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Alteration of Acrylonitrile-Methylacrylate-Butadiene Terpolymer by *Nocardia rhodochrous* and *Penicillium notatum*†

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[^{14}C]Barex-210, a terpolymer of acrylonitrile, methylacrylate, and butadiene, was tested for bioconversion. Powdered samples of polymer, each specifically ^{14}C labeled at different carbon atoms of the polymer, were incubated with either *Nocardia rhodochrous* or *Penicillium notatum* in an enriched growth medium for various periods of time. After 6 months of incubation, the ^{14}C-labeled polymer was transformed from a high-molecular-weight material completely soluble in dimethyl formamide (DMF) into both a lower-molecular-weight form still soluble in DMF and a second form that was no longer soluble in DMF. The amount of ^{14}C-labeled carbon atoms converted into DMF-insoluble material was 8% of the backbone carbon-carbon atoms and 12% of the side-chain nitrile and acrylate atoms from the acrylonitrile-methylacrylate copolymer and 60% of the elastomer (acrylonitrile-butadiene copolymer) atoms. Metabolism of the polymer was not established from measurements of metabolic $^{14}CO_2$. Evolution of $^{14}CO_2$ amounted to only 0.3, 0.6, 1.8, and 3.3% of these four fractions, respectively. Although the transformation of high-molecular-weight polymer into DMF-insoluble material was rapid in the early stages of microbial growth, the accompanying CO_2 evolution was much slower. Further evidence of polymer alteration was indicated by the infrared spectrum of the insoluble material, which showed a disappearance of the nitrile and methylacrylate peaks.

Barex-210 (Vistron Corp., Cleveland, Ohio), a recently introduced high-molecular-weight polymer, is mainly acrylonitrile copolymerized with lesser amounts of methylacrylate and butadiene. The resin has been tested by a number of food and beverage companies for use as a plastic packaging material (4, 12) and, if extensively used, this plastic could add to the solid waste problem. Since proposed approaches to solid waste disposal include bioconversion, it is of value to determine the potential for degradation of this particular polymer.

The common plastic materials such as polyethylene, polyvinyl chloride, and polystyrene and their chemical components have been evaluated as carbon sources for microbial growth (6-10, 13). Klausmeier and Jones (10) showed that by use of enriched culture, selected microorganisms could degrade almost all plasticizers regardless of their chemical structure. The more resistant plasticizers were utilized by microorganisms only when extraneous sources of nutrient were provided and when the molecular weight of the plasticizer was sufficiently low (8). High-molecular-weight plasticizers, however, were quite resistant to bioconversion.

Recently a strain of *Nocardia rhodochrous* has been shown to be able to metabolize nitrile compounds such as acetonitrile and acrylonitrile (2). Intra- and extracellular enzymes were involved in nitrile metabolism; one of them, oxynitrilase, has been isolated from a soil pseudomonad by Hook and Robinson (5, 11).

The present study was undertaken to estimate the extent and nature of bioconversion of Barex-210 polymer by selected soil microorganisms, including *N. rhodochrous*. The study concentrates on the extent of utilization of this polymer when ideal conditions for microbial growth are provided by supplementing the test media with additional exogenous sources of carbon and nitrogen. Samples of this polymer with ^{14}C label at different chemical groups of the molecule were used to provide insight into the sequence of bioconversion.

MATERIALS AND METHODS

Source of chemicals. The chemicals used were analytical grade. Dimethyl formamide (DMF) was spectrophotometric grade from Fisher Scientific Co.,

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Springfield, N.J. The reagents used for liquid scintillation counting were obtained from Fisher Scientific Co.

Barex-210 samples and ^{14}C positions. The polymers were synthesized by emulsion polymerization by Standard Oil of Ohio (4), using radiolabeled monomers obtained from Mallinckrodt Nuclear Co., St. Louis, Mo. The process uses two separate copolymerizations which are then grafted by chain extension. The first copolymer consists of 75% (wt/wt) acrylonitrile and 25% (wt/wt) methylacrylate; the second consists of 30% (wt/wt) acrylonitrile and 70% (wt/wt) butadiene. These are block grafted in the ratio of 13:1, respectively. The resulting terpolymer has approximately 17% nitrogen by weight derived from acrylonitrile. The elastomer portion (butadiene-acrylonitrile) provides only about 3% of the total nitrogen (5.1%, wt/wt).

