anaerobic conditions. The dechlorination activity of soil was promoted by repeated application of benthiocarb, but it ceased within a few days after disappearance of benthiocarb in the soil. The activity was closely related to the phosphate concentration in soil. The dechlorination of benthiocarb was presumed to be caused by gram-positive facultative anaerobic bacteria reactive in a narrow range of temperature and pH. The dechlorination microbes in the reactive-soil suspension were able to dechlorinate only limited benthiocarb analogs but not all other chlorinated phenyl compounds examined. Separation of the bacteria from soil particles was unsuccessful.

In the present study, the microbial dechlorination of benthiocarb in several liquid media was investigated in order to obtain more detailed information of its characteristics, especially of nutritional factors and to find appropriate media for growing the dechlorination microbes, using the highly diluted suspension of a soil activated by repeated application of benthiocarb.

MATERIALS AND METHODS

1. Chemicals and Soils

Benthiocarb and dechlorobenthiocarb used were previously described. Other chemicals were obtained commercially.

Ohshiro paddy (hereafter described as Ohshiro) and Nagoya University Farm upland I (Nagoya) soils, dechlorination-reactive, and Anjo paddy (Anjo), Tochigi paddy (Tochigi)
and Chigasaki paddy (Chigasaki) soils, dechlorination-inactive, were used. Their sampling places, properties and dechlorination activity were reported previously.†2)

2. Media
Media used were prepared according to a textbook.5)
1) Bouillon medium for anaerobes: 10 g bouillon, 10 g peptone, 5 g NaCl and 0.3 g cysteine in 1 l water.
2) V. L. basal medium: 10 g peptone, 5 g yeast extracts, 2 g bouillon, 5 g NaCl and 0.3 g cysteine in 1 l water.
3) Mineral salt medium-(1): 1.0 g K₂HPO₄, 0.5 g KNO₃, 0.2 g MgSO₄, 0.1 g CaCl₂, 0.1 g NaCl, 0.01 g FeCl₃ and 1 ml microelement solution (1.0 g AlCl₃, 0.5 g KI, 0.5 g KBr, 0.5 g LiCl, 7.0 g MgCl₂·4H₂O, 11 g H₂BO₃, 1.0 g ZnCl₂, 0.05 g SnCl₂·2H₂O, 1.0 g NiCl₂, 5.0 g CoCl₂, 0.5 g BaCl₂, 0.5 g Na₂MoO₄, 0.1 g Na₂BO₃·H₂O in 3.6 l water) all in 1 l water. The concentration of mineral salts in the medium was varied, or yeast extract (Kyokuto) or Bacto Casamino acid (Difco, mixture of amino acid) was added into the medium.
4) Mineral salt medium-(2): 2.46 g (NH₄)₂SO₄, 2.38 g KH₂PO₄, 5.65 g K₂HPO₄·3H₂O, 1.0 g MgSO₄·7H₂O, 0.0064 g CuSO₄·5H₂O, 0.0011 g FeSO₄·7H₂O, 0.0079 g MnCl₂·4H₂O, 0.015 g ZnSO₄·7H₂O and the 1 ml microelement solution described in 1 l water. Yeast extract or Casamino acid was added to the medium according to the purpose.
5) Soil extract media: One kilogram each of the 5 soils listed in Table 1 was put in 1 l of water in a 2-l Erlenmeyer flask, shaken for 5 min, and then autoclaved at 120°C for 20 min. The content was filtered through a filter paper (Toyo No. 2) by suction. The filtrate was made up to 1 l and 0.2 g of K₂HPO₄ was dissolved therein.
All media was adjusted to pH 7.0 with 0.1 N NaOH or 0.1 N HCl when necessary.
The following media were also subjected to further examination.
1) Ohshiro soil extract to which KNO₃ was added at 0.09, 0.18 or 0.36% or glycine at 0.07, 0.14 or 0.28% and CaCl₂ at 0.02 or 0.039%.
2) The Chigasaki soil extract containing 0.07, 0.11 or 0.16% glucose, and with or without 0.001% FeCl₃ or 0.0006% AlCl₃.
3) Yeast extract—mineral salt medium-(A). To mineral salt medium-(1), yeast extract was added at 0.1%, or 14 amino acids and 4 vitamins (B₁, B₂, B₆ and biotin) of the yeast extract components at a concentration corresponding to the contents of yeast extract, separately or in combination of amino acids and vitamins were added.
4) Yeast extract—mineral salt medium-(B). To mineral salt medium-(1), the yeast extract was added at 0.1%, or 14 amino acids and 4 vitamins (B₁, B₂, B₆ and biotin) of the yeast extract components at a concentration corresponding to the contents of yeast extract, separately or in combination of amino acids and vitamins were added.
5) Yeast extract—mineral salt medium-(C). To the mineral salt medium-(1) of concentration 0.5, 2 or 4 times higher than mineral salt medium-(1) the yeast extract was added at 0.05, 0.1 or 0.2%.
These media were adjusted to pH 7.0.

