Enzyme from Soil Bacterium Hydrolyzes Phenylcarbamate Herbicides

Abstract. *An enzyme preparation from* Pseudomonas *sp., isolated from a soil culture by an enrichment technique, liberated 3-chloroaniline from the herbicide isopropyl* N*-(3-chlorophenyl) carbamate. Aniline, 3-chloroaniline, and 3,4-dichloroaniline were detected when the enzyme preparation was incubated with several alkyl esters of the phenylcarbamates and chlorophenylcarbamates. No chloroaniline was detected when the 3-(*p*-chlorophenyl)-1,1-dimethylurea (monuron) was used as a substrate. The substrate specificity of the isolated enzyme suggests that it catalyzes the initial hydrolysis of many biologically active phenylcarbamates in soils.*

Esters of the phenylcarbamic acids constitute an important class of pesticides. Degradation of these compounds has been attributed to the activity of microorganisms (*1*). We now describe some of the properties of an enzyme, isolated from a soil microorganism, which liberates 3-chloroaniline from the herbicidally active isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) and from several structurally related phenylcarbamates. The enzyme was obtained from a soil bacterium identified as a species of *Pseudomonas*.

Soil microorganisms responsible for decomposing CIPC were isolated from soil cultures by an enrichment technique. Organisms isolated from enriched cultures were incubated in a mineral salts solution (*2*) containing CIPC at a concentration of 100 parts per million (ppm), weight to volume. The solubility of CIPC in this medium is 108 ppm. Degradation by microorganisms was determined by assaying for chloride-ion liberation (*3*), aniline production (*4*), and $C^{14}O_2$ production from isopropyl-C^{14}- and ring-C^{14}-CIPC (*5*).

The *Pseudomonas* sp. was cultured in 10-liter volumes of media containing excess amounts of CIPC as a primary source of carbon. The CIPC (5.0 g), dissolved in 10 ml of 95 percent ethanol and sterilized by filtration, was added to the autoclaved nutrient culture solution. The culture solution was continuously agitated so that it would continue to be saturated with CIPC as rapidly as the carbon source was removed by metabolic degradation.

Cells were harvested by continuous-flow centrifugation at 3°C. All subsequent manipulations were conducted at 0° to 3°C, unless otherwise stated. Twice-washed cells were treated with high-frequency sound for 7 to 10 minutes, and the disrupted cells were separated from the supernatant by centrifugation at 10,000*g* for 10 minutes. The crude enzyme preparations in the supernatant were stable when stored at −5°C for several weeks.

Table 1. 3-Chloroaniline production from four phenylcarbamates incubated with cell-free extracts from a *Pseudomonas* sp.

Carbamates	3-Chloroaniline (μg/20 min)
Isopropyl *N*-(3-chlorophenyl)	21.5
2-Chloroethyl *N*-(3-chlorophenyl	13.0
2(1-Chloropropyl) *N*-(3-chlorophenyl)	10.0
2-Ethylhexyl *N*-(3-chlorophenyl)	3.0

Enzyme activity was demonstrated by incubating the cell-free supernatant with CIPC and then assaying for 3-chloroaniline. Assay solutions consisted of 2.5 ml of CIPC (1.15 μmole), 0.4 ml of tris-HCl buffer (*p*H 8.5, 0.01*M*), and 0.1 ml of dialyzed enzyme (1.3 mg of protein). Protein was determined by the biuret procedure (*6*), with bovine serum albumin as a standard. Dialysis of the enzyme overnight removed all contaminants that produced interfering color with the azo dye in controls (boiled enzyme). Chloroaniline production was linear with respect to time for the first 30 minutes with enzyme concentrations up to 2.0 mg protein in the assay solution. Assays conducted for 20 minutes at 30°C with other carbamates yielded sufficient 3-chloroaniline to be detected colorimetrically (*4*). The colorimetric procedure is not sensitive at concentrations below 2 μg of 3-chloroaniline per 3 milliliters of assay solution. The enzyme was active over a *p*H range of 7 to 10, with maximum activity in the range 8.0 to 9.0.