The reactions normally proceed to nearly 100% completion with the resulting graft terpolymer having a minimum molecular weight of about 100,000. The structure of the graft polymer is shown in Fig. 1 with the locations of ^{14}C labels for each of the six polymers of [^{14}C]Barex-210. The polymer purity for each batch is given in the legend of Fig. 1, as determined by gel filtration chromatography (see below). Traces of residual monomers and of low-molecular-weight (about 500 to 1,000) oligomers can remain from incomplete polymerization. Two batches were found to contain low-molecular-weight oligomers, but no monomers were detected by radioassay. The specific activity of the six batches ranged from 1.5×10^6 to 2.0×10^6 dpm per g of polymer, and the specific carbon atoms were 10 to 20% randomly labeled.

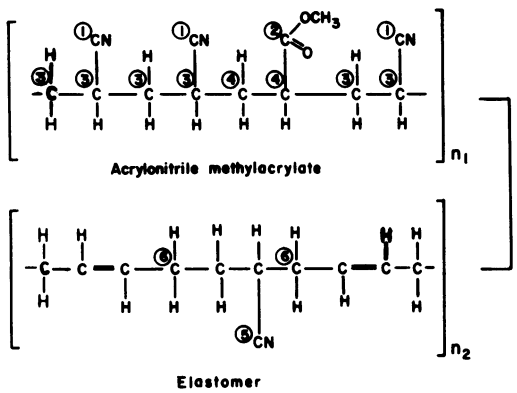

FIG. 1. *Structure of Barex-210 components (^{14}C-polymers) showing position of ^{14}C label in six polymers and percentage of high-molecular-weight purity of each polymer. (1) $H_2C{=}CH^{14}CN$ in acrylonitrile-methylacrylate copolymer, 100%. (2) $H_2C{=}CH^{14}COOCH_3$ in acrylonitrile-methylacrylate copolymer, 100%. (3) $H_2{}^{14}C{=}{}^{14}CHCN$ in acrylonitrile-methylacrylate copolymer, 100%. (4) $H_2{}^{14}C{=}{}^{14}CHOOCH_3$ in acrylonitrile-methylacrylate copolymer, 100%. (5) $H_2C{=}CH^{14}CN$ in acrylonitrile-butadiene copolymer, 92%. (6) $H_2{}^{14}C{=}CHCH{=}CH_2$ in acrylonitrile-butadiene copolymer, and showing one randomly labeled carbon atom, 96%. For Barex-210: $n_1 = 93\%$, $n_2 = 7\%$, polymerized by block grafting of n_1 to n_2.*

Microorganisms. *Penicillium notatum* isolated from soil was used for a series of long-term studies. For more defined studies, *N. rhodochrous* LL100-21 (2) was used. Four-day vegetative cultures were used as inocula in all experiments. Five percent (vol/vol) inoculum volumes were used.

Composition of growth media. *Penicillium* nutrient medium contained (per liter): KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 22.5 mg; $CaCl_2$, 27.5 mg; $FeCl_3 \cdot 7H_2O$, 0.25 mg; sucrose, 1 g; glucose, 1 g; NH_4NO_3, 1 g; pH 6.0. The medium was sterilized by autoclaving.

Basal salt broth contained (per liter): NaCl, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; K_2HPO_4, 1.03 g; KH_2PO_4, 0.75 g; $CaCl_2$, 25 mg; ethylenediaminetetraacetic acid, 15 mg; $ZnSO_4 \cdot 7H_2O$, 6.7 mg; $MnSO_4 \cdot 4H_2O$, 1.7 mg; $FeSO_4 \cdot 7H_2O$, 1.5 mg; $CoCl_2 \cdot 6H_2O$, 0.49 mg; $CuSO_4$, 0.47 mg; $NaMoO_4 \cdot 2H_2O$, 0.45 mg; pH 7.0. The $MgSO_4 \cdot 7H_2O$ and $CaCl_2$ were autoclaved separately and added aseptically to the autoclaved medium. This medium was used for maintenance and growth studies of *N. rhodochrous*, with acetonitrile (1%, vol/vol) as the source of carbon and nitrogen (2).

