Blumberg, W. R., Peisach, J., *Ann. N.Y. Acad. Sci.* **222**, 539 (1973).

Crawford, R. L., *Appl. Microbiol.* **30**, 439 (1975a).

Crawford, R. L., *J. Bacteriol.* **121**, 531 (1975b).

Dagley, S., Geary, P. J., Wood, J. M., *Biochem. J.* **109**, 559 (1968).

Dagley, S., *Am. Sci.* **63**, 681 (1975).

Dagley, S., "Essays in Biochemistry", Vol. 12, Campbell, P. N., Ed., Biochemical Society, London, 1976, pp 81–137.

Debrunner, P. G., Münck, E., Que, L., in "Iron-Sulfur Proteins", Vol. III, Lovenberg, W. E., Ed., Academic Press, New York, N.Y., in press (1977).

Durand, R., Zenk, M. H., *FEBS Lett.* **39**, 218 (1974).

Flamm, W. G., Crandall, D. I., *J. Biol. Chem.* **238**, 389 (1963).

Fujisawa, H., Hayaishi, O., *J. Biol. Chem.* **243**, 2673 (1968).

Fujisawa, H., Uyeda, M., Koiima, Y., Nozaki, M., Hayaishi, O., *J. Biol. Chem.* **247**, 4414 (1972).

Fujisawa, H., Hiromi, K., Uyeda, M., Nozaki, M., Hayaishi, O., *J. Biol. Chem.* **247**, 4422 (1972).

Gaber, B. P., Miskowski, V., Spiro, T. G., *J. Am. Chem. Soc.* **96**, 6868 (1974).

Grinstead, R. R., *Biochemistry* **3**, 1308 (1964).

Hayaishi, O., Hashimoto, Z., *J. Biochem.* (*Tokyo*) **37**, 371 (1950).

Hesp, B., Calvin, M., Hosokawa, K., *J. Biol. Chem.* **244**, 5644 (1969).

Hendrickson, W. A., Klippenstein, G. L., Ward, K. B., *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2160 (1975).

Hopper, D. J., Chapman, P. J., *Biochem. J.* **122**, 19 (1970).

Howell, L. G., Spector, T., Massey, V., *J. Biol. Chem.* **247**, 4340 (1972).

Jensen, L. H., in "Iron-Sulfur Proteins", Vol. II, Lovenberg, W. E., Ed., Academic Press, New York, N.Y., 1973, Chapter 4.

Kita, H., *J. Biochem.* (*Tokyo*) **58**, 116 (1965).

Koch, S., Holm, R. H., Frankel, R. B., *J. Am. Chem. Soc.* **97**, 6714 (1975).

Kojima, Y., Fujisawa, H., Nakazawa, A., Nakazawa, T., Konetsona, F., Tanuchi, H., Nozaki, M., Hayaishi, O., *J. Biol. Chem.* **242**, 3270 (1967).

Lipscomb, J., Howard, J., Lorsbach, T., Wood, J. M., Federation Proceedings in Oxygenase Session (1976).

McCord, J. M., Kelle, B. B., Fridovich, I., *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1024 (1971).

Nakazawa, T., Nozaki, M., Hayaishi, O., Yamano, T., *J. Biol. Chem.* **243**, 119 (1969).

Nozaki, M., Kagamiyama, H., Hayaishi, O., *Biochem. Biophys. Res. Commun.* **11**, 65 (1963).

Nozaki, M., Ono, K., Nakazawa, T., Kotoni, S., Hayaishi, O. *J. Biol. Chem.* **243**, 2682 (1968).

Nozaki, M., Kotoni, S., Ono, K., Senoh, S., *Biochim. Biophys. Acta* **220**, 213 (1970).

Nozaki, M., "Non-heme Iron Dioxygenase in Molecular Mechanisms of Oxygen Activation", Haysishi, O., Ed., Academic Press, New York, N.Y., 1974, pp 142–144, Chapter 4.

Ohta, Y., Ribbons, D. W., *FEBS Lett.* **11**, 189 (1970).

Okamura, M. Y., Klotz, I. M., in "Inorganic Biochemistry", Eichhorn, G. L., Ed., Elsevier, Amsterdam, 1973, Chapter 11.

Oosterhuis, W. T., *Struct. Bonding* (*Berlin*) **20**, 59 (1974).