The 3-chloroaniline was verified as the enzymic end product by flame ionization gas chromatography. The methylene chloride extract of the enzyme assay solution and an authentic sample of 3-chloroaniline were compared with respect to retention time and cochromatography on a 1.8-m column. The stationary phase of the column was ½ percent Ucon LB550-X on chromosorb W(60–80 mesh).

The enzyme preparation acts upon phenylcarbamates other than CIPC. Five carbamates, including the isopropyl *N*-phenylcarbamate (IPC), were dissolved in aqueous solutions at concentrations equimolar to that of CIPC ($4.54 \times 10^{-4}M$), with the aid of a sonic oscillator. Five other carbamates were put into solution at their maximum solubility but at concentrations less than $4.54 \times 10^{-4}M$. The low-solubility carbamates examined included α-carbo(2,4-dichlorophenoxyethoxy)ethyl *N*-(3-chlorophenyl)carbamate; isopropyl *N*-(3,4-dichlorophenyl)carbamate; *sec*-butyl *N*-(3,4-dichlorophenyl)carbamate; and α-carboisopropoxyethyl-*N*-(3-chlorophenyl)carbamate. Aniline, 3-chloroaniline, or 3,4-dichloroaniline was detected from all ten carbamates. Chloroaniline production from four of the 3-chlorophenylcarbamates is shown in Table 1. No chloroaniline was detected when the enzyme was incubated with 3-(*p*-chlorophenyl)-1,1-dimethylurea (monuron).

The mechanism of the enzymic reaction is uncertain at this time. Hydrolysis of the ester linkage of CIPC to give isopropyl alcohol and the 3-chlorophenylcarbamic acid is one possibility. The 3-chlorophenylcarbamic acid is unstable and would spontaneously yield 3-chloroaniline and CO_2. Enzymic attack at the amide linkage in the carbamate cannot be ruled out, however, as a site of action. The products of this type of reaction would be 3-chloroaniline and the isopropyl ester of carbonic acid. Some evidence against the latter hypothesis is the failure of the enzyme to attack monuron. Chloroaniline production from CIPC was inhibited by di-isopropylfluorophosphate (DFP). A $10^{-6}M$ solution of DFP gave 42 percent inhibition. This compound is an inhibitor of esterases in general as well as of endopeptidases that exhibit esterase activity.

The broad specificity of the enzyme preparation on several carbamates suggests that the reaction is a key step responsible for the detoxification of many biologically active phenylcarbamates. Studies with the intact organisms indicate that 3-chloroaniline does not tend to accumulate in the system since about half of the radioactivity from CIPC uniformly labeled with C^{14} in the ring could be recovered as $C^{14}O_2$ after a 3-hour incubation period.

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References and Notes

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3 November 1964

Pressure-Induced Color Mutation of Euglena gracilis

Abstract. *Photosynthetic cultures of* Euglena gracilis *Z which were illuminated during growth were more resistant to the lethal effect of high hydrostatic pressures than nonphotosynthetic cultures grown in the dark. A high percentage of mutants permanently lacking chlorophyll and with altered carotenoids was obtained after subjecting cultures to high pressure. A minimum pressure of 500 atmospheres was critical for color mutation and morphological change. The highest effective pressure used was 1000 atmospheres.*

The photosynthetic apparatus of *Euglena* is subject to destruction by a number of environmental factors. Chlorotic substrains have been derived after treatment of cultures with drugs such as streptomycin (*1*), erythromycin (*2*), and pyribenzamine (*3*) after growth at high temperature (*4*) and after exposure to ultraviolet radiation (*5*). The application of high pressure has now been found to induce in *Euglena* a permanent loss of the ability to photosynthesize and produce chlorophyll.

Euglena gracilis, strain Z (*6*), was cultured in Difco euglena broth (*7*). The cultures were grown at 27° to 29°C in the dark or under constant illumination of 2700 lux. After 5 days, cells were harvested by centrifugation at 1000*g*, washed, suspended in distilled water, and left overnight so that paramylum reserves would be depleted. The suspensions were then adjusted photometrically to contain approximately 1×10^6 cells per milliliter and were dispensed into plastic tubes (Beckman polyallomer tubes) which were then sealed with serum bottle stoppers. Sodium acetate, introduced into the sealed tubes by means of a hypodermic syringe, was used as a substrate in a final acetate concentration of 0.05*M*. In all these procedures special precautions were taken to maintain aseptic conditions.

