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Evidence for Cometabolism in Sewage

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A procedure was developed to demonstrate cometabolism in models of natural ecosystems. The procedure involves showing the formation of metabolic products in high yield and the lack of incorporation of substrate carbon into cellular constituents. Samples of four ^{14}C-labeled herbicides (trifluralin, profluralin, fluchloralin, and nitrofen) were incubated with sewage aerobically and under discontinuous anaerobiosis for 88 days, and fresh sewage was added at intervals. Products were formed from each of the herbicides in nonsterile, but not in sterile, sewage. The yield of recovered products reached 87% for profluralin and more than 90% for fluchloralin and trifluralin, and the number of products ranged from 6 for nitrofen to 12 for fluchloralin. Concentrating the sewage microflora 40-fold greatly enhanced the rate of conversion. None of the radioactivity from the herbicide entered the nucleoside pool of the sewage microflora. The lack of incorporation of substrate carbon into cells and the almost stoichiometric conversion of the substrate to organic products indicate that members of the microbial community were cometabolizing the test compounds.

Microorganisms may modify chemicals that they cannot utilize as sources of energy or as nutrients. This process is known as cometabolism or, if the reaction involves an oxidative sequence, cooxidation. Because the conversion does not provide the active organisms with either energy or nutrients, no population increase occurs in response to the presence of the compound, and a product is formed that accumulates (1, 16). This lack of population increase, if it occurs in nature, may be of great importance because the absence of replication of a small population suggests that the reactions it carries out will occur slowly. Moreover, the accumulation in nature of metabolic products derived from toxic chemicals is of ecological concern because the product may still have the properties of the precursor that were responsible for the toxicity.

Cometabolism has been shown to occur in pure culture (12, 14) and in mixed culture (2, 6, 17) with a number of organic compounds, including some of environmental importance. It also has been suggested that cometabolism takes place in natural ecosystems (1, 11), but direct evidence in support of this view has yet to be obtained. In a number of instances, however, the slow biological transformation of a compound in a natural ecosystem, the accumulation of products that are not readily metabolized in that ecosystem, and the failure to isolate a microorganism able to use the compound as a nutrient or energy source suggest that cometabolism is indeed occurring in nature (9, 15, 19).

This study was designed to develop a method to show cometabolism in models of natural ecosystems and to use that method to demonstrate cometabolism in sewage.

MATERIALS AND METHODS

Chemicals. The compounds used were fluchloralin [*N*-(2-chloroethyl)-α,α,α-trifluoro-2,6-dinitro-*N*-propyl-*p*-toluidine; synonym, basalin], profluralin [*N*-(cyclopropylmethyl)-α,α,α-trifluoro-2,6-dinitro-*N*-propyl-*p*-toluidine; synonym, tolban], trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine; synonym, treflan), and nitrofen (2,4-dichlorophenyl *p*-nitrophenyl ether; synonym, Tok), and the unlabeled and ^{14}C-labeled compounds were obtained from Eli Lilly & Co., Indianapolis, Ind., BASF Wyandotte Corp., Parsippany, N.J., Ciba-Geigy Corp., Greensboro, N.C., and Rohm and Haas Co., Philadelphia, Pa., respectively. Fluchloralin and profluralin were uniformly ring labeled, trifluralin was labeled in the trifluoromethyl group, and nitrofen was uniformly labeled in the nitrophenyl ring; the specific activities were 94.2, 52.4, 10, and 0.79 μCi/mg, respectively. The radiochemical purity of the compounds was determined by thin-layer chromatography (TLC) with a hexane-acetone (9:1) solvent system on silica gel sheets (Eastman Kodak, Rochester, N.Y.). The plates were then exposed (5) for 4 weeks to Kodak SB-5 X-ray film, and the film was developed according to the producer's specifications (8). This chromatographic and autoradiographic technique also was used in studies of product formation. More than 99% of the radioactivity in the original preparation of the parent chemicals was present in the spot to which the parent compounds migrated.

