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Metabolism of Cyclohexane-Diol-(1, 2)-*Trans* by a Soil Bacterium (I)

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SUMMARY

Cyclohexane-diol-(1,2)-*trans* was shown to be metabolized by an adaptive enzyme system in *Pseudomonas sp.* C-4. CH-dione was formed from CHD by dehydrogenase (s), and its cyclohexane ring was cleaved by a hydrase, forming adipic acid *via* adipic acid semialdehyde.

The enzymes participating in CHD metabolism were named CHD dehydrogenase, (CH-one-ol dehydrogenase), CH-dione hydrase and adipic acid semialdehyde dehydrogenase, respectively.

Of these enzymes, the dehydrogenases required DPN as a cofactor and the hydrase reqiured some unknown cofactor.

INTRODUCTION

The benzene ring of catechol is cleaved, forming *cis*, *cis*-muconic acid, by pyrocatechase, which requires ferrous ion as its only cofactor. Pyrocatechase, which was discovered in this laboratory (Hayaishi and Hashimoto, 1950), has been purified to a considerable extent, and its characteristics have been reported recently (Suda and Hashimoto, 1951; Tokuyama *et al.*, 1956; Tokuyama *et al.*, 1957). It is of interest to know whether the cyclohexane ring of cyclohexanediol-(1, 2) is metabolized in a similar way to the benzene ring of catechol.

Although *Acetobacter suboxidans* metabolizes cyclohexane-diol-(1, 2)-*cis* and-*trans* and releases cyclohexanone-ol into the culture medium, studies on subsequent metabolism have not been reported (Posternak *et al.*, 1955).

This communication describes the pathway of degradation of cyclohexane-diol-(1, 2)-*trans* by a soil bacteria, *Pseudomonas sp.* C-4, and some of the characteristics of the enzymes involved in this metabolism.

MATERIALS AND METHODS

1. *Bacterial source and extraction of enzymes*

Pseudomonas sp. C-4 was isolated from soil with the following medium: 3.0 g CHD, 3.0 g KH_2PO_4

The abbriviation used in this paper are: CHD, cyclohexane-diol-(1,2); CH-one-ol, cyclohexanone-(1)-ol-(2); CH-dione, cyclohexanedione-(1,2); Tris, tris-(hydroxymethyl) aminomethane chloride.

3.0 g Na_2HPO_4, 0.2 g $MgSO_4$ and 0.001 g $FeSO_4 \cdot 7H_2O$ (per liter). The pH was adjusted to 7.2. Inocula were grown on 0.5 per cent peptone broth supplemented with 0.3 per cent CHD, at 30°C with shaking. After 14 hours incubation, the cells were collected (average yield, 7 g wet weight of cells per liter of medium). The pellet was ground with alumina W-800 (Wako Pure Chemicals), extracted with 5 volumes of 0.02 M Tris-HCl buffer (pH 7.5) and centrifuged at 6,000 × g for 15 minutes. The supernatant was centrifuged again at 105,000 × g for 30 minutes and the supernatant was used as the crude extract. Similar extracts were obtained by disruption of cells in 9 KC Raytheon sonic oscillator.

Nucleic acid was removed from the crude extract by the addition of 0.1 volume of 5 per cent streptomycin and the extract was fractionated using saturated ammonium sulphate solution (pH 6.5). The enzyme activities in each fraction were estimated. The crude extract was centrifuged at 105,000 × g for 30 minutes and then treated with 0.1 volume of 5 per cent streptomycin, as described above. To the supernatant, saturated ammonium sulfate (pH 6.5) was added. Activity was shown as a total units in each fraction. Activity units are: for dehydrogenases, increase of optical density at 340 mμ per minutes; for CH-dione hydrase, μl of CO_2 released from bicarbonate-CO_2 medium (pH 7.5) in the first 15 minutes. For the measurement of CH-dione hydrase activity in each fraction, 0.2 ml of condensed dialysate was added to the reaction medium. The results are presented in Table 1.