Growth procedures. Incubations were done in a temperature (30 ± 1°C)- and humidity (60% relative)-controlled room. Erlenmeyer flasks (250 ml) containing 50 ml of growth medium and 200 mg of ^{14}C-polymer were fitted with gas traps and either incubated on a gyratory shaker (model G2, New Brunswick Scientific Co., Inc., Edison, N.J.) (170 strokes/min) or mixed by the use of a stirring bar on a stirrer housed in the incubator. Expired $^{14}CO_2$ from samples was collected in a phenethylamine trap by continuous flushing with compressed air previously scrubbed through a 5 N KOH trap.

Harvesting procedures and sample preparation. The entire flask contents were transferred after appropriate growth periods to a centrifuge tube and centrifuged at 2,000 × *g* for 1 h. The volume of supernatant liquid was measured, and a portion was counted for ^{14}C activity in Bray (1) cocktail. The remaining volume was transferred into a flask and freeze-dried (model B64 freeze dryer, New Brunswick Scientific Co., Inc.). The residue in the centrifuge tube was transferred into another flask and freeze-dried. These samples were then extracted with DMF as outlined below.

Analytical procedures. A 20- to 30-mg portion of freeze-dried sample was extracted five times with 20 ml of DMF in a screw-cap 22-ml glass vial containing a stirring bar (0.5 by 1 cm) to provide mixing. Five consecutive extractions were performed for alternating periods of 7, 16, 7, 17, and 7 h. After each extraction, the vial was centrifuged at 500 × *g* for 15 min, and the DMF layer was removed. The DMF extracts were pooled and counted for ^{14}C activity in Bray (1) cocktail. Extraction of freshly made mixtures of polymer and *N. rhodochrous* showed that more than 97% of polymer was extracted by this procedure. Confirmation of complete polymer extraction was indicated by infrared spectroscopy (model 257 infrared spectrophotometer, The Perkin-Elmer Corp., Norwalk, Conn.) (Fig. 2A, C). Based on radioassay, less than 3% (wt/wt) of polymer remained in the microbial residue. An in-

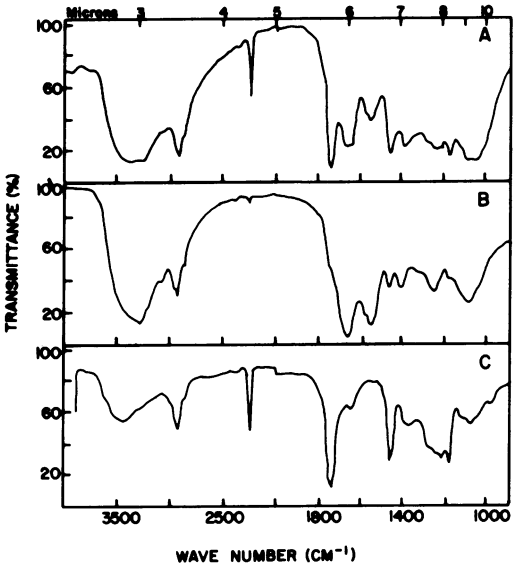

FIG. 2. *Infrared spectra of polymer and microorganism mixtures before and after DMF extraction. (A) 1 mg of Barex-210 ([14C-polymer 6) plus 2 mg of freeze-dried N. rhodochrous (or P. notatum) before DMF extraction. (B) Freeze-dried residue remaining after DMF extraction of the same polymer-microorganism mixture. The same spectrum was obtained for the DMF-nonextractable material after DMF extraction of freeze-dried centrifuged pellets of polymer incubated for 9 months with either organism. (C) Freeze-dried DMF extract of same polymer-microorganism mixture. —C≡N peak at 4.55 μm; —COOCH3 peaks at 5.77 and 6.8 μm. Samples were mixed with 100 mg of KBr, pelleted, and analyzed.*

frared spectrum indicated the lack of nitrile groups (4.6 μm) and methylacrylate groups (5.8 and 6.8 μm) in these residues (Fig. 2B). About 4% (wt/wt) of the microbial mass was also extracted in DMF by the above procedure. A control sample (a sample of polymer incubated in uninoculated growth medium) was routinely tested for nonbiological changes in each experiment.