Table 1 Elementary composition of soil extracts.

<table>
<thead>
<tr>
<th>Soil extract</th>
<th>Dechlorination activity†</th>
<th>C (mg/ml)</th>
<th>N (mg/ml)</th>
<th>C/N</th>
<th>Relative contents b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Ohshiro</td>
<td>†</td>
<td>0.320</td>
<td>0.120</td>
<td>2.67</td>
<td>1.00 1.00 1.00</td>
</tr>
<tr>
<td>Nagoya Univ.</td>
<td>†</td>
<td>0.091</td>
<td>0.047</td>
<td>1.93</td>
<td>0.42 0.88 1.10</td>
</tr>
<tr>
<td>Anjo</td>
<td>+</td>
<td>0.133</td>
<td>0.090</td>
<td>4.47</td>
<td>0.16 1.13 1.54</td>
</tr>
<tr>
<td>Chigasaki</td>
<td>–</td>
<td>0.109</td>
<td>0.375</td>
<td>0.29</td>
<td>1.63 0.25 1.71</td>
</tr>
<tr>
<td>Tochigi</td>
<td>–</td>
<td>0.374</td>
<td>0.250</td>
<td>1.50</td>
<td>1.11 0.25 0.82</td>
</tr>
</tbody>
</table>

* The activity after inoculation of activated Ohshiro soil suspension. The levels of activity were introduced from Fig. 2.
† Relative contents of elements were indicated on the basis as 1.00 for Ohshiro soil extract.
3. Elementary Analysis of Soil Extracts

Ten milliliters of the soil extract was dried up at 60°C on aluminum foil in a sample boat. The dried sample was analyzed for the total carbon and nitrogen using a Yanagimoto MT-500 CN Corder. For the determination of Al, Si, P, S, Cl, K, Ca, Ti, Mn and Fe, 100 μl of the soil extract was absorbed into filter paper (Toyo No. 2, diameter 5 mm) and dried. The elements in the dried paper were determined using a Kevex Unispec TM System-700 X-ray Emission Spectrograph. The results are shown in Table 1.

4. Preparation of Dechlorination-reactive Soil Suspension

Ohshiro soil (10 g) in a test tube (2.7 x 20 cm) was activated for dechlorination as described in our previous report. The soil was suspended into the sterilized water 100 or 1000 times the quantity.

5. Dechlorination in Media Inoculated with the Suspension

Benthiocarb was dissolved at 10 ppm into each medium in a 1-l flask, and 18 ml of the medium was placed in test tube (18 x 200 mm) and autoclaved. The medium in the tube was mixed with 2 ml of soil suspension described above. The tube was closed with a rubber stopper, and incubated at 28°C for designated periods.

6. Determination of Benthiocarb and Dechlorobenthiocarb

The incubation mixture in the tube was vigorously shaken and a 2-ml portion was quickly taken out into a 10-ml test tube with a glass stopper. The content was shaken with 2 ml of n-hexane for 30 min. Benthiocarb and dechlorobenthiocarb in the hexane extract were determined by a gas chromatograph equipped with a N-P FID according to the described method.