Table 1. Distribution of Enzyme Activities in Ammonium Sulfate Fraction

Ammonium sulphate saturation	0-33%	33-45%	45-55%	55-70%
CHD dehydrogenase	0.088	3.42	0.536	0.715
Adipic acid semialdehyde dehydrogenase	0.088	0.380	0.952	3.770

Ammonium sulphate saturation	0-20%	20-40%	40-60%	60-80%
CH-dione hydrase	99	2802	5473	928

2. *Substrates and co-enzymes*

CHD-*trans* was kindly supplied by Sumitomo Chemicals Co.

Cyclohexanone-(1)-ol-(2) was synthesized from cyclohexanone. Commercial cyclohexanone was distilled twice and 80-100°C fraction was collected (14 mm Hg). To this cyclohexanone, water was added and then a calculated amount of bromine was added dropwise while stirring the mixture in an ice water bath. After the reaction ceased, bromocyclohexanone was extracted from the aqueous layer with benzene and washed twice with water. After drying the extract with Na_2SO_4, the benzene was removed, and the bromo-cyclohexanone was hydrolysed in an aqueous solution of K_2CO_3 at room temperature for 6 hours, and the crystals formed were dried over silica gel and recrystallized from absolute ethanol (m.p. 104°C). Elementary analysis gave values consistent with the cyclohexanone-(1)-ol-(2).

Thoretical; C: 63.16 %, H : 8.77 %
Fonud; C: 62.7 %, H : 8.97 %
Formula; $C_6H_{10}O_2$

Cyclohexane-dione-(1,2) was synthesized from cyclohexanone by oxidation with SeO_2 (Hach *et al.*, 1959), and stored in a refrigerator in the dark.

Adipic acid semialdehyde was synthesized from adipic acid as follows. Adipic acid diester, prepared from adipic acid, was hydrolysed to mono-ethylester, and mono-ethyl mono-chloroadipate was obtained by a reaction of this compound with SO_2Cl_2, and reduced with H_2 using silk-paladium as a catalyst (Izumi, 1959). The adipic acid semialdehyde ethyl ester was hydrolysed by boiling with Amberlite IR-120 (H^+), and ethanol was removed. This was stored in the deep-freeze.

DPN was purchased from Sigma Chemicals Co.
Adipic acid and all other chemicals were obtained from commercial sources.

3. *Enzyme assay*

For the assay of dehydrogenases, the reaction mixture contained (in μ moles); DPN, 0.5; nicotinamide, 30; substrate, 30; pyrophosphate buffer (pH 8.0), 100 and an appropriate amount of enzyme in a 3 ml quartz cell (1 cm light path) and the volume was adjusted to 3.0 ml with water. The reaction was initiated by the addition of the substrate and the increase in absorption at 340 mμ was read every 15 sec. at room temperature (24-26°C) using Hitachi spectrophotometer, model EPU-2. For the control, the substrate was replaced by the same volume of water.

Acid production was measured manometrically at 30°C. The following components were added in Warburg flask (in μ moles); $NaHCO_3$, 63; CH-dione, 10; and an appropriate amount of crude extract and H_2O to a total volume of 3.0 ml. The gas phase was CO_2: N_2 or CO_2: air (5:95, V/V). The pH was 7.5 The control flask contained no substrate. Activity was expressed as μl of CO_2 released in the first 15 minutes of incubation.

Total reducing capacity was estimated with Fehling's solution modified by Nelson (1944).

4. *Paper chromatography*

Adipic acid semialdehyde was formed from CH-dione enzymatically in the following reaction mixture: 1.0 mM of phosphate buffer (pH 8.0). 10 μM of CH-dione, 3.0 ml of condensed dialysate, 1.3 mg of protein, prepared by $(NH_4)_2SO_4$ fractionation (33-45 per cent) and by streptomycin treatment, and H_2O to a final volume of 30 ml. After 90 minutes of inclubation at 30°C the reaction was stopped by adding 20 ml of 10 per cent TCA. From the centrifuged supernatant, adipic acid semialdehyde hydrazone was prepared by the addition of half a volume of 0.1 per cent 2,4-dinitrophenyl hydrazine (in N HCl). The mixture was incubated for 20 minutes at 30°C, stored in a refrigerator overnight and then the hydrazone was extracted with ethyl acetate. The solvent system used and the Rf values of the hydrazone obtained are shown in Table 2.