Incubation. Unlabeled samples of the four chemicals, all of which are herbicides, were added singly to primary effluent obtained at the Ithaca, N.Y., sewage treatment plant to yield a final concentration of 100

μg/ml. For samples incubated aerobically, 1.0-liter Erlenmeyer flasks, each containing 250 ml of the amended sewage, were shaken at 40 rpm on a rotary shaker. For samples incubated under discontinuous anaerobiosis, the treated sewage samples (250 ml) were contained in 500-ml suction flasks that were sealed with rubber stoppers, with a latex venting tube running from the side arm to a water trap. The O_2 present in these flasks was no more than 85 mg, and it was probably readily consumed microbiologically; however, the contents of the flasks were exposed to air each time a sample was taken. Labeled compounds in amounts sufficient to provide approximately 2.5 μCi were also added to each flask. The incubation was performed by maintaining duplicate samples in the dark at 22°C. Sterile controls, in which the sewage was sterilized by autoclaving and the herbicides or glucose was sterilized by passage through 0.22-μm membrane filters (Millipore Corp., Bedford, Mass.), were included with all treatments.

Samples were taken on days 0, 18, 34, 55, and 88 in amounts sufficient to provide 0.4 μCi for a determination of the incorporation of the label into nucleosides and 0.1 μCi for a determination of substrate loss and product formation. Fresh sewage, which served as a source of carbon, and organisms were added on days 11, 22, 33, 44, 55, 66, and 77 to all of the nonsterile treatments. The amount of sewage added was 1/10 the volume of the liquid then present in the flasks. In an experiment in which sewage was concentrated 40-fold and incubated aerobically at 22°C in 25-ml Erlenmeyer flasks in the dark for 24 h, 3.2 and 0.8 ml were removed at 0 and 24 h for a determination of the incorporation of the label into nucleosides and for a measurement of substrate loss and product formation.

Sample processing. To note substrate loss and product formation, all samples were acidified with 1 drop of 0.1 N HCl and extracted with twice their volume of CH_2Cl_2. This volume was reduced to 0.5 ml, and a 25-μl portion was then spotted on TLC sheets. The spotted plates were chromatographed and autoradiographed as described above. Distinct spots corresponding to the dark regions on the X-ray film were cut out of the sheets, and the radioactivity of the spots was quantified by liquid scintillation counting with a Beckman LS-100 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.), with ACS scintillant (Amersham Corp., Arlington Heights, Ill.) as the counting fluid. Counts of random 25-μl samples of the CH_2Cl_2 extracts reduced to 0.5 ml and added directly to the scintillation fluid differed by no more than 5% from the total counts of the same sample after chromatographic separation.

The other portion of the samples was converted to nucleosides, presumably from the organisms present in the sample, in the following manner. The samples were centrifuged for 20 min at 16,000 × *g*, and the resulting pellet was washed twice with distilled water and recentrifuged. The pellet was suspended in 3.0 ml of distilled water and treated 12 times in a sonic oscillator (Sonifier Cell Disrupter, model W185D; Heat Systems Ultrasonics, Plainview, N.Y.) for 20-s periods, with 10-s intervals between exposures. This period for sonic treatment was determined by measuring the increase in ribose concentration (10) in relation to the time of sonic treatment of a sewage sample. The pellet treated in this way was mixed with 4.0 mg of pronase (Calbiochem, La Jolla, Calif.) and incubated at 30°C for 1.0 h. The enzymatically treated pellet was then centrifuged at 47,000 × *g* for 30 min, and the supernatant fluid was incubated with 4.0 mg of pronase for 1.0 h. The pronase was added to remove any protein associated with the nucleic acids.

After the second pronase treatment, the samples were brought to 100°C for 15 min to inactivate the enzyme. The samples were then cooled and incubated on a shaker at 30°C with immobilized micrococcal nuclease (Worthington Biochemicals Corp., Freehold, N.J.). The nuclease (1.0 mg) was attached to 2.0 g of CNBr-activated Sepharose 4B support resin (Sigma Chemical Co., St. Louis, Mo.) by recommended methods (18) and placed inside four sealed packets of 10-μm nylon mesh (Small Parts, Miami, Fla.). The samples were outside the packets. This step yielded free 3′-nucleotides. After the nuclease treatment, the packet was washed with 1.0 ml of distilled water, and the water was retained to keep nucleotides present in the water. The solution of 3′-nucleotides was dried at 100°C, 1.0 mg of alkaline phosphatase (Sigma Chemical Co.) in 1.0 ml of distilled water was added to the dried material, and this mixture was incubated for 12 h at 30°C. The enzyme converted the nucleotides to nucleosides. Portions (250 μl) of the solution containing the free nucleosides were then spotted on polyethyleneimine cellulose F TLC sheets (VWR Scientific Co., Rochester, N.Y.), and the chromatographic separation was performed with a solvent system of distilled water (20). Adenosine, uridine, cytosine, inosine, hypoxanthine, and guanosine (Sigma Chemical Co.) were used as standards. Autoradiography of the sheets was then performed for 6 weeks in the manner described above.