Que, L., Jr., Lipscomb, J. D., Zimmerman, R., Münck, E., Orme-Johnson, N. R., Orme-Johnson, W. H., *Biochim. Biophys. Acta* **452**, 320 (1976).

Rao, K. K., Evans, M. C. W., Cammack, R., Hall, D. O., Thompson, C. L., Jackson, P. J., Johnson, C. E., *Biochem. J.* **129**, 1063 (1972).

Schultz, E., Engle, F. E., Wood, J. M., *Biochemistry* **13**, 1768 (1974).

Schultz, E., Wood, J. M., *Biochim. Biophys. Acta*, in press (1977).

Stenkamp, R. E., Sieker, L. C., Jensen, L. H., *Proc. Natl. Acad. Sci. U.S.A.* **73**, 349 (1976).

Strickland, S., Massey, V., *J. Biol. Chem.* **248**, 2944 (1973).

Takemori, S., Yasau, H., Mihara, K., Suzuki, K., Katagiri, M., *Biochim. Biophys. Acta* **191**, 58 (1969).

Takemori, S., Komiyama, T., Katagiri, M., *Eur. J. Biochem.* **23**, 178 (1971).

White-Stevens, R. H., Kamin, H., *J. Biol. Chem.* **247**, 2358 (1972).

Zabinski, R. M., Münck, E., Champion, P. M., Wood, J. M., *Biochemistry* **11**, 3212 (1972).

# Biodegradation of Natural and Man-Made Recalcitrant Compounds with Particular Reference to Lignin

Ronald L. Crawford,[*] Don L. Crawford, Christina Olofsson, Loulou Wikstrom, and John M. Wood

Novel procedures are described for study of the biological decomposition of natural and man-made recalcitrant molecules. Six different lignocelluloses were prepared containing $^{14}C$ in primarily their lignin components. Degradation of these lignin-labeled lignocelluloses by the microfloras of soil and lake water was observed by monitoring evolution of $^{14}CO_2$ from incubation mixtures. The standard most-probable-number technique for enumeration of specific microbial groups within natural habitats was adapted for the enumeration of degraders of recalcitrant, insoluble, or other organic compounds. Evolution of $^{14}CO_2$ from $^{14}C$-labeled substrates was shown to be an acceptable, readily recognizable transformation for use in scoring dilution replicates positive or negative for degradation of a specified compound. Use of this radioisotopic modification of the most-probable-number technique allowed enumeration of lignin degraders, 2,4-D (2,4-dichlorophenoxyacetate) degraders, and hexachlorobiphenyl degraders in several soil and water environments.

There are many chemical structures in the biosphere that are to various degrees resistant to microbiological

[1]Freshwater Biological Institute, University of Minnesota, Navarre, Minnesota 55392 (R.L.C., J.M.W.), the University of Idaho, Department of Bacteriology and Biochemistry, Moscow, Idaho 83843 (D.L.C.), and the Royal Institute of Technology, Stockholm, Sweden (C.O., L.W.).

degradation in soil and water. Such compounds are often identified as being "recalcitrant" (Alexander, 1965a,b, 1967, 1975). These biologically resistant molecules may enter the biosphere either through the activities of industrial societies or via natural, biological processes (Alexander, 1975).

Probably the most common slowly decomposed, natural compound on the earth is the plant polymer lignin. Lignin is a structural polymer found in all vascular plants where it performs numerous, essential physiological functions

Table I. Media Compositions for Most-Probable-Number Determinations

| Medium component | Quantity per flask or tube for medium no. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Basal, mineral-salts medium[a] | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL |
| Yeast extract | | | | | | | 2 mg | 2 mg | 2 mg | 2 mg |
| Glucose | 20 mg | | | 20 mg | | | | | | |
| Benzoic acid | | 20 mg | | | 20 mg | | | | | |
| L-Tyrosine | | | 20 mg | | | 20 mg | | | | |
| L-[U-$^{14}$C]Tyrosine | | | | | | 0.1 µCi | | | | |
| D-[U-$^{14}$C]Glucose | | | | 0.1 µCi | | | | | | |
| [U-$^{14}$C]Hexachlorobiphenyl | | | | | | | 0.2 µCi | | | |
| [COOH-$^{14}$C]Benzoic acid | | | | | 0.1 µCi | | | | | |
| [2,side chain-$^{14}$C]-2,4-Dichlorophenoxyacetic acid | | | | | | | | 0.1 µCi | | |
| Maple [$^{14}$C]lignocellulose[b] | | | | | | | | | 2 mg | |
| Cattail [$^{14}$C]lignocellulose[c] | | | | | | | | | | 2 mg |

[a] See Crawford (1975). [b] 3800 dpm. [c] 6000 dpm.

