

## Mechanism of Biodegradation of Paraquat by *Lipomyces starkeyi*

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**The biodegradation of ring-<sup>14</sup>C- and methyl-<sup>14</sup>C-labeled paraquat by the soil yeast *Lipomyces starkeyi* was studied in vitro. It was found that the degradation of paraquat (acting as a sole source of culture nitrogen) resulted in the accumulation in the extracellular medium of radiolabeled acetic acid. The culture also evolved radiolabeled CO<sub>2</sub>. The results suggest that the degradation of paraquat by *L. starkeyi* is associated with the integrity of the cell wall and that disruption or removal of the wall results in a complete loss of degradative capability. A mechanism for the degradation of paraquat by this organism is postulated.**

Paraquat (1,1'-dimethyl-4,4'-bipyridylum dichloride) is a broad-spectrum contact herbicide that is immobilized on clay soil fractions shortly after application (28). The adsorption of paraquat by clay colloids renders the herbicide unavailable for microbial degradative attack, and consequently, paraquat may remain unaltered, albeit biologically inert, in the soil for many years with negligible rates of loss from the environment (20, 24). In the short period of time between its application and deactivation by soils, however, paraquat may be degraded by soil microorganisms (3). Studies on the microbial degradation of herbicides in soils have often proved difficult (9), and owing to the adsorption of paraquat, studies on paraquat degradation in situ have been particularly so. Thus, the majority of studies have been carried out in vitro.

Several microorganisms have been reported as being capable of the in vitro degradation of paraquat (2, 4, 8, 12, 25, 30). In the majority of the cases, however, paraquat degradation was shown to be extremely variable in both extent and rate, and evidence of only one degradation product other than CO<sub>2</sub> has been reported, the *N*-methyl betaine of isonicotinic acid (8). The latter product has, however, been shown to be a major intermediate in the photolytic degradation of paraquat (23).

A strain of the soil yeast *Lipomyces starkeyi* is capable of rapid, efficient, and complete in vitro degradation of paraquat when the herbicide is used as a sole source of culture nitrogen (1, 2). This aerobic degradation is effected over a wide range of pHs and temperatures (1). The only product of paraquat degradation identified was CO<sub>2</sub>, and no mechanism for the degradation of paraquat was proposed.

The results in this paper suggest that the degradation of paraquat by *L. starkeyi* is associated with the integrity of the cell wall and that disruption or removal of the wall results in a complete loss of degradative capability. Using radiolabeled paraquat, we showed that paraquat is degraded by *L. starkeyi*, resulting in the accumulation in the extracellular medium of the final degradation product, acetic acid.

### MATERIALS AND METHODS

**Chemicals.** methyl-<sup>14</sup>C-labeled paraquat (specific activity, 33 mCi/mmol) was obtained from the Radiochemical Centre.,

Amersham, United Kingdom. Ring-<sup>14</sup>C-labeled paraquat, 1,1'-dimethyl-2,6,2',6'-[<sup>14</sup>C]4,4'-bipyridylum dichloride (specific activity, 15.8 mCi/mmol), was kindly donated by Imperial Chemical Industries, Ltd., Plant Protection Division, Jealotts Hill Research Station, Bracknell, Berkshire, United Kingdom. All other chemicals were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, United Kingdom. β-1,3-Glucanase was a gift from Ray Gibson, Cadbury Schweppes Ltd., Reading, United Kingdom.

**Organism.** A culture of *L. starkeyi* Lod. and Rij. (15) was obtained from Imperial Chemical Industries.

**Culture.** The microorganism was cultured on a nitrogen-free defined medium of the following composition: glucose, 5 g; salts solution A, 10 ml; MgCl<sub>2</sub> · 6H<sub>2</sub>O, 0.2 g; EDTA, 37.2 mg; and distilled water to 1 liter (pH was adjusted to 6.8 with 1 M HCl). Salts solution A was as originally formulated by Evans et al. (7). Paraquat (10 to 100 mg) was added, as required, as a nitrogen source.