One study was conducted with uniformly labeled glucose (specific activity, 1.55 mCi/mg; Schwarz Bio Research, Inc., Orangeburg, N.Y.) in place of the herbicides. In this study, 100 ml of raw sewage was incubated aerobically or aerobically and then anaerobically with a mixture of labeled and unlabeled glucose added to a final concentration of 100 μg/ml, with each flask containing 2.5 μCi. The samples were incubated in 250-ml Erlenmeyer flasks on a shaker at 40 rpm for the aerobic treatments and in 125-ml side-arm flasks with facilities for gas venting in the anaerobic treatments. Samples were taken at 0 and 48 h in tests with unconcentrated sewage and at 0 and 24 h in tests with concentrated sewage particulates, and the nucleic acids in the samples were digested to the nucleosides. Radioactive adenosine was recovered from the TLC plate by scraping off the area cochromatographing with the authentic chemical, and this labeled adenosine was treated with 1.0 mg of adenosine deaminase (Sigma Chemical Co.) in 10 mM phosphate buffer, pH 7.0. After a 12-h incubation at 29°C, radioactive inosine was sought by evaporating the reaction mixture, spotting portions on a TLC plate, chromatographing the sample as described above, and scraping material from the plate that cochromatographed with the authentic chemical. The radioactivity was determined with the liquid scintillation spectrometer.

RESULTS

To determine whether the method allowed for measuring the incorporation of the radiolabel into nucleosides, an initial experiment was performed with radioactive glucose. Results showing the amounts of ^{14}C from glucose that were incorporated in 48 h into nucleosides present in the sewage, presumably in microbial cells, are given in Table 1. At most, 0.87% of the added radioactivity was recovered in any single nucleoside. The spot on the TLC plates that cochromatographed with adenosine was confirmed to be that nucleoside when incubation of the material removed from that spot with adenosine deaminase led to the complete destruction of the compound and the appearance of radioactivity in a product that cochromatographed with inosine.

The results of experiments in which sewage was incubated with fluchloralin, nitrofen, profluralin, and trifluralin for 88 days are given in Table 2. At zero time and in the sterile controls, all of the treatments contained a compound(s) with less than 0.1% of the ^{14}C label that was counted. This compound(s), which was not present in the original chemical preparations, did not migrate on the TLC plates and is considered to have been formed spontaneously. In all instances, except in the initial period in the aerobic incubation with trifluralin, the number of products increased with time. The percentage of the recovered ^{14}C label present in the products increased with time for aerobic and discontinuous anaerobic incubations with the four test compounds, indicating increased substrate loss and product formation. In the aerobic treatments with fluchloralin and profluralin and in the anaerobic treatments with fluchloralin and trifluralin, the substrate was almost totally destroyed. The radioactivity not found in the products in Table 2 was present in the spots corresponding to the parent chemicals. The R_f values of the 116 separate products were consistently lower than those of parent compounds, which were 0.86, 0.88, 0.82, and 0.83 for nitrofen, fluchloralin, trifluralin, and profluralin, respectively, suggesting a greater affinity for water of the products than of the parent compounds.

Portions (4.0 liters) of sewage were concentrated 40-fold by centrifugation at 10,000 × *g* for 15 min. The resulting pellets were suspended in 100 ml of distilled water, and 8.0 ml of the suspension in test tubes received labeled and unlabeled herbicides at a concentration of 100 μg/ml, with 1.0 μCi of ^{14}C per tube. These samples were incubated for 24 h. Under these conditions, 21 products appeared, and these were generated in a much shorter time than in the previous experiment (Table 3). These products were also more hydrophilic than the parent compounds.

TABLE 1. *Incorporation of [^{14}C]glucose into sewage nucleosides*

Nucleoside	Label recovered as nucleoside (cpm)
Adenosine	1,460
Cytosine	1,910
Guanosine	1,800
Inosine	362
Uridine	602

In both the long-term incubations and the short-term experiment, the ^{14}C label from the herbicide was not incorporated into the nucleic acid fraction of the sewage. Thus, autoradiographs of the TLC plates, on which were placed the nucleoside-containing digestion products derived from the herbicide-treated sewage, showed no radioactivity at the spots to which the authentic nucleosides migrated. The autoradiograms were allowed to develop for a total of 6 weeks.