(Sarkanen and Ludwig, 1971). Lignin gives plants rigidity, binding plant cells together so as to impart resistance toward impact, compression, and bending. Lignin acts through its recalcitrance as a barrier to prevent invasion of plant tissues by pathogenic microbes. It also acts as a barrier to permeation of water across cell walls of xylem tissue. Lignin is a vital component of the biospheric carbon cycle since it ranks second only to the celluloses in abundance as a naturally occurring biopolymer (Harkin, 1967). Even under laboratory conditions lignin biodegradation proceeds at a relatively slow rate in soil and water. Extensive mineralization of lignins added to soil may require several months, as was recently confirmed using novel, radioisotopic assay procedures (Crawford and Crawford, 1976; Kirk et al., 1975).

Of the many man-made, biologically resistant molecules that enter natural environments, chlorinated aromatic hydrocarbons have caused particular concern. Compounds such as DDT ($p,p'$-dichloro-2,2-diphenyl-1,1,1-trichloroethane) and the polychlorinated biphenyls (PCB's) persist in the environment for decades where they accumulate in food webs, often producing lethal effects in animals or making natural food resources such as fish unsafe for human consumption (Alexander, 1975).

It is thus important that the methodology available for study of the biodegradation of recalcitrant molecules such as lignin and the PCB's be improved. For example, the single most important problem that has restricted progress in the study of lignin biodegradation during the past half-century has been weaknesses in available methodology (Kirk et al., 1975; Kirk, 1971). Until very recently there was not even available an unequivocal assay for determining the rate and extent of lignin degradation by mixed populations or pure cultures of microorganisms (Crawford and Crawford, 1976; Kirk et al., 1975). Similar methodological problems have often restricted study of biodegradation of recalcitrant, man-made compounds, though probably not to the same extent as for lignin biodegradation.

In our laboratory we have been attacking these problems of insufficient methodology. In this paper some of the progress we feel we have made during the past year in improving the available methodology for studying the biodegradation of recalcitrant molecules within natural environments will be discussed.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of [lignin-$^{14}$C]-Lignocelluloses. The lignin components of various lignocelluloses were selectively labeled with $^{14}$C by feeding plants aqueous solutions L-[U-$^{14}$C]phenylalanine through their cut stems (Crawford and Crawford, 1976; Brown and Neish, 1955; Higuchi et al., 1967). A small limb from a hardwood or softwood (approximate diameter, 1.5 cm) typically received 50 µCi of radioactive amino acid (<0.1 µmol of phenylalanine) dissolved in 2 mL of 0.01 M phosphate buffer (pH 6.8). Labeled lignocelluloses were freed of extractives and milled to 40 mesh as previously described (Crawford and Crawford, 1976). Extractive-free lignocelluloses were analyzed for distribution of $^{14}$C by a modified Klason fractionation procedure (Moore and Johnson, 1967; Pearl, 1967). Klason lignin is the water-insoluble material remaining after digestion of lignified tissue with cold 72% $H_2SO_4$, dilution, and refluxing with dilute acid (Pearl, 1967).

Counting of Radioactivity. $^{14}$C was trapped and quantified as previously described (Crawford and Crawford, 1976).

Biodegradation of [lignin-$^{14}$C]Lignocelluloses. Soil and water samples were incubated with labeled substrates in sterile flasks equipped with sealed ports for periodic aeration and flushing of the gaseous phase, as described by Crawford and Crawford (1976).