For the isolation of adipic acid, the following reaction mixture was used; phosphate buffer (pH 7.5) 3 mM, CH-dione 400 μM, crude extract 200 mg, total volume 60 ml. After 2.5 hours incubation at 30°C, adipic acid was extracted from the reacrion mixture with ethyl acetate after the protein had been removed with 4 per cent TCA. The Rf values of adipic acid are shown in Table 2. As the color developing reagent for adipic acid, aqueous solution of sodium 2-(2,4-dinitrophenyl)-azo-

Table 2. The Rf Values of the Reaction Products

	Solvent system	Rf values	
		Authentic sample	Reaction product
2, 4-Dinitrophenyl hydrazone of adipic acid semialdehyde	n-butanol, ethanol, N NH_4OH (7, 1, 2)	0.62	0.62
	iso-amyl alcohol, ethanol, N NH_4OH (7, 3, 2)	0.55	0.56
	petroleum ether, ethanol (20, 80)	0.92	0.93
Adipic acid	petroleum ether, chloroform, gracial acetic acid (20, 1, 5)	0.46	0.46
	n-propanol, conc. NH_4OH (6, 4)	0.55	0.54
	n-butanol, formic acid, H_2O (7, 3, 2)	0.88	0.83

1-naphthol-3,6-disulfonate was used at pH 7.0 (Kobayashi, 1957). With the dye, acids show up as yellow spots against a blue background. The color is stable for several hours.

RESULTS

1. *Preliminary experiments with washed cell suspension*

The enzymes required for the metabolism of CHD were formed adaptively in cells of *Pseudomonas sp.*, C-4. The bacterial cells were grown for 14 hours on peptone broth medium supplemented with CHD (B), or without CHD (A). The washed cells were suspended in distilled water at a concentration of 2 mg dry weight per ml. The Warburg flasks contained (in μ moles) ; phosphate buffer (pH 7.2), 200; substrate, 3; cells 2 mg (dry weight) in 1.0 ml; and water to a final volume of 3.0 ml. The center well contained 0.2 ml of 20 per cent KOH.

The resting cells started to oxidize CHD after a lag period of 50 minutes. The lag period to CH-one-ol and adipic acid was 50 minutes and 70 minutes, respectively. CH-dione was not oxidized even after 240 minutes (Fig. 1).

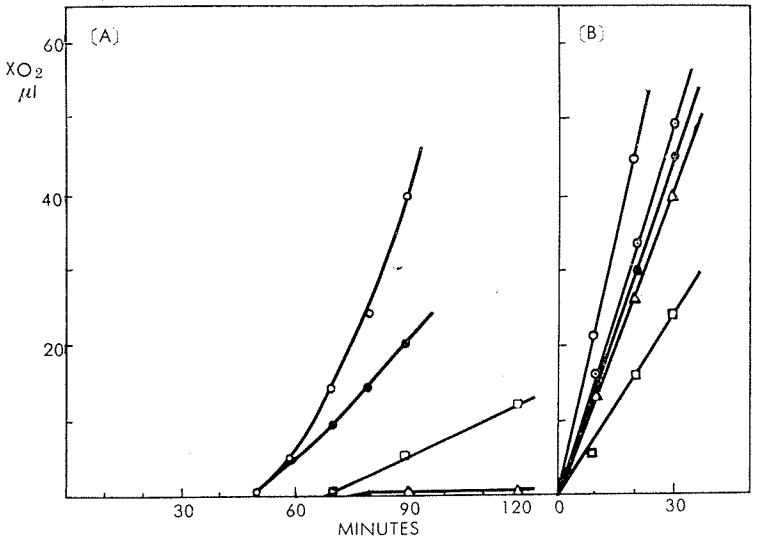

Fig. 1. Oxidation of Various Substrates by Cells of *Pseudomonas sp.*, C-4

Substrates; ○ - - - ○, CHD; ● - - - ●, CH-one-ol; △ - - - △, CH-dione; ⊙ - - - ⊙, adipic acid semialdehyde; □ - - - □, adipic acid
Temperature, 30°C
(A) : cells grown in broth
(B) : cells grown in broth containing CHD

