Influence of Pesticides on the Presence and Activity of Nitrogenase in *Azotobacter vinelandii*

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The influence of various pesticides on the growth of *Azotobacter vinelandii* was tested under nitrogen fixing conditions. Growth was stimulated in nearly all cases. The presence of nitrogenase in bacterial extracts was greatly reduced. Inhibition experiments showed that organochlorine compounds have no influence on the reduction of acetylene. Derivatives of 2,4-dinitrophenol inhibit in a specific way.

Chemicals used as pesticides are almost exclusively synthetic organic compounds. The safe use of these compounds depends largely on an understanding of their mode of action (Casida, 1973). Biological N_2 fixation is one of the most important processes carried out by microorganisms. It is therefore interesting to have information on the influence and possible mode of action of pesticides on this process. In this paper we report on their influence on bacterial growth, on the synthesis of the nitrogenase enzyme complex, and on the acetylene reducing activity of nitrogenase formed in the absence of pesticides.

MATERIALS AND METHODS

Growth Conditions. *Producing Strain.* The following strains of *Azotobacter vinelandii* have been tested: ATCC 9104, ATCC 12518, and ATCC 13705.

Medium. The American Type Culture Collection (Rockville, Md.) recommends for the culture of these microorganisms its basal medium no. 190 ($FeSO_4$ (0.005 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.2 g), and tap water (900 ml)) with addition of an african violet soil extract (African violet soil (77.0 g), Na_2CO_3 (0.2 g), and distilled water (200 ml), autoclave 1 hr, filter through paper before using in media) or 2% of a sugar. As this type of soil was not at our disposal we used a garden soil according to a suggestion of Brandon (1974).

The medium is prepared by adding to 900 ml of basal medium 100 ml of soil extract and adjusting the pH to 7.6. After sterilization a sterile solution of sugar is added for a final concentration of 1–2%. As no enzyme activity was registered in a medium with soil extract we successfully substituted for this extract the following solution of molybdenum and vanadium ($Na_2MoO_4 \cdot 2H_2O$ (1 g), $VOSO_4 \cdot 5H_2O$ (0.5 g), and distilled water (1000 ml)) for the soil extract.

The results reported in Table I show that the strains 12518 and 13705 are roughly equivalent in bacterial growth and show a higher nitrogenase specific activity than strain 9104, the use of glycerol or sodium citrate is to be avoided, and the concentration of 2% of sugar is better than 1%. The medium finally adopted was basal medium containing 900 ml, glucose 2% (100 ml), and Mo and V solution, 1 ml.

Conditions of Culture. Cultures were performed in stainless steel 20-l. fermentors. The agitation was effected with a vertical propeller rotating at 60 rpm. Aeration was accomplished through a ring sparger fixed at the bottom of the fermentor. After some trials we adopted an aeration of 1 vol of air/1 vol of culture per min at a temperature of 27°.

The inoculum, which represents 20% (v/v) of the culture, is a 48-hr-old culture obtained in agitated erlenmeyer flasks (1 l., containing 150 ml of medium) incubated at 27°, on a rotary shaker set at 110 rpm and 8 cm stroke. The growth experiments described in Table I were done once. The reliability of the experiments can, however, be inferred from experiments where different bacterial strains have been used with the same composition of the medium (see Table I).

Enzyme Isolation. The isolation was carried out essentially according to the method described by Burns et al. (1970). Cells were washed in phosphate buffer, pH 7 (25 m*M*), centrifuged, and suspended in Tris buffer, pH 7.4 (50 m*M*). Using a French press (Milner et al., 1950) at 210 kg/cm^2, the bacterial cells were disrupted two times. The suspension was used, without centrifugation, for the experiments described in Table I. The supernatant obtained after centrifugation for 20 min at 10,000*g* is the crude extract. This crude extract was used for the experiments described in Table II. It had a specific activity of 5 nmol of C_2H_4/min per mg of protein. The experiments from Tables III and IV were done with heated extract. This was obtained by heating the crude extract for 10 min at 60° under an atmosphere of hydrogen followed by centrifugation for 2 hr at 45,000*g*. The heated extract had a specific activity of 7 nmol of C_2H_4/min per mg of protein.

Activity Measurements. The activity of the nitrogenase-enzyme complex was measured as reduction of acetylene (Hardy et al., 1968) and protein was determined with the biuret reagent (Gornall et al., 1949). Bovine serum albumin was used as a standard. All activity measurements were done in duplicate and no difference was found within experimental error.