Most-Probable-Number Determinations. Most-probable-number (MPN) determinations based upon observations of turbidity in a defined growth medium were performed essentially as described by Alexander (1965b). MPN determinations based upon $^{14}CO_2$ evolution as the recognizable transformation were also performed essentially by the procedure of Alexander, with the following modifications. Erlenmeyer flasks (50 mL) replaced culture tubes. Each flask received 20 mL of basal medium (14; Table I) containing 0.01% (w/v) yeast extract and a $^{14}$C-labeled substrate (Table I). The flasks were autoclaved, cooled, and plugged with sterile serum caps. As with turbidimetric MPN's, tenfold dilutions were used as inocula with five replicates per dilution (Alexander, 1965b). The 10° soil dilutions were prepared by suspending 10 g of moist soil in 90 mL of sterile water. Final MPN's were corrected to represent organisms per milliliter of water or gram dry weight of soil. After inoculation from dilutions using sterile syringes, flasks were incubated at 30 °C while still capped. After the desired incubation period (1–3 weeks), the gaseous phase within each flask was flushed through a $^{14}CO_2$ trapping/counting solution (Crawford and Crawford, 1976) and the percent of added $^{14}$C recovered as $^{14}CO_2$ determined. Control flasks were identical with experimental flasks except that they remained uninoculated. Flasks showing evolution of $^{14}CO_2$ significantly greater than that from control flasks were scored as positives for degradation of the particular $^{14}$C-labeled substrate added to the growth medium, except that with

Table II. Distribution of $^{14}$C in Various [*lignin*-$^{14}$C] Lignocelluloses

| Plant | dpm/mg | % dpm in Klason lignin | % dpm acid soluble | % dpm recovered |
|---|---|---|---|---|
| Hemlock (*Tsuga canadensis*) | 6500 | 52 | 36 | 88 |
| Virginia Pine (*Pinus virginianum*) | 4400 | 56 | 35 | 91 |
| Red Cedar (*Juniperus virginiana*) | 6000 | 42 | 32 | 74 |
| White Oak (*Quercus albus*) | 6900 | 45 | 40 | 85 |
| Red Maple (*Acer rubrum*) | 1900 | 70 | 26 | 96 |
| Black Gum (*Nyssa sylvatica*) | 6000 | 48 | 46 | 94 |
| Cattail (*Typha latifola*) | 3000 | 93 | 2 | 95 |

[*lignin*-$^{14}$C]lignocelluloses 2% recovery of $^{14}$C as $^{14}$CO$_2$ was established as the minimum recovery before ascribing lignin degrading ability to any particular flask (Crawford and Crawford, 1976). To prevent oxygen depletion over the more extended incubation periods, flasks were flushed at weekly intervals with sterile, CO$_2$-free air (Crawford and Crawford, 1976) and their incubation continued while collecting $^{14}$CO$_2$ evolution data in a cumulative manner. The table of Cochran (1950) as reported by Alexander (1965b) was used to calculate MPN values.

RESULTS AND DISCUSSION

Previously we reported a new procedure for the study of lignin biodegradation by pure or mixed cultures of microorganisms (Crawford and Crawford, 1976). Natural lignocelluloses were prepared containing $^{14}$C in primarily their lignin components, and lignin degradation was observed in numerous soils by monitoring evolution of $^{14}$CO$_2$ from these [*lignin*-$^{14}$C]lignocelluloses. In experiments detailed previously we used $^{14}$C-labeled oak, maple, and cattail. Here we report preparation and biodegradation studies of [*lignin*-$^{14}$C]lignocelluloses from a number of additional species of trees, including both hardwoods and softwoods. Table II lists our labeled woods and presents data concerning distribution of $^{14}$C within the labeled lignocellulose complexes.

As in our previous study (Crawford and Crawford, 1976), in all cases examined here (Table II) the majority of $^{14}$C incorporated into the plant tissues resides in the acid-insoluble (Klason lignin) components of the lignocelluloses. The values shown here for percent of label located in the acid-soluble wood components are higher than those reported previously (Crawford and Crawford, 1976), particularly for white oak and red maple. These higher values probably result from the relatively high and variable content of these plants of acid-soluble lignin (Musha and Goring, 1974; Casey, 1970). For example, Migita and Kawamura (1944) reported that the 72% sulfuric acid soluble lignin varied between 5 and 20% of the Klason lignin for tropical hardwoods and between 25 and 60% for temperate hardwoods. Casey (1970) reported 72% sulfuric acid soluble lignins as high as 43–79% (% total lignin) for certain hardwood species. These observations once again point out the drawbacks of the Klason procedure, which gives at best only a rough estimate of lignin content. Support for the location of greater than 90% of the $^{14}$C in the lignin component of these lignocelluloses is readily obtained by showing that lignin-degrading organisms such as the white-rot fungus *Polyporus versicolor* readily evolve large recoveries (60–70% of incorporated $^{14}$C) of $^{14}$CO$_2$ from our [$^{14}$C]lignocelluloses, while highly cellulolytic organisms such as the actinomycete *Thermomonospora fusca* (Crawford and Crawford, 1976; Crawford, 1974) which cannot degrade lignin grow well on the labeled material but do not catalyze evolution of significant amounts (<2% recovery) of $^{14}$CO$_2$ (Crawford and Crawford, 1976).