Cells adapted to CHD began to consume oxygen without any lag period after the addition of CHD, CH-one-ol, CH-dione, adipic acid semialdehyde or adipic

acid. This suggests that these substances are all metabolic intermediates formed from CHD by the cells. Cells also became adapted to CH-dione after 50 minutes lag period, when the induction medium was supplemented with 0.05 per cent casamino acids and 0.05 per cent yeast extract. The cells adapted to semialdehyde, adipic acid, CHD and to CH-one ol. The reason for this phenomenon is not yet clear.

These results suggest that *Pseudomonas sp.*, C-4 metabolizes CHD sequentially as follows;

CHD → CH-one-ol → CH-dione → Adipic acid semialdehyde → Adipic acid.

The enzyme involved in each step is called cyclohexane-diol dehydrogenase, cyclohexanone-ol dehyrogenase, cyclohexane-dione hydrase and adipic acid semialdehyde dehydrogenase, respectively. The first two enzymes could be the same one.

2. *Cyclohexane-diol dehydrogenase*

A crude bacterial extract oxidized CHD and decolorized methylene blue and 2,6-dichlorophenol-indophenol only in the presence of DPN. DPN could not be replaced by TPN.

The optimal pH of this enzyme was at 7.8-8.0 (pyrophosphate buffer) (Fig. 2).

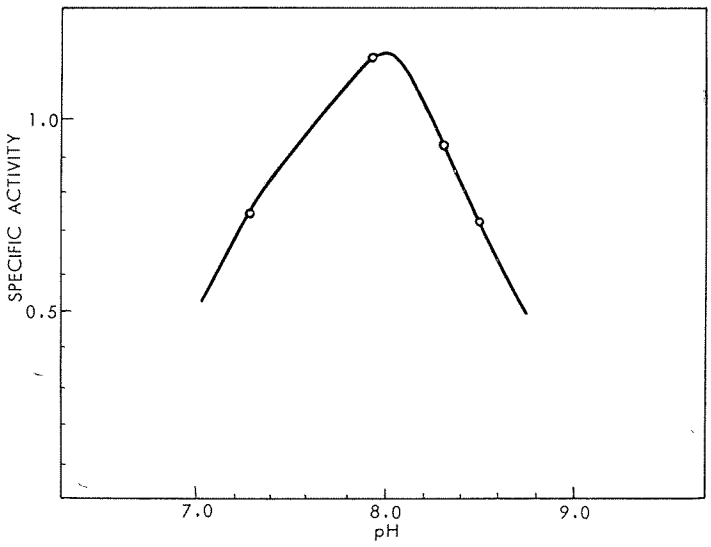

Fig. 2. CHD Dehydrogenase Activity as a Function of pH

The content of reaction mixture was as described in "Materials and Methods." 191 μg of protein from the 33-55 % $(NH_4)_2SO_4$ fraction was used as the enzyme. Activities are shown as the absorbancy change at 340 mμ per mg of protein.

Ninety per cent of the enzyme activity was inhibited by 5×10^{-6} M p-chloromercuribenzoate. Seventy four per cent of the inhibited activity was restored

by the addition of 5×10^{-5} M glutathione and 88 per cent by pre-incubation of the enzyme with CHD.

The stoichiometry of the dehydrogenation reaction can be measured by the oxygen uptake on the addition of the extract of *Clostridium sporogenes*, which serves to regenerate DPN from DPNH formed. Fifty mg of lyophilyzed *C. sporogenes* cells were suspended in 3.0 ml of 0.02 M Tris buffer (pH 7.4). After 16 hours, the suspension was centrifuged and the clear supernatant was used as the extract. The Warburg flasks contained (in μ moles) ; pyrophosphate buffer (pH 8.0), 200; nicotinamide, 30; DPN, 5; *C. sporogenes* extract, 0.5 ml; dialysed crude extract from CHD adapted cells, 0.5 ml; and water to a final volume of 3.0 ml. The center well contained 20 per cent KOH.