RESULTS

1. Dechlorination of Benthiocarb in Several Bacterial Media

The dechlorination of benthiocarb was compared among 10 different bacterial media inoculated with 10,000 times diluted suspension of the activated soil containing dechlorination microbes. As shown in Fig. 1, the dechlorination occurred after a lag period of approximately 5 days of incubation in the Ohshiro soil extract medium and the mineral salt medium-(1) containing 0.1% yeast extract. In the mineral salt medium-(1) which contained 0.4% Casamino acid or 0.05% starch, the dechlorination started after 14 days. The reaction, how-
ever, did not occur in usual bacterial media such as bouillon medium, V.L. basal medium, the mineral salt media-(1) and -(2), and mineral salt medium-(2) which contained yeast extract or Bacto Casamino acid. The dechlorination did not occur either when the microbial soil suspension was not inoculated.

2. The Dechlorination in Different Soil Extracts

The microbial soil suspension was inoculated to the phosphate-amended extracts of 5 soils having different dechlorination activity. The results are shown in Fig. 2. When the suspension was inoculated at 0.1%, the dechlorination started after 7 days of incubation in the extracts of Ohshiro and Nagoya soils which had strong dechlorination activity, whereas it started after 13 days in the Anjo soil extract. On the contrary, the dechlorination did not occur in the extracts of Tochigi and Chigasaki soils which had no dechlorination activity, even if the soil suspension containing dechlorination microbes was inoculated. When the activated soil was inoculated at 0.01%, the differences in the activity were much more remarkable.

The elementary analysis of soil extracts (Table 1) showed that the contents of total nitrogen and Ca were lower in the extracts of active Ohshiro and Nagoya soils than in those of inactive Tochigi and Chigasaki soils. The C/N ratios and Fe and Al contents were higher in the former than in the latter. P, Si, S, Cl, K and Mn contents did not differ largely between them. In this results, P and K contents have no meaning because enough amount of K₂HPO₄ was amended to the soil extracts.

3. Effects of Carbon, Nitrogen and Ferrous Contents in Soil Extracts on Dechlorination

As shown in Fig. 3, when a nitrogen source was amended to the Ohshiro soil extract, dechlorination in the medium containing glycine at 0.07% or 0.14% or KNO₃ at 0.09% was greatly promoted compared to the case of non-application of a N source. The lag period remained almost the same with or without application of a N source. Dechlorination occurred at lower rates or did not occur when additional N source was amended. The application of CaCl₂ at 0.02 or 0.039% into Ohshiro soil extract had no effect on dechlorination.

On the other hand, as shown in Fig. 4, the Chigasaki soil extract was inactive for dechlorination whether or not glucose was amended.
at 0.07%. Dechlorination occurred only to a small extent when glucose was amended at 0.11% or 0.16%. Dechlorination in the extract medium containing glucose, however, was greatly promoted when Fe was applied. Aluminum amendment produced almost no effect. Amendment of smaller amounts of glucose delayed the start of reaction, but did not lower the reaction rate.

4. Effects of Yeast Extract Component in the Mineral Salt Medium on Dechlorination

As shown in Fig. 5, dechlorination progressed rapidly after 8 days of incubation in mineral salt medium-(1) when yeast extract was amended at 0.1%, or when only the amino acids and vitamins or amino acids of yeast extract components were amended in the corresponding amounts to the yeast extract. The reaction did not occur when only vitamins were added. The length of lag period and the dechlorination rate were almost the same when the reaction occurred, but the reaction proceeded most smoothly when the yeast extract was added.

5. Effects of Organic and Inorganic Nutrient Contents in the Medium on Dechlorination

Figure 6 shows the dechlorination in the mineral salt media at various concentrations of mineral salt medium-(1) containing various amounts of yeast extract. When yeast extract was amended at a concentration half (0.05%) of the standard application, dechlorination took place rapidly after the shortest lag period at the highest reaction rate. As the yeast concentration increased, the reaction rate decreased and the start was delayed. At half concentration of the mineral salts, the start was delayed but the rate remained the same as at the standard concentration. The reaction did not occur by amendment of yeast extract at 0.4% at the standard concentration of the medium and in the media at twice or higher concentrations of the standard mineral salts.