Figure 1 presents data concerning the degradation of six different [*lignin*-$^{14}$C]lignocelluloses by the microflora of
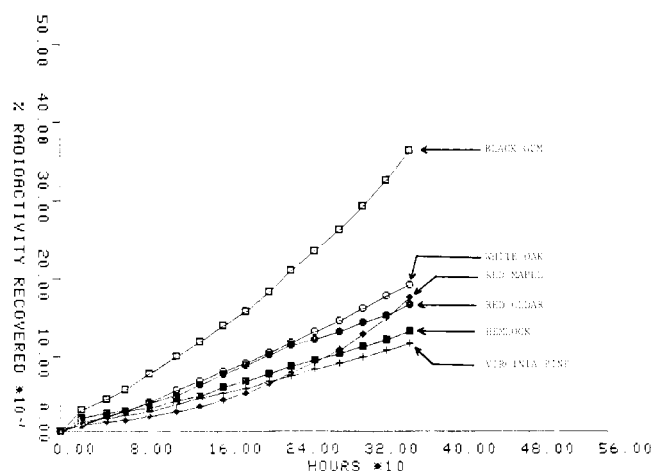


Figure 1. Degradation of [*lignin*-$^{14}$C]lignocelluloses by the microflora of a forest soil taken from an oak/maple woods.
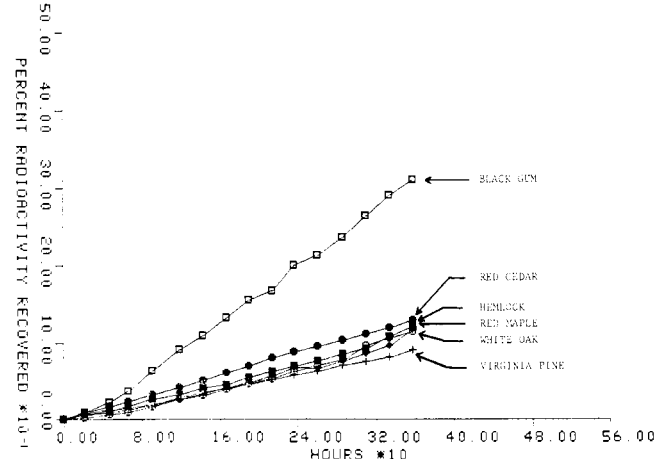


Figure 2. Degradation of [*lignin*-$^{14}$C]lignocelluloses by the microflora of a forest soil taken from a virgin hemlock stand.

a soil humus fraction taken from beneath a maple/oak forest in the Shenandoah National Park, Va. Figure 2 summarizes data generated from a similar experiment using soil from the $A_0$ horizon of a virgin hemlock stand in the same park. As observed previously (Crawford and Crawford, 1976; Kirk et al., 1975) lignin degradation proceeds at a relatively slow rate in these temperate, forest-floor soils. These types of experiments again point out the promising potential for using [*lignin*-$^{14}$C]lignocelluloses to study the cycling of lignin carbon in particular environments such as the forest ecosystem.

In order to enrich and isolate microorganisms that are able to degrade recalcitrant molecules it is desirable to identify those habitats which harbor the highest numbers of the desired organisms. Thus, we have adapted the standard MPN procedure (Alexander, 1965b) so that it may be used to enumerate degraders of recalcitrant, insoluble compounds such as lignin and hexachlorobiphenyl. An absolute requirement of the MPN procedure is that

Table III. Most-Probable-Numbers of Microorganisms as Determined by Turbidity and $^{14}CO_2$ Evolution

| Substrate | Medium[a] no. $^{14}CO_2$ | Turbidity | Inoculum | MPN turbidity[d] | MPN $^{14}CO_2$ evolution |
|-----------|------|-----------|----------|------------------|--------------|
| Glucose | 4 | 1 | Lake water[b] | $2.3 \times 10$ | $3.3 \times 10$ |
| Benzoic acid | 5 | 2 | Lake water[b] | 1.0 | 2.0 |
| Tyrosine | 6 | 3 | Lake water[b] | 4.9 | 2.3 |
| Glucose | 4 | 1 | Soil[c] | $2.3 \times 10^6$ | $2.4 \times 10^6$ |

[a] See Table I. [b] Lafayette Bay, Lake Minnetonka, Minn. [c] Marsh-edge soil. [d] MPN per mL of water or g of dry soil.