As shown in Table 3, one mole of oxygen was consumed per mole of CHD.

Table 3. Oxygen Uptake with CHD

CHD, added	Oxygen uptake	
	Total	Ratio
Micromoles	micromoles	O_2/CHD
2.0	2.2	1.10
5.0	5.3	1.06
10.0	10.1	1.01

The reaction product of the dehydrogenation reaction was studied. On the addition of a neutral aqueous solution of NH_2OH and Ni^{++} to the reaction mixture, a pink precipitate was formed immediately. This suggests that the substance formed is an aliphatic dioxo compound (Feigel, 1954). From this result, it is very likely that CH-dione is the reaction product. To prove it, CHD was incubated with DPN and *C. sporogenes* extract as described in Table 3. After the completion of the reaction, protein was removed by the additon of 3 per cent TCA. The aliquot was passed through the column of Amberlite IR-120 (H^+), 0.6×10 cm and aliquot of the effluent was passed through Amberlite IR-400 (Cl^-), 0.6×9.7 cm, because CH-dione could not be adsorbed by Amberlite IR-120 (H^+) nor Amberlite IR-400 (Cl^-). The aliquot of the effluent from the latter column was used to take the absorption spectrum. As CH-dione has an absorption peak at 260-262 mμ, and its molar extinction coefficient at 260 mμ is 6.5×10^3, the absorption spectrum of the reaction product was compared with it. The reaction product showed a similar absorption spectrum as that of the authentic CH-dione (Fig. 3).

The significance of CH-one-ol was examined as an intermediate between CHD and CH-dione. Synthetic CH-one-ol was reduced by the crude extract with DPNH but not oxidized by DPN or TPN, and no dioxi compound was formed.

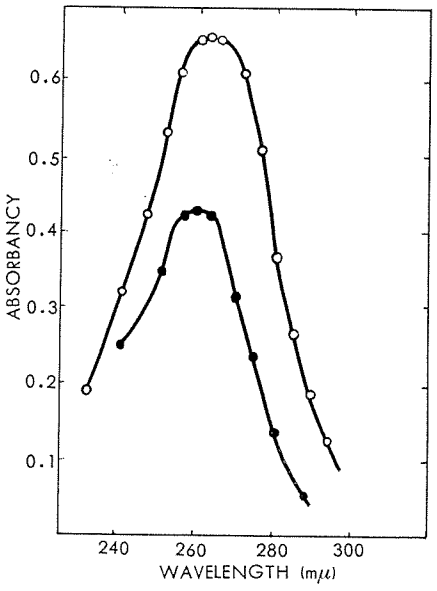

Fig. 3. Absorption Spectrum of CH-dione and that of the Reaction Product

○ - - - ○, authentic CH-dione;
● - - - ●, the effluent from the column

3. *Cyclohexane-dione hydrase*

A crude extract from CHD-adapted cells cleaved CH-dione to form an acid. The amount of CO_2 released in the first 15 minutes from bicarbonate-CO_2 medium was dependent on the amount of crude extract added (Fig. 4).

A half mole of CO_2 was released from one mole of CH-dione with the extract (Table 4).

Table 4. Relationship between the Amount of CH-dione Added and the Amount of CO_2 Released from a Bicarbonate Medium

CH-dione, added	CO_2 produced	
	Total	Ratio
Micromoles	Micromoles	CO_2/CH-dione
5.0	2.4	0.54
10.0	4.4, 4.9	0.44, 0.49
12.5	6.8	0.48

The Warburg flasks contained, 63 μM of sodium bicarbonate, 7.1 mg of crude extract, 0.2 ml of CH-dione, and water to a final volume of 3.0 ml. The gas phase was CO_2: air (5 : 95, V/V).