The residue remaining in the vial was suspended in 1 ml of water and processed in a biological oxidizer (Harvey Instrument Co., Paterson, N.J.). In this process, the ^{14}C-labeled residues were catalytically oxidized at 900°C in an atmosphere of O_2 to $^{14}CO_2$, which was then trapped by flushing with O_2 through 15 ml of CO_2-trapping phosphor solution containing (per liter): 2,5-diphenyloxazole (PPO), 6 g; 1,4-bis-[2]-(5-phenyloxazolyl)benzene (POPOP), 0.2 g; methoxyethanol, 350 ml; toluene, 400 ml; and phenylethylamine, 250 ml.

The activity obtained from scintillation counting (model 3380, Packard Instrument Co., Inc., Rockville, Md.) was corrected for counting efficiency as determined by automatic external standardization and the use of a previously prepared quench curve. The percentage of ^{14}C recovery from the biological oxidizer was determined from each set of assay by use of standards.

After radioassay of the DMF extracts, selected extracts were concentrated and subjected to column gel filtration chromatography. Sepharose R CL-6B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was used in a glass column (1.2 by 90 cm, 73 cm high), and 100% DMF was the eluting solvent. A portion of DMF-extracted sample equivalent to 1 to 5 mg of polymer was normally chromatographed; 4-ml fractions were eluted and counted for ^{14}C activity. Recovery of radiolabeled ^{14}C was about 94% from this column.

RESULTS

Displacement of ^{14}C label from different segments of the polymer. The conversions to DMF-insoluble polymer after incubation with *N. rhodochrous* are shown in Fig. 3. The expired $^{14}CO_2$ from each polymer-microorganism incubation mixture is presented in Fig. 4. When the medium was enriched to support extensive growth of *N. rhodochrous*, the microorganism could alter the chemical nature of the polymer [^{14}C]Barex-210. Some carbon was released as $^{14}CO_2$ from all of the labeled polymers, but most label remained in DMF-extractable or -nonextractable material. Varying quantities of ^{14}C were transformed from different segments of the polymer. Growth of *N. rhodochrous* for 1 week to 9 months (Fig. 3 and 4) showed that the microorganism primarily transformed the elastomeric (acrylonitrile-butadiene) part of ^{14}C-polymer 5 or 6. The major portion (acrylonitrile-

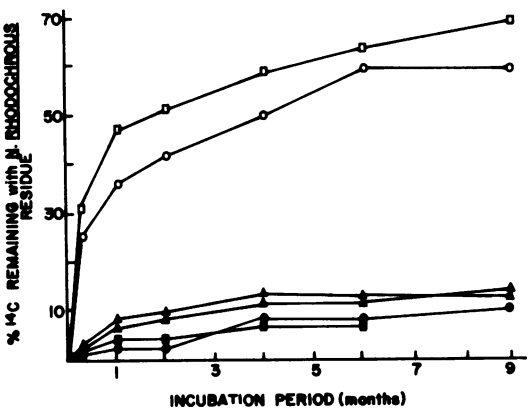

FIG. 3. *Percentage of ^{14}C remaining with the pelleted fraction after incubation of N. rhodochrous with 200 mg of [^{14}C]Barex-210 and extraction with DMF. Experiment was that shown in Fig. 4, although incubation of one set of growth flasks was continued for an additional 3 months. The entire contents of a single flask was analyzed after gas sampling. Symbols for ^{14}C-polymers: (△) 1; (▲) 2; (●) 3; (■) 4; (□) 5; (○) 6. Refer to Fig. 1 for the position of the ^{14}C label in each polymer.*