Chemicals. The following substances have been used: calcium cyanide from K&K; Protex (sodium 2,2-dichloropropionate) and Dinocap (4,6-dinitro-2-(*l*-methylheptyl)-phenylcrotonate) from Protex, Belgium; cyanuric acid from Eastman Kodak; Thiram (tetramethylthiuram disulfide); dimethoate (*O,O*-dimethyl *S*-(*N*-methylcarbamoyl)-methyl phosphorodithioate) from Bayer; Orthocide (*N*-trichloromethylmercapto-4-cyclohexane-1,2-dicarboximide) from Californian Spray Chemical Corp.; DNOC (4,6-dinitro-*O*-cresol) from Fluka; Nirit supra (2,4-dinitrophenyl thiocyanate) and PCNB (pentachloronitrobenzene) from Hoechst; Tricarbamix: zineb (zinc ethylenebisdithiocarbamate) 45%, maneb (manganous ethylenebisdithiocarbamate), 15%, and ferbam (iron salt of dimethyldithiocarbamic acid), 15%, from Du Pont de Nemours. The following compounds were obtained from Supelco Inc.: Cortilan (1,2,4,5,6,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan, contains 40% related compounds), diazinon (*O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidyl) thiophosphate), DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane), Kelthane (1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol), malathion (*S*-(1,2-dicarbethoxyethyl) *O,O*-dimethyl dithiophosphate), endosulfan (6,7,8,9,10,10-hexa-

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Table I. Choice of a Nitrogenase Enzyme Complex Producing Strain and the Composition of Medium

ATCC strain	Supplement to basic medium[a]	Yield of wet bacteria, g/10 l. of medium	Sp act.[b]
9104	Soil extract	24	0.00
9104	Soil extract, Mo + V	36	0.00
12518	Soil extract, Mo + V	35.5	0.00
13705	Soil extract, Mo + V	39	0.00
9104	Mo, V, 1% sucrose	61.5	0.202
12518	Mo, V, 1% sucrose	80	0.31
13705	Mo, V, 1% sucrose	79	0.45
12518	Mo, V, 2% sucrose	133	0.31
12518	Mo, V, 2% mannitol	120	0.26
12518	Mo, V, 2% glycerol	65	0.057
12518	Mo, V, 2% glucose	165	0.37
12518	Mo, V, 2% sucrose, 1% Na citrate	2	0.00

[a] See Material and Methods section for the details. [b] Specific activity of nitrogenase enzyme complex in nanomoles of C_2H_4/minute per milligram of protein.

Table II. Influence of Various Pesticides on the Yield of Bacterial Mass of *Azotobacter vinelandii* and the Synthesis of Nitrogenase Enzyme Complex

Compound	Applied dose, g/10 l.	Yield of wet bacteria, g/10 l. of medium	Sp act.[a]
None		165	5
Soluble in culture medium			
Ammonium thiocyanate	1.2	474	0
Calcium cyanide	0.2	74	0
Potassium cyanate	1.2	174	0.04
Protex	3.2	313	0.08
Thiram	3.2	140	0.07
Dimethoate	6.4	45	0.74
Poorly soluble in culture medium			
Orthocide	1.3	342	0.65
Cortilan	1.3	785	0.54
Cyanuric acid	Sat.	200	0.26
Diazinon	Sat.	457	5
Dinocap	1.3	350	0.91
DDT	0.13	770	0.17
DNOC	0.13	735	0.76
Kelthane	1.3	890	0.04
Malathion	Sat.	620	0.32
Nirit Supra	1.3	394	0.5
PCNB	Sat.	645	2.7
Endosulfan	Sat.	340	1.13
Tricarbamix	1.3	490	0.13

[a] Specific activity of nitrogenase enzyme complex in nanomoles of C_2H_4/minute per milligram of protein.

Table III. Effect of Organic Solvents on the Activity of Nitrogenase[a]

Solvent	% inhibition as a function of solvent composition, vol % 1	5	10
Cyclohexanone	20	100	100
Acetone	15	43	70
Ethanol	2	20	65
Dimethylformamide	0	12	55
Dimethyl sulfoxide	0	8	30

[a] Zero percent inhibition when no organic solvent is present.

chloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide), Azodrin (*O,O*-dimethyl 2-methylcarbamoyl-1-methylvinyl phosphate). All other chemicals were obtained from Merck, Darmstadt.

RESULTS

Growth Experiments. Table II gives the influence of various compounds on the yield of bacteria expressed in grams of cell paste obtained with a 16-hr culture under identical conditions. Products soluble in the medium were applied at a dose adequate to obtain a significant inhibition of the bacterial growth. Slightly soluble products were applied at a dose adequate to obtain a saturated solution.

Influence on the Synthesis of Nitrogenase. Bacteria that were grown in the presence of pesticides at doses indicated in Table II were broken and the specific acitivity of their extracts was measured. Since the bacterial cells were thoroughly washed there is no possible direct action of residual products on the activity of the enzyme. The results of these experiments are included in Table II.