Table IV. Data Used to Calculate MPN of Maple Lignin Degraders in a Forest Soil ($A_0$ Horizon)[a]

| Dilution | % $^{14}C$ recovered as $^{14}CO_2$ for each replicate after 1 week incubation | | | | |
|----------|------|------|------|------|------|
| $10^0$ | 4.7 | 6.3 | 4.2 | 2.9 | 4.2 |
| $10^1$ | 3.8 | 2.9 | 5.5 | 2.1 | 3.7 |
| $10^2$ | 4.6 | 4.9 | 4.1 | 2.6 | 2.0 |
| $10^3$ | 2.6 | 3.5 | 3.0 | 2.4 | 2.6 |
| $10^4$ | 1.2 | 1.2 | 1.3 | 2.1 | 2.1 |
| $10^5$ | 0.8 | 1.0 | 1.3 | 0.7 | 0.8 |
| Control | 0.35 | 0.01 | | | |

[a] Minimum value for scoring positive = 2.0%; incubation temperature = 30 °C; MPN = $6.22 \times 10^4$ lignin degraders-/g of dry soil. $^{14}C$ was trapped and quantified as previously described (Crawford and Crawford, 1976). Vials were counted for 20 min. Counting efficiency was approximately 90%. The soil used in this experiment contained approximately 21.2% water on a weight basis.

Table V. MPN of Lignin Degraders as Determined by $^{14}CO_2$ Evolution from [$lignin$-$^{14}C$] Lignocellulose

| Substrate[a] | Inoculum | MPN[b] | Medium[e] |
|-----------|----------|--------|--------|
| Maple lignin | Lake water[d] | 7.9 | 9 |
| Cattail lignin | Lake water[d] | $7.0 \times 10$ | 10 |
| Maple lignin | Marsh water | $4.9 \times 10$ | 9 |
| Maple lignin | Forest soil | $6.2 \times 10^4$ | 9 |
| Cattail lignin | Marsh edge soil[c] | $>2.0 \times 10^6$ | 10 |

[a] 2 mg of [$lignin$-$^{14}C$]lignocellulose/20 mL of basal medium. [b] 2% recovery scored positive; MPN/g of dry soil or mL of water. [c] Too few dilutions. [d] Lafayette Bay, Lake Minnetonka, Minn. [e] See Table I.

Table VI. MPN of 2,4-Dichlorophenoxyacetic Acid and Hexachlorobiphenyl Degraders in Surface Waters of Lake Itasca, Minn. and a Marsh-Edge Soil

| Substrate | Medium[a] | MPN[b] | Inoculum |
|-----------|------|--------|----------|
| 2,4-D | 8 | 70 | Lake Itasca[c] |
| Hexachlorobiphenyl | 7 | 0 | Lake Itasca[c] |
| 2,4-D | 8 | 140 | Marsh-edge soil |
| Hexachlorobiphenyl | 7 | 0 | Marsh-edge soil |

[a] See Table I. [b] MPN by $^{14}CO_2$ procedure; per g of dry soil or mL of water. [c] Surface sample (0 m).

there be a readily recognizable transformation of a specific growth medium in order to enumerate any particular group of microorganisms following their "extinction dilution". For example, if one wishes to enumerate organisms that are able to grow on glucose as a sole source of carbon and energy he may use as a readily recognizable transformation the development of turbidity in a defined medium containing only inorganic salts and glucose. Since complete, aerobic decomposition of organic compounds by microorganisms yields cell mass, $H_2O$, and $CO_2$, it should be possible to enumerate degraders of organic compounds by using evolution of $^{14}CO_2$ from a $^{14}C$-labeled substrate as the readily recognizable transformation. Table III summarizes data showing that one may indeed equate $^{14}CO_2$ evolution from a $^{14}C$-labeled compound with the ability of members of the microflora of soil or water to grow on that compound as sole source of carbon and energy. MPN's obtained by the standard turbidity procedure are almost identical with MPN's obtained using the same substrates except using $^{14}CO_2$ evolution instead of turbidity to score replicates positive or negative (Table III).