**Spheroplast production.** Spheroplasts of *L. starkeyi* cells were prepared as follows. Cells from 250-ml cultures were harvested at the late-log phase of growth, washed with distilled water, and incubated at 30°C for 15 min in 40 ml of 0.1 M mercaptoethanol-25 mM EDTA (disodium salt)-50 mM dithiothreitol-0.2 M Tris hydrochloride buffer (pH 9.0). The cells were collected by centrifugation (13,000 × *g*) for 20 min and washed twice in a solution of 1.0 M sorbitol in 50 mM sodium citrate buffer (pH 5.9). The washed cells were suspended in 40 ml of the latter buffer to which had been added 250 mg of lyophilized β-1,3-glucanase (11) and incubated at 30°C for 1 h. After incubation, the spheroplasts were collected by centrifugation (2,000 × *g*) for 5 min. The spheroplasts were washed twice in sodium citrate-phosphate buffer (pH 5.9) containing 0.1 M sorbitol and used immediately after washing. Such a procedure has been shown previously to result in intact and viable spheroplasts (11).

**Preparation of cell extract.** Cells of log-phase *L. starkeyi* were collected by centrifugation (13,000 × *g*) for 20 min and washed with 20 mM potassium phosphate buffer (pH 7.3) containing 150 mM NaCl and 1 mM MgCl<sub>2</sub>. Washed cells were disrupted by liquid shear homogenization (Stanstead Fluid Power Ltd., Stanstead, Essex, United Kingdom) at a pressure of 1,100 to 1,275 kg/cm<sup>2</sup>. Cell debris was removed by centrifugation (20,000 × *g*) at 4°C for 20 min.

**Assays. (i) Paraquat.** Paraquat was assayed by the method of Calderbank and Yuen (5). A sample (0.9 ml) of cell-free supernatant from the culture was added to 0.1 ml of 1.5%

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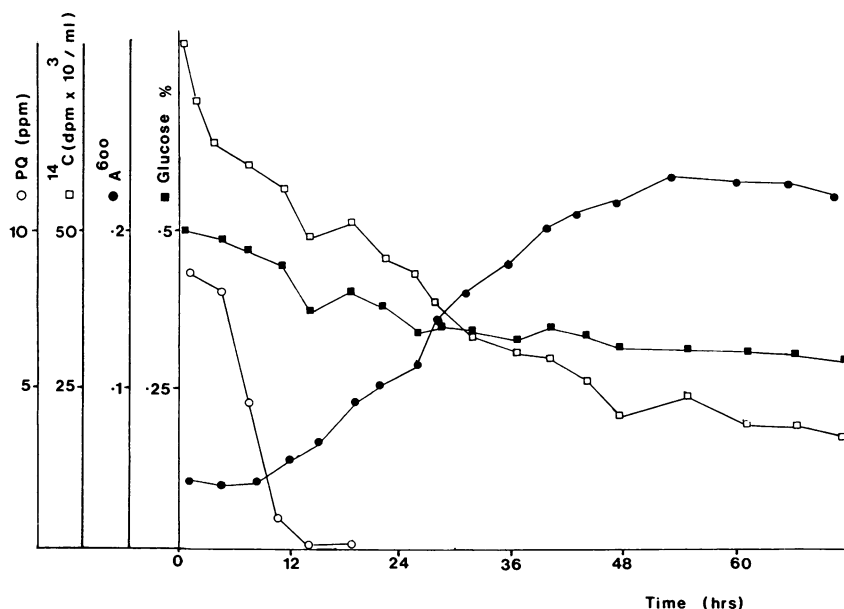


FIG. 1. Changes in culture parameters after the degradation of *methyl*-<sup>14</sup>C-labeled paraquat by *L. starkeyi*. PQ, Paraquat.

(wt/vol) sodium dithionite in 1 M NaOH. The  $A_{600}$  was measured after 5 min at 25°C.

(ii) **Glucose.** Glucose was assayed by using a commercially prepared glucose assay system (glucose colorimetric diagnostic assay kit 510; Sigma).

(iii) **Protein.** Protein was assayed by the method of Lowry et al. (16), and methylamine was assayed by the method of Dubin (6).