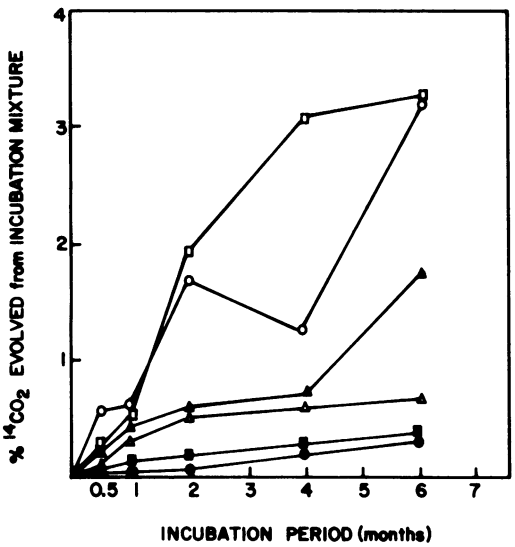

FIG. 4. *Percentage of $^{14}CO_2$ evolved during the incubation of N. rhodochrous with 200 mg of [^{14}C]-Barex-210 in basal medium. Symbols are as in Fig. 3. Experiment was that shown in Fig. 3. Refer to Fig. 1 for the position of the ^{14}C label in each polymer.*

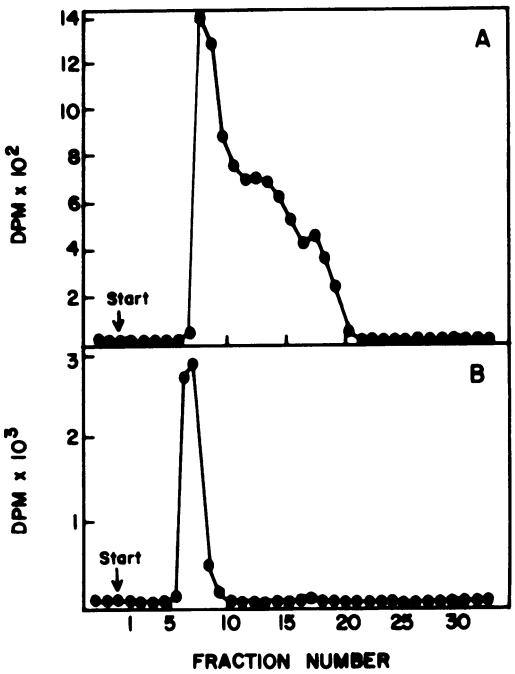

FIG. 5. *Gel filtration column chromatography of DMF-extracted [^{14}C]Barex-210 (^{14}C-polymer 1). (A) 200 mg of polymer incubated for 6 months with P. notatum in fungal medium; (B) 200 mg of polymer incubated alone for 6 months or with P. notatum for 1 day. Similar results were found with N. rhodochrous. Less than 1% low-molecular-weight polymer was present in the control sample.*

methylacrylate) of the polymer, which constitutes 93% (wt/wt) of the weight of polymer, was utilized less extensively, with 2 to 15% transformation from side-chain functional groups, $^{14}C{\equiv}N$ and $^{14}COOCH_3$ (^{14}C-polymers 1 and 2) and 2 to 8% transformation from backbone carbons (^{14}C-polymers 3 and 4).

A comparison of the DMF-extractable material for polymer incubated with or without *P. notatum* was made, using column gel filtration chromatography (Fig. 5). Extracted polymer that had been incubated with *P. notatum* for a period of 9 months showed the presence of lower-molecular-weight (about 1,000 to 100,000) compounds retaining the functional groups of $-C{\equiv}N$ and $-COOCH_3$, as indicated by infrared spectroscopy. By radioassay, 8% of the polymer remained with the microbial residue as DMF-nonextractable material which was nearly devoid of $-C{\equiv}N$ and $-COOCH_3$ peaks (Fig. 2B). The presence of lower-molecular-weight polymers supports the finding that a small fraction of the carbon-carbon bonds of the backbone chain are being broken. No significant change (less than 1%) occurred in the structure of the polymer extracted from the uninoculated control or from a 1-day incubation mixture of *P. notatum* plus polymer.

DISCUSSION

Acrylonitrile-methylacrylate-butadiene terpolymer (Barex-210) in a powdered form was resistant to alteration by microorganisms when it was the sole source of carbon or nitrogen. Incubation of this polymer with microorganisms in an enriched medium, however, resulted in polymeric changes. The amount of polymeric transformation increased with increased incubation time. Displacement of ^{14}C differed according to the position of the ^{14}C label in each segment of polymer. Although a powdered form of the polymer was used, the polymer was resistant, but not impervious to microbial attack. It is thus possible that bioconversion may result to a limited degree if this polymer is added to landfills or disposal systems or is otherwise subjected to microbiological contact in the natural environment.