DISCUSSION

Cometabolism has heretofore not been directly demonstrated in a natural ecosystem. The results presented here show that microorganisms in a model of at least one ecosystem do not get carbon from a substrate that they metabolize and that they form organic products in quantities almost equal to the amount of substrate transformed; however, small amounts of energy may have been obtained by the microflora. These results were obtained with a highly heterogeneous community that was incubated long enough for growth to have occurred at the expense of the test substrates, and the test system was given regular additions of organisms and fresh carbon sources in the form of sewage. Moreover, organisms able to grow on the test compounds have yet to be isolated, and the microbial conversions in natural ecosystems are characteristically slow (10, 17, 21). These findings are thus consistent with the view that the chemicals are transformed by cometabolism and not by growth-linked processes.

The procedure developed here to demonstrate cometabolism relies on the assumption that carbon from a substrate that is cometabolized does not become incorporated into cellular constituents. Nucleosides were chosen as the cell constituent for analysis because they are easily chromatographed with water as the solvent and because there were few nucleosides to detect.

Considerable attention has been given to the products formed in nature from trifluralin (9, 19,

TABLE 2. *Cometabolism of four herbicides in sewage*

Aeration status	Chemical	^{14}C recovered in products (%)[a] at day:			
		18	34	55	88
Aerobic	Fluchloralin	12 (2)[b]	22 (5)	92 (6)	91 (11)
	Profluralin	3.5 (2)	27 (2)	66 (6)	87 (9)
	Trifluralin	3.0 (3)	7.0 (2)	ND[c]	49 (9)
	Nitrofen	<0.1 (1)	1.2 (2)	30 (5)	40 (6)
Discontinuously anaerobic	Fluchloralin	ND	32 (4)	90 (7)	95 (12)
	Profluralin	0.5 (2)	2.8 (2)	17 (2)	23 (5)
	Trifluralin	3.4 (2)	5.7 (2)	82 (2)	91 (7)
	Nitrofen	ND	1.3 (2)	1.8 (2)	11 (4)

[a] Percentage of that added at day 0.

[b] Values within parentheses are the numbers of discrete radioactive spots found on the TLC plates, other than those corresponding to the parent material.

[c] ND, Not determined.

TABLE 3. *Modification of herbicides after 24 h by sewage concentrated 40-fold*

Chemical	Recovered ^{14}C present in products (%)	No. of products
Fluchloralin	91	9
Profluralin	34	4
Trifluralin	40	4
Nitrofen	48	4

21). Many products are formed from trifluralin in soil (9), and the pathways of breakdown may vary with the oxygen status of the environment (19). Carter and Camper (4) reported an increase in the numbers of a pseudomonad when trifluralin was present as the sole added carbon source, but they stated that the herbicide was only partially metabolized; a more rigorous test of growth needs to be performed, with oligocarbophilic development rigorously excluded. The transformations of fluchloralin, nitrofen, and profluralin have received some attention, and products have been found after incubating fluchloralin in soil (13), nitrofen in flooded and nonflooded soils (15), and profluralin in soil and culture (3; K. Strelka, M.S. thesis, Clemson University, Clemson, S.C., 1977). These herbicides were not found to be extensively degraded in soil, and profluralin was reported to be cometabolized in pure culture (21). In this study, the continual presence of a source of O_2, in contrast with the discontinuous anaerobiosis, affected both the rate of product formation and the number of products generated microbiologically. These data are consistent with reports of differences in the pathways of degradation of trifluralin and profluralin under aerobic and anaerobic conditions (3, 5, 21).

Cometabolism characteristically leads to a slow rate of product formation if the number of cells bearing the responsible enzymes is small. Cometabolism can sometimes be enhanced by the addition of substrate analogs (7) and by the addition of chemicals of dissimilar structure (17). In the present investigation, however, cometabolism was enhanced by merely increasing the density of organisms. If such an approach can be made practical, it should be possible to increase the rate of destruction of at least certain toxic chemicals that otherwise may be only slowly transformed when released into soils or natural waters.

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