DISCUSSION

In this study, the 10,000 times diluted suspension of soil which had been highly activated by repeated applications of benthiocarb was used for inoculation of the microbes, because the separation of microbes from soil particles was difficult. The microbial suspension was inoculated to various kinds of bacterial media and extracts of several soils active or inactive for benthiocarb dechlorination. The results (Fig. 1) suggested that the composition of
media affected the growth of dechlorinating microbes to a large extent but not their activity, since the lag period varied greatly while the reaction rate did not differ significantly. Dechlorination did not occur in usual bacterial media such as bouillon and V.L. basal media. The reaction occurred rapidly in mineral salt medium-(1) containing yeast extract or Bacto Casamino acid which is an amino acid mixture obtained from casein hydrolysates. The findings suggest that dechlorinating microbes require both organic and inorganic nutrients for their activity or growth in media. However, dechlorination did not occur in mineral salt medium-(2) containing yeast extract or Bacto Casamino acid. This may be due to the high concentration of inorganic salts in mineral salt medium-(2), as described later.

Dechlorination in the reactive-soil extract medium occurred rapidly when the highly diluted (1000 times) suspension was inoculated (Fig. 1). The result indicated that a great population of dechlorination microbes existed in the activated soil. A lag period of at least 4 days, however, may suggest that the microbes need time to proliferate before starting dechlorination in the medium.

In our previous study, dechlorination occurred in Ohshiro and Nagoya University Farm I soils but not in Tochigi and Chigasaki soils, even if the activated soil was amended. In this study, when the diluted suspension of active soil was inoculated to the extracts of Tochigi and Chigasaki soils, the lag period varied to a great extent according to the dechlorination activity of the soil (Fig. 2). The difference became more remarkable when smaller amounts of the soil extracts were inoculated. The results indicate that the dechlorination activity in the media corresponded to the activity in the soils, and that the factors influencing the dechlorination in the soils also exist in the soil extracts. The dechlorination activity is largely correlated to the phosphorus content of soils, and is influenced greatly by pH values of the suspension. From the factors influencing the dechlorination activity in soil, however, the phosphate content and pH values were excluded from the meaning, because enough amount of K2HPO4 was added to the soil extracts and their pH was adjusted to 7. It seems, that was also involved in the activity. When glycine was added at 0.07 or KNO3 at 0.09% to the medium of reactive Ohshiro soil extract, which had lower N content and higher C/N ratio than inactive soil extracts (Table 1), dechlorination was greatly promoted (Fig. 3). The rate, however, again decreased as more N
was amended. The most suitable N content in the medium of Ohshiro soil extract was 0.25 mg/ml, and it was the same N content of inactive Chigasaki soil extract medium, in which the activity appeared after adding glucose to give a higher C/N ratio (Fig. 4). Therefore, for the dechlorination activity, the C/N ratio seems to be more important than N content.

The extracts of inactive Chigasaki and Tochigi soils contained larger quantities of Ca (Table 1). The Ca content, however, does not seem to be related to the growth and activity of the dechlorination microbes, because the amendment of Ca to the Ohshiro soil extract had no effect to the lag period and the dechlorination rate (Fig. 3).

The limiting factor for dechlorination in inactive soils was Fe content, which was very low in the extracts of inactive Chigasaki and Tochigi soils (Table 1). When Fe was added together with glucose to the Chigasaki soil extract medium, the dechlorination occurred at high rates (Fig. 4). Glucose is probably necessary for the proliferation of the dechlorination microbes, because the start of reaction was delayed largely when the amendment of glucose was decreased. Aluminum application affected neither the lag period nor the reaction rate, although the extracts of inactive soils contained much smaller amount of Al than those of active soils. The Al content, however, seemed to contribute to the continuation of dechlorination. The results of this study and our previous study2 indicated that Fe and P were important elements for dechlorination of benthiocarb in soil.