**Analytical methods.** (i) **Gas-liquid-phase chromatography.** Gas-liquid-phase chromatography was performed with a Pye GCV chromatograph (Pye Unicam) in combination with a Hewlett-Packard 3380 A integrator under the following conditions: 10% polyethylene glycoladipate (mesh size, 80 to 100; Phase Separations, Queensferry, United Kingdom) in a glass column (150 cm by 4 mm) at 110°C at a gas flow rate of 42 ml of helium per min. Detection was by a flame ionization detector at 220°C. A number of carboxylic acids were examined by this system, including acetic acid (retention time [RT], 4.60 min), propionic acid (RT, 6.90 min), isobutyric acid (RT, 7.82 min), butyric acid (RT, 10.69 min), isovaleric acid (RT, 13.07 min), valeric acid (RT, 18.71 min), isocaproic acid (RT, 26.46 min), and caproic acid (RT, 32.29 min). The <sup>14</sup>C-labeled product was found to cochromatograph (RT, 4.64 min) with the acetic acid standard (RT, 4.60 min) on this system. The identification of acetic acid as the final degradation product was confirmed by high-pressure liquid chromatography, the <sup>14</sup>C-labeled product cochromatographing with acetic acid as shown below.

(ii) **High-pressure liquid chromatography.** Samples were analyzed by high-pressure liquid chromatography with a Waters chromatography system (Waters Associates, Inc., Milford, Mass.) with a reverse-phase  $\mu$ Bondapak C<sub>18</sub> (Waters Associates) column (100 by 3 mm). Sample components were detected with a differential refractometer (model R401; Waters Associates). The mobile phase was double-distilled filtered water and was pumped through the column at 500 lb/in<sup>2</sup> at an isobaric flow rate of 1 ml/min. Under these conditions, an acetic acid standard and the radiolabeled product were found to cochromatograph (elution volumes, 4.30 and 4.26 ml, respectively).

(iii) **TLC.** Thin-layer chromatography (TLC) was performed by various combinations of the following systems. Chromagram silica G and Polygram sil G/UV254 (Eastman Kodak, Hemel Hempstead, Hertfordshire, United Kingdom) and DC Kieselgel F254 and DC cellulose (E. Merck AG, Darmstadt, Federal Republic of Germany) were developed with the following solvent systems: (i) 5 M NH<sub>4</sub>Cl; (ii) ethyl acetate; (iii) *N*-butanol-acetic acid-H<sub>2</sub>O (4:1:2); (iv) 0.1 M potassium phosphate buffer (pH 6.8)-ethanol (50:50). Separated sample components on developed TLC plates were visualized by either UV irradiation at 254 or 366 nm or spraying with Dragendorff reagent.

DC Kieselgel F254 and solvent system iv were found to be optimal for the analysis of culture supernatants, with the following compounds employed as standards: homarine hydrochloride ( $R_f$  0.79), 4,4'-bipyridyl ( $R_f$  0.91), 1-methyl-2-pyridone ( $R_f$  0.95), and [*methyl*-<sup>14</sup>C]paraquat ( $R_f$  0.76).

**<sup>14</sup>C counting.** Sample radioactivity was determined by liquid scintillation counting. Samples (50 to 1,000  $\mu$ l) were added to 6 ml of scintillation fluid (Aqualuma; LKB Instruments, Inc., Rockville, Md.) and counted in triplicate over 300 s in an LKB Rackbeta 1215 liquid scintillation counter fitted with a DPM package. Solid or particulate materials such as crude culture homogenates or cell pastes were dissolved in Lumasolve (LKB) following the instructions of the manufacturer. Samples of high ionic strength were counted with Rialuma (LKB).

**autoradiography.** TLCs of samples containing [<sup>14</sup>C]paraquat or its degradation products were used to expose autoradiographs. The developed TLC plates were laid against X-ray film (X-Omat H; Kodak) separated by a layer of clingfilm. The autoradiographs were exposed over an 18- to 36-h period at 4°C and were subsequently developed according to the instructions of the manufacturer.