Influence of Solvents on the Activity of the Isolated Enzyme. The various solubilities of the products prompted us to search for solvents that are not inhibitory to the enzyme. Table III indicates that ethanol, dimethylformamide, and especially dimethyl sulfoxide can be used for inhibition experiments, without any important effect on the activity of the enzyme.

Influence of Pesticides on the Activity of the Isolated Enzyme. The pesticides tested have been classified in three groups according to their solubility in water, ethanol, and dimethyl sulfoxide. The extent of their inhibition of the activity of the nitrogenase enzyme complex is tabulated in Table IV.

DISCUSSION

It is evident from Table II that nearly all compounds studied increase the yield of the bacterial mass. On the other hand, it is seen that the nitrogenase enzyme complex specific activity falls below 20% of the value observed when no pesticides were added to the culture medium. Notable exceptions are diazinon and to a lesser extent PCNB. Growth stimulation in the presence of pesticides

Table IV. Extent of Inhibition of Specific Activity of Nitrogenase Enzyme Complex by Several Compounds[a]

Compound	Use	Concen, m*M*	% inhibition[b]
Soluble in water			
Ammonium thiocyanate	H	3.5	30
Potassium cyanate	H	3.5	30
Cyanuric acid	I	0.1–1	0
Dinocap	F	0.5	50
DNOC	I	1	50
Soluble in ethanol			
DDT	I	0.1–1	0
Diazinon	I	0.1–1	0
Kelthane	A	0.1–1	0
Endosulfan	I	0.1–1	0
Dimethoate	I	0.1–1	0
Soluble in dimethyl sulfoxide			
Orthocide	F	0.7	50
Cortilan	I	0.1–1	0
Malathion	I	0.8	15
Nirit Supra	F	0.7	50
PCNB	F	0.1–1	0
Tricarbamix	F	0.3	50
Protex	H	0.1–1	0
Azodrin	I	0.1–1	0
Thiram	F	0.1–1	0

[a] Common name is used (Perkow, 1971). H = herbicide, F = fungicide, I = insecticide, A = acaricide. [b] Percent inhibition is indicated at the concentration given in the concentration column. When no inhibition is observed, the range of concentration tested is given.

has been frequently observed (Alexander, 1969). The increase of the yield of bacterial mass observed for some pesticides (see Table I) should be compared with the stimulatory effect of pesticides that has been observed upon ammonification, nitrification, and CO_2 evolution. Our results are at variance with those of Mackenzie and MacRae (1972). Using *Azotobacter vinelandii* in pure culture, these authors found no influence of DDT, lindane, Dalapon-Na, and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) upon the growth and nitrogen-fixing capacity. Very recently, Vlassak and Heremans (1974) have observed a stimulatory effect of aldrin upon acetylene reduction under conditions where *Clostridium* species were active. We conclude, therefore, that the synthesis of the nitrogenase enzyme complex is not affected by pesticides, at least in the case when no combined nitrogen is present in the molecule. The latter compounds would act in a similar manner as ammonium (Daesch and Mortenson, 1972).

A possible explanation for the reduced activity in the bacterial extracts may be found in the influence of pesticides on the protection against oxygen damage provided by the bacterial membrane (Oppenheim and Marcus, 1970). It is not unreasonable to assume that pesticides change the membrane properties so that the protection becomes less effective. Yates (1970), on the other hand, has shown that NADH dehydrogenase can function as a protector against oxygen damage while other enzymes tested do not. From Table IV it seems that 75% of the compounds used do not inhibit more than 50% of the enzymic activity in the concentration range tested.

Concerning the possible relation between the chemical nature of the pesticides and their influence on the activity of the isolated enzyme, some interesting points can be observed. First, it is noted that the organochlorine compounds do not inhibit. This is surprising compared to the specific inhibition found for DDT on the sodium potassium dependent ATPase (Janicki and Kinter, 1971) and on methanogenesis (McBride and Wolfe, 1971). Two compounds, Dinocap and dinitro-*o*-cresol (DNOC), have been investigated in more detail because of their use as uncouplers in oxidative phosphorylation (Casida, 1973). These compounds act as competitive inhibitors for the ATP transformation by the enzyme (our unpublished observation). Vlassak et al. (1975) reported that DNBP, a similar compound, is a competitive inhibitor for the substrate ATP of nitrogenase. A final point to be discussed is the influence of various solvents on the activity of nitrogenase. The same general pattern of inhibition is found as for the above-mentioned ATPase. It is of interest in this context to note that cyclohexanon, a commercial solvent for DDT, completely inhibits nitrogenase while DDT itself does not! All solvent inhibitions are sigmoidal, a fact that can be correlated with the cooperative interactions in nitrogenase which have been ascribed to protein–subunit interactions (Van Rossen et al. 1973).

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