Dechlorination rapidly occurred in mineral salt medium-1 only when yeast extract was added (Fig. 1). The proliferation rate of dechlorination microbes, which was presumed from the length of lag periods, greatly differed when yeast extract was applied at 0.1% and Casamino acid at 0.4% to the mineral medium (Fig. 4). Addition of amino acid components of yeast extract either with or without vitamins did not affect the lag period (Fig. 5). The results may indicate that the proliferation of microbes requires the appropriate concentration of organic nutrients. Addition of amino acid components of yeast extract did not complete the reaction in the medium to the addition of yeast extract. Some factors other than amino acids may contribute to the smooth reaction. Addition of major vitamins did not affect the reaction either.

Furthermore, dechlorination occurred in mineral salt medium-1 but not in mineral salt medium-2 containing the same amount of yeast extract, although the mineral concentration in the latter medium was approximately 10 times higher than in the former (Fig. 1). In combinations of different concentrations of yeast extracts and mineral salts in the medium, the concentrations of yeast extracts and mineral salts and the ratio between them greatly affected the lag period and the reaction rate of dechlorination (Fig. 6). The medium containing the mineral salts of the standard concentration and the yeast extract of concentration half of the standard level showed the shortest lag period and the highest rate of dechlorination. If the concentration of either minerals or yeast extract or both was twice or more, dechlorination was greatly suppressed or did not occur.

The concentration of phosphate in soil was also related to the dechlorination of benthio-carb.2 The reaction was sharply affected by pH values and temperature.2 Therefore, enough amount of phosphate was added to the media examined in this study and the media were adjusted to the optimum pH 7. The reaction, however, did not occur in the soil extract media prepared from dechlorination-inactive soils. In this case, the limiting factor was the deficiency in concentration of Fe, which affected the proliferation of the dechlorination microbes. The N concentration or the C/N ratio in soil extract affected the activity of the microbes. The concentration and ratio of organic nutrients and minerals in the media sharply influenced the proliferation of the microbes. Our previous reports noted that the dechlorination microbes were active only under limited conditions and they could dechlorinate only benthio-carb and its few analogs.4 In addition to such properties, the nutritional requirement for their proliferation and dechlorination activity was very strict as well. The characteristics may reflect the fact that the rice dwarfing caused by benthio-carb application occurs only in specific
soils under limited conditions.

From this study, the mineral salt medium-
(1) amended with yeast extract at half con-
centration of the common media and the soil
extract media prepared from dechlorination-
active soil were selected for further investi-
gation of the microbes.

ACKNOWLEDGMENTS

We wish to express our thanks to Kumiai Chemi-
cal Industry Co., Ltd. for supplying benthiocarb and
dechlorobenthiocarb.

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要 約

ペンチオカープ脱塩素菌の栄養条件*

文 永熙，鶴塚昭三

ペンチオカープ脱塩素活性を高めた土壤の無機懸
濁液を脱塩素菌源として各種培地に接種し、ペンチオカ
ープ脱塩素反応を比較した。脱塩素活性を有する土壤の
抽出液にリン酸塩を添加したものおよび無機塩塩地に酵
母エキスまたはアミノ酸混合物を添加した培地では、反
応はあるラグタイムのものまたは急速に進行した。しかし、一
般細菌用のブイヨン培地、V.L.基本培地、無機塩塩培地で
は反応は起きなかった。培地中の無機塩類および有機栄
養分の濃度は、ラグタイムの長さおよび反応速度に大きく
影響した。各種土壤の抽出液中の脱塩素活性は、も
との土壤の活性とよく一致した。培地の塩素濃度は活性
に大きく影響した。また鉄含量は脱塩素菌の増殖に必須
の要因であった。培地の栄養源分および無機塩類の濃
度およびその比率もまた脱塩素菌にとって重要な因子で
あった。

* 土壌中におけるペンチオカープの脱塩素に関する研
究（第 5 報）