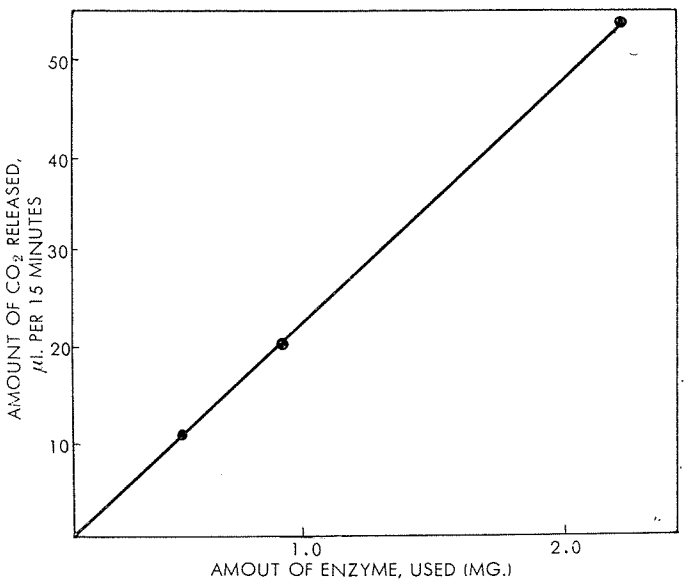

Fig. 4. Effect of Protein Concentration on CO_2 Production from Bicarbonate Buffer

The conditions were as described in "Materials".

Along with the acid production, an increase in total reducing capacity was observed. The acid formation was measured as described in Table 4, except 7.2 mg of protein from the crude extract was used. To estimate the increase of total

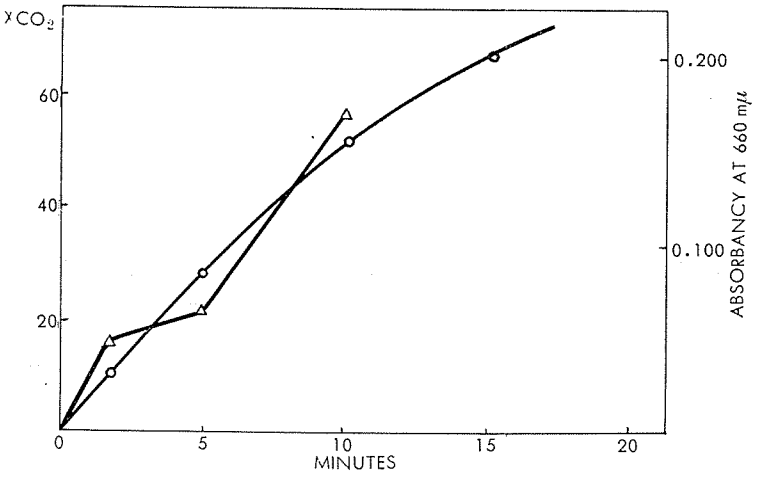

Fig. 5. Relationship between Formation of Acids and Increase of Total Reducing Capacity

○ - - - ○, CO_2 production ; △ - - - △, optical density at 660 mμ by Nelson's method

reducing capacity, an aliquot taken from the reaction mixture was mixed with Nelson's copper reagent (Nelson, 1944). In the latter case, components were the same as that for acid fromation, except Tris buffer (pH 7.5) was used. The results are shown in Fig. 5.

For the identification of the reaction product, 2,4-dinitrophenyl hydrazine was added to the deproteinized reaction mixture, phenyl hydrazone formed was chromatographed as described above. The Rf values of the phenyl hydrazone were identical with those of authentic adipic acid semialdehyde (Table 2).

The cofactor requirement of the cyclohexane-dione hydrase was studied. The acid-forming activity of the crude extract was lost by overnight dialysis against distilled water 4°C. The activity was restored by a supplementation of a dialysate from the crude extract. The effect of the dialysate was not replaced by glutathione or thiamine pyrophosphate, Mg^{++} and pyruvate, and not checked by 10^{-3} M of EDTA nor 3×10^{-3} M *p*-chloromercuribezoate. The activity was not lost by the treatment of dialysate with charcoal. Further attempt of the isolation of the active pricniple has not been performed yet.