Since experiments with simple substrates such as glucose, benzoate, and tyrosine established that evolution of $^{14}CO_2$ from a $^{14}C$-labeled substrate is an acceptable, easily measured transformation for use in MPN determinations, we have used this technique to obtain MPN's for degraders of complex, recalcitrant molecules. Table IV summarizes data used to calculate a MPN for degraders of maple lignin in a forest soil. The MPN calculated from these data, when corrected for dilution factor and soil moisture, was $6.22 \times 10^4$ lignin-degrading organisms per gram of dry soil. Similarly obtained MPN's for other natural environments are presented in Table V.

As expected, aqueous environments which contain less lignocellulose than soils also contain fewer countable lignin degraders than do soils. The success of our preliminary experiments, reported here, indicates that our radioisotopic MPN procedure should be a useful tool for microbial ecologists who wish to study the distribution of lignin degraders within natural environments. The procedure should also be adaptable to other, less recalcitrant poly-

mers such as cellulose, chitin, and starch. Though not done in experiments reported here, it will be necessary in some instances to acidify MPN replicates prior to flushing and trapping of evolved $^{14}CO_2$, particularly if the MPN medium utilized has an alkaline pH.

Table VI summarizes results of MPN determinations for degraders of chlorinated, aromatic compounds in the surface waters of Lake Itasca, Minn. and a marsh-edge soil. The herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) has proven to be a relatively biodegradable, man-made compound (Alexander, 1965a, 1967, 1975); therefore, it comes as no surprise to find countable populations of 2,4-D degraders in soil and lake water. Hexachlorobiphenyl is a highly persistent, industrial chemical. In our very limited survey of soils and lake waters we have found few or no microorganisms able to convert [U-$^{14}C$]hexachlorobiphenyl to $^{14}CO_2$ in significant amounts. The preliminary experiments summarized in Table VI indicate that the $^{14}CO_2$ evolution adaptation of the MPN technique will be of value to investigators who desire to enumerate degraders of pesticides or industrial chemicals within natural habitats such as soil and water. As with all procedures that involve "viable counts" of microorganisms within natural environments, our modification of the MPN procedure should be used with caution so that the well-known liabilities of viable counting techniques in general are recognized (Schmidt, 1973). Experimental questions that utilize this procedure should be specific and supporting techniques should be utilized whenever possible.

ACKNOWLEDGMENT

and its research facilities available. In particular, we wish to thank Richard Gray, Sr., without whose efforts the Freshwater Biological Institute would not exist.

LITERATURE CITED

Alexander, M., *Adv. Appl. Microbiol.* **7**, 35–80 (1965a).
Alexander, M., in "Methods of Soil Analysis", Black, C. A., Ed., Part 2, American Society of Agronomy, Inc., Madison, Wisc., 1965b, pp 1467–1472.
Alexander, M., *New Sci.*, 439–440 (Aug 31, 1967).
Alexander, M., *Microb. Ecol.* **2**, 17–27 (1975).
Brown, S. A., Neish, A. C., *Nature (London)* **175**, 688–689 (1955).
Casey, J. M., M.S. Thesis, University of Washington, Seattle, 1970.
Cochran, W. C., *Biometrics* **6**, 105–116 (1950).
Crawford, D. L., *Can. J. Microbiol.* **20**, 1069–1072 (1974).
Crawford, R. L., *J. Bacteriol.* **121**, 531–536 (1975).
Crawford, R. L., Crawford, D. L., *Appl. Environ. Microbiol.* **31**, 714–717 (1976).
Harkin, J. M., in "Oxidative Coupling of Phenols", Battersby, A. R., and Taylor, A. I., Ed., Marcel Dekker, New York, N.Y., 1967, pp 243–321.
Higuchi, T., Ito, Y., Kawamura, I., *Phytopathology* **6**, 865–881 (1967).
Kirk, T. K., *Annu. Rev. Phytopathol.* **9**, 185–210 (1971).
Kirk, T. K., Connors, W. J., Bleam, R. D., Hackett, W. F., Zeikus, J. G., *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2515–2519 (1975).
Migita, N., Kawamura, I., *J. Agric. Chem. Soc. Jpn.* **20**, 348 (1944).
Moore, W. E., Johnson, D. B., "Procedures for the Analysis of Wood and Wood Products", U.S.D.A. Forest service, Forest Products Laboratory, Madison, Wisc., 1967.
Musha, Y., Goring, D. A. I., *Wood Sci.* **7**, 133–134 (1974).
Pearl, I. A., "The Chemistry of Lignin", Marcel Dekker, New York, N.Y., 1967.
Sarkanen, K. V., Ludwig, C. H., Ed., "Lignins: Occurrence, Formation, Structure, and Reactions", Wiley-Interscience, New York, N.Y., 1971.
Schmidt, E. L., *Bull. Ecol. Res. Commun. (Stockholm)* **17**, 453–454 (1973).