The polyallomer tubes, sealed in water-filled plastic bags, were then placed in steel pressure vessels (*8*) and subjected to pressure in the dark for 20 minutes to 4 hours. The experimental pressures used were 10, 100, 500, 670, and 1000 atmospheres (atm). Control cultures were maintained at ambient pressure, approximately 1 atm. When pressure was released, 1-ml samples were removed with a hypodermic syringe and dispensed into 9 ml of sterile culture medium in screw-capped tubes. A standard dilution series was then prepared to ascertain viability after exposure to pressure. Samples were also streaked on the surface of Difco euglena agar plates. Within 30 minutes after return to ambient pressure, samples were examined under the microscope and compared with controls.

Euglena exposed to 10 or 100 atm for as long as 2 hours showed no obvious morphological difference from controls, appearing normal in all respects. After being subjected to 500 atm for ½ hour or longer, the *Euglena* were mostly immotile but essentially normal in size and shape. Cells which had been subjected to 1000 atm were all rounded and immotile after 2 hours of exposure. However, after only 20 minutes at 1000 atm, although all cells were immotile, not all were rounded. In terms of cell morphology, therefore, it seems that changes in motility and shape depend upon the amount of pressure applied and the duration of application.

In viability tests, cell suspensions subjected to 10 and 100 atm for periods up to 4 hours, regardless of whether the cultures were illuminated during growth, showed no difference from controls. Two hours of exposure to 1000 atm proved to be 100 percent lethal for dark-grown *Euglena*; however, of the initial inoculum from *Euglena* grown in light, 1×10^{-6} to 1×10^{-5} cells remained viable. The presence of the photosynthetic apparatus apparently made some of the cells more resistant than others to the lethal effect of high hydrostatic pressure. This difference in resistance was verified by experiments in which pressure and time were varied. The results are summarized in Table 1.

Several color mutants of *Euglena*, incapable of forming chlorophyll, were isolated from cultures subjected to high pressure. One mutant, designated PR-1, was isolated from liquid culture and has been carried through more than 14 serial subcultures under illumination without chlorophyll being resynthesized. Monochromatic microscopy (*9*) failed to reveal the presence of mature chloroplasts in this and subsequently isolated mutants. Small greenish bodies which might have been proplastids or simply pigment-lipid globules were visible. That these bodies did not contain chlorophyll was demonstrated by the absence of a chlorophyll peak at 665 mμ in alcohol extracts examined in a Beckman DK recording spectrophotometer.

Table 1. Viability of *Euglena gracilis* Z exposed to high hydrostatic pressure. Initial inoculum was 1×10^6 cells/ml. The results are expressed as percentages.

Time (hours)	Pressure (atm)		
	1, 10, 100, and 500	670	1000
Cultures grown in darkness			
½ to 1	100		0
3	100		0
Cultures grown in light			
½ to 1	100	10	1
3	100	0.1	0.0001

To determine whether the appearance of a mutant containing no chlorophyll was due to an induction phenomenon or to the chance selection of a colorless, viable mutant already present in the population, samples from control cultures and from a culture of PR-1 were streaked on plates containing Difco euglena agar. After 8 days under illumination, the controls showed 100 percent green colonies and PR-1 showed 100 percent white colonies. This demonstrated that: (i) there were no colorless mutants already present in the stocks, and (ii) if such cells were present, they would have grown on the agar plates, as attested to by the growth of PR-1.

Although all colonies were green, the control plates contained at least two variants: one deep green, which synthesized chlorophyll rapidly, and the other a lighter green, which synthesized chlorophyll slowly. The latter variant also became deep green after 12 to 14 days. Whether the color mutants are derived chiefly or solely from one or the other of these variants is not known. After being subjected to 1000 atm for 20 minutes or 2 hours, cells were inoculated into liquid culture media, incubated in the light until just-visible growth appeared, and then streaked on agar plates. About 20 percent of the colonies on the agar appeared opaque white in reflected light. Some of these were variegated and had