4. *Adipic acid semialdehyde dehydrogenase*

This dehydrogenase is present in an extract of cells adapted to CHD or CH-dione and requires DPN as a cofactor. TPN was not tested. The pH optimum is 8.0 (pyrophosphate buffer).

The reaction product was identified by paper chromatography and was shown to be adipic acid (Table 2).

DISCUSSION

Pseudomonas sp. C-4, which can grow on CHD as the sole carbon source, was isolated. Metabolic pathway of CHD by this organism is proposed as shown in Fig. 6.

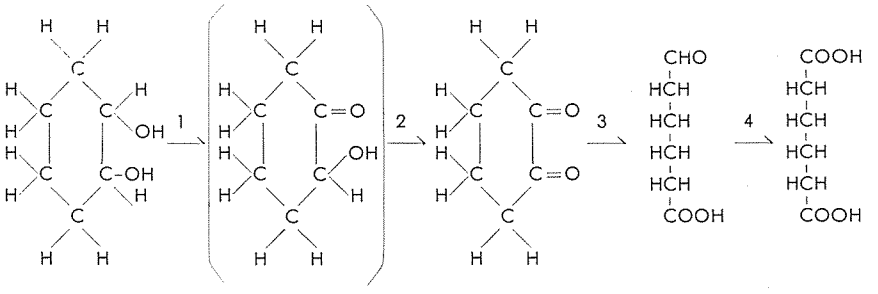

Fig. 6. Scheme for the Metabolism of CHD by a Soil Bacterium, *Pseudomonas sp.* C-4

The enzyme which catalyses reaction 1 is named CHD dehydrogenase. This enzyme is SH enzyme and requires DPN as a cofactor. The CHD used here was the *trans*-isomer and *cis*-isomer was not stuidied.

The cells adapted to CHD oxidize CH-one-ol without any lag period, and two moles of DPN are required to oxidize one mole of CHD-one-ol dehyrogenase, which catalyses the reaction 2, is inferred. It is still not known why synthesized CH-one-ol did not work as a substrate for the dehydrogenation reaction. But it may be reasonable to propose CH-one-ol as an intermediate in between CHD and CH-dione, because there are two dehydrogenation steps between them. On the other hand, the same ammonium sulphate fraction as that of CHD dehydrogenase catalyses the dehydrogenation of cyclohexanol depending on DPN, but not inositol. This suggests that the substrate of CHD dehydrogenase is not restricted to CH-*di*-ol but CH-*mono*-ol can also serve as its substrate. If the dehydrogenation of CH-one-ol was catalysed by CHD dehydrogenase, two moles of DPN may also be reduced by one mole of CHD. To get the answer on whether or not CH-one-ol dehydrogenase is distinct from CHD dehydrogenase, further purification of enzymes is necessary.

The enzyme which catalyses reaction 3 is called CH-dione hydrase. This reaction proceeds anaerobically and irreversibly. For this reaction, some cofactor, which is in the dialysate from crude extract, is required. Although a reaction mechanism of this enzyme is not elucidated yet, no oxygen uptake was shown to CH-dione even by a crude extract, and thiamine pyrophosphate and Mg^{++} could not replace the effect of the dialysate, so that the formation of the hydrated coumpound might be assumed to be an intermediary step of this reaction.

Reaction 4 is catalysed by adipic acid semialdehyde dehydrogenase and DPN is required as a cofactor. In this reaction, adipic acid is formed from adipic acid semialdehyde.

As shown in this communication, the metabolism of the cyclohexane ring of CHD is quite different from that of the benzene ring of catechol. Another experiment (unpublished) showed that the metabolism of these two substances did not interfere with each other. The adaptive formation of each of these enzyme system was independently induced by its own substrate. The substrate of one system, even it was added to the medium in a high concentration, did not inhibit the induced formation of the other enzyme system. Moreover, neither pyrocatechase nor CHD dehydrogenase was inhibited by CHD or catechol, respectively.

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