# Degradation of Insoluble Lignin by Chloride Monoxide

Henry I. Bolker,* Heather E. W. Rhodes, and Kuen Sing Lee

Moistened spruce cuoxam and periodate lignins, treated with $Cl_2O$ in $CCl_4$, were ring chlorinated, as deduced from infrared spectra, and became soluble in NaOH solution, $Me_2SO$, dioxane, and THF. The extent of solubility depended upon the quantity of $Cl_2O$ applied. Molecular weights (by sedimentation equilibrium) of the dissolved lignins were a function of the fraction dissolved. GPC on Styragel of the soluble chlorolignins showed broad, skewed distributions, each having a single maximum at the low molecular weight end. Similar fractions chromatographed on Sephadex G-50 exhibited bimodal chromatograms. The high molecular weight peak was demonstrated to be an artifact, and the distribution given by Styragel chromatography is probably correct. It is concluded that the original cuoxam lignin, and hence lignin in wood, was a three-dimensional, cross-linked, infinite network polymer gel.

Of all the polymers of nature, the plant polymer lignin, composed of phenylpropane building blocks arranged in complex patterns, has alone continued to resist substantial efforts to unravel the details of its molecular architecture and to elucidate the chemical processes by which it can be separated from its accompanying polysaccharides. Research on the delignification of plant tissue—a process of prime commercial importance—usually focusses either on the organic chemistry of the degradative reactions, or on the properties of the polymeric degradation products.

Among the latter investigations, McNaughton et al. (1967) have found that in delignification at 150–180 °C by means of a solution of sodium hydroxide and sodium sulfide (i.e., kraft pulping), the lignin dissolved early in the process has a low molecular weight. As the extraction proceeds, the molecular weight of the dissolved material becomes progressively higher. Similar behavior has been observed during the acidolysis of sprucewood in methanol (Kosikova and Skamla, 1968), in the preparation of milled-wood (Björkman) lignin from pinewood (Bogomolov et al., 1974), and in other reactions (Yean and Goring, 1964; Rezanowich et al., 1963; Albrecht and Nicholls, 1976).

When a new delignifying agent, chlorine monoxide ($Cl_2O$; the anhydride of HOCl), was brought to light (Bolker and Liebergott, 1972), we undertook, as reported here, to determine whether the soluble products of its attack on lignin would exhibit the same pattern. The significance of this aspect of our investigation is that increasing molecular weight with increasing solubility of lignin is predicted by our previous work (Bolker and Brenner, 1970). It led to the conclusion that the relationship could be explained if lignin in wood is an infinite network of cross-linked chains and is solubilized by the cleavage of cross-links.

There have been other, substantially different, explanations proposed for the increase in molecular weight of the dissolved lignin as the delignification reaction proceeds. The most common explanation (Alekseev et al., 1969; 1971; Lacan and Matasovic, 1966; Alekseev and Reznikov, 1970; Chupka et al., 1970), which might be called the "condensation theory", is that lignin in wood consists of finite macromolecules with reactive sites that "condense", i.e., form intermolecular bonds, when the lignin is extracted. Since "condensation" occurs under both acidic and alkaline conditions, the concept, and its terminology as applied to lignin, probably arose by analogy with the chemistry of phenol–formaldehyde resins. Whatever its origin, the theory implies that isolated, soluble lignins, when further exposed to extracting reagents and conditions, would either increase in molecular weight (if all the reactive sites had not been used up during the original isolation), or would not change (if they had). Adler et al. (1968) found, however, that there is a marked decrease in the molecular weight of Björkman (milled-wood) lignin upon mild hydrolysis. A decrease in the molecular weight

Pulp and Paper Research Institute of Canada and Department of Chemistry, McGill University, Montreal, Quebec, Canada H3C 3G1.