Despite the microbially related changes in the polymer, actual metabolism of structural carbon (as ^{14}C) by *N. rhodochrous* or *P. notatum* could not be completely established. Had ^{14}C been utilized by the microorganisms, it would have been used either for the production of energy with the subsequent release of $^{14}CO_2$ or for the synthesis of microbial material. The data on the release of $^{14}CO_2$ from medium containing poly-

mer plus microorganism (Fig. 4) showed that only 0.3 to 3.3% of ^{14}C in the polymer was released as $^{14}CO_2$. Although above experimental error, these values represent only a relatively small amount of expired CO_2. Such amounts may arise from residual radiochemical impurities in the original batches of the polymer or can be considered to be a very limited metabolism of the polymer by the microorganism. Although the DMF-nonextractable material retained the ^{14}C label, the nonsolubility of this material and the disappearance of the —C≡N and —$COOCH_3$ peaks indicates a significant chemical change in the polymer.

During the incubation periods of from 1 week to 9 months, using *N. rhodochrous*, 2 to 15% of the ^{14}C was transformed to DMF-nonextractable material from the acrylonitrile-methylacrylate copolymer segment of the polymer, and 20 to 70% from the acrylonitrile-butadiene copolymer segment was transformed. Since the release of 0.5 to 3% $^{14}CO_2$ was not proportional to these changes, it is clear that added nutrients provided the main source of energy for growth. It is possible that during the incubation period the polymer was subjected to microbial enzymes, e.g., oxidases (3), creating cross-linkages in the butadiene fraction, which rendered the polymer insoluble in DMF without direct utilization by the microorganism. Both extra- and intracellular enzymes released during cell lysis could play a role in the chemical changes of the polymer.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Bray, G. A.** 1960. A simple efficient scintillation for aqueous solution in a liquid scintillation counter. Anal. Biochem. **1**:279–285.
2. **DiGeronimo, M. J., and A. D. Antoine.** 1976. Metabolism of acetonitrile and propionitrile by *Nocardia rhodochrous* LL100-21. Appl. Environ. Microbiol. **31**:900–906.
3. **Fonken, G. S., and R. A. Johnson.** 1972. Chemical oxidations with microorganisms. Marcel Dekker Inc., New York.
4. **Henahan, J. F.** 1971. James D. Idol, Jr. Setting the world of nitrile chemistry afire. Chem. Eng. News **49**:16–18.
5. **Hook, R. H., and W. G. Robinson.** 1964. Ricinine nitrilase. II. Purification and properties. J. Biol. Chem. **239**: 4263–4267.
6. **Jones, G. B., and T. LeCampoin-Alsumard.** 1970. Biodegradability of polyurethanes by marine fungi. Int. Biodeterior. Bull. **6**:1–10.
7. **Klausmeier, R.** 1966. The effect of extraneous nutrients on biodeterioration of plastics, p. 232–243. *In* S. C. I. Monograph no. 23, Microbiological deterioration in the tropics.
8. **Katz, S. E., and C. A. Fassbender.** 1968. System for determination of the potential degradability of urea-aldehyde condensates used as fertilizers. J. Assoc. Off. Anal. Chem. **14**:975–980.
9. **Katz, S. E., and C. A. Fassbender.** 1968. Method for evaluating the potential availability of water-insoluble nitrogen source in mixed fertilizers. J. Assoc. Off. Anal. Chem. **15**:1286–1290.
10. **Klausmeier, R., and W. A. Jones.** 1961. Microbial degradation of plasticizers. Dev. Ind. Microbiol. **2**:47–53.
11. **Robinson, W. G., and R. H. Hook.** 1964. Ricinine nitrilase. I. Reaction product and substrate specificity. J. Biol. Chem. **239**:4257–4262.
12. **Salame, M., and S. P. Nemphos.** 1973. The nature and properties of high acrylonitrile copolymer resins, p. 21–28. *In* Proceedings of the Symposium on Environmental Impact of Nitrile Barrier Containers. Monsanto Co., St. Louis.
13. **Wendt, T. M., A. M. Kaplan, and M. Greenberger.** 1970. Weight loss as a method for estimating the microbial deterioration of PVC film in soil fungi. Int. Biodeterior. Bull. **6**:137–143.