## RESULTS

The changes in culture parameters with the degradation of paraquat are shown in Fig. 1 and 2 when *methyl*-<sup>14</sup>C- and ring-<sup>14</sup>C-labeled paraquat were used as sole nitrogen sources, respectively. After a lag period, the length of which was

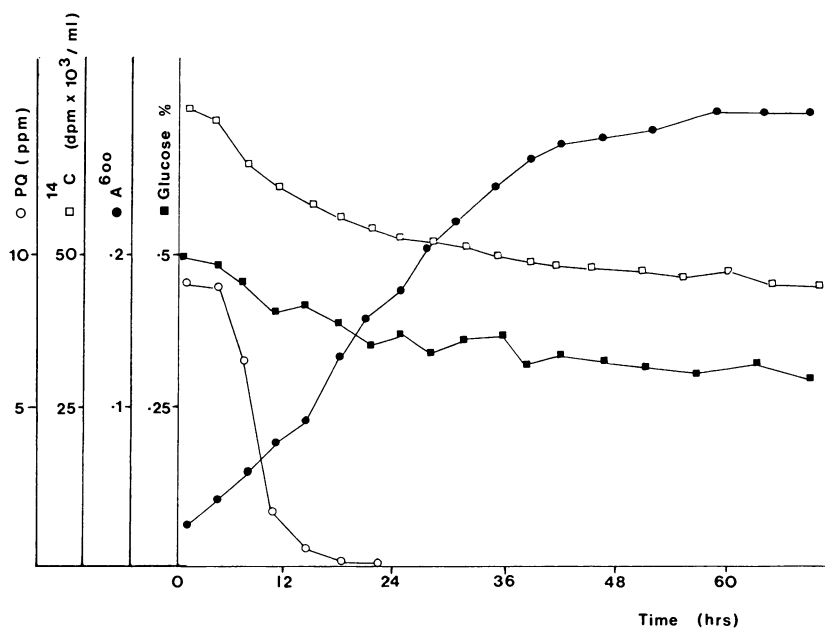


FIG. 2. Changes in culture parameters after the degradation of ring-<sup>14</sup>C-labeled paraquat by *L. starkeyi*. PQ, Paraquat.

determined by the state of induction of paraquat-catabolizing mechanisms in the inoculating cells (1), paraquat was rapidly degraded. Increase in culture optical density ( $A_{600}$ ) at the expense of glucose occurred only slowly and appeared to be independent of paraquat concentration. Approximately 75% ( $60 \times 10^3$  dpm/ml) of the original ( $80 \times 10^3$  dpm/ml) culture cell-free <sup>14</sup>C content was lost from the culture after the degradation of *methyl*-<sup>14</sup>C-labeled paraquat (Fig. 1). The degradation of ring-<sup>14</sup>C-labeled paraquat (Fig. 2) resulted in the loss from the culture of approximately 40% ( $30 \times 10^3$  dpm/ml) of the original ( $75 \times 10^3$  dpm/ml) <sup>14</sup>C content. Analysis of the *methyl*- and ring-labeled cells showed them to contain 9 and 4% of the original <sup>14</sup>C concentration, respectively. Over 95% of the <sup>14</sup>C lost from the culture was identified as <sup>14</sup>CO<sub>2</sub> by employing a calcium hydroxide CO<sub>2</sub> trap and determining the <sup>14</sup>C content of the resultant calcium carbonate precipitate by scintillation counting.

After the degradation of all of the ring-<sup>14</sup>C-labeled paraquat present in the medium, the nature of the <sup>14</sup>C-containing degradation product remaining in the cell-free supernatant was determined. Purification and concentration of the <sup>14</sup>C-labeled product were effected with a strong basic anion-exchange resin, Amberlite IRA 410 (BHD, Poole, Dorset, United Kingdom), as follows.

A sample (50 ml) of cell-free supernatant derived from a culture in which all the paraquat had been utilized was concentrated to 15 ml by rotary evaporation at 700 mm Hg (93,325 Pa) and 35°C. This concentrate (representing approximately 60% of the <sup>14</sup>C introduced into the culture) was then applied to a 20-ml Amberlite IRA 410 column previously equilibrated with 0.5 M NaOH. The column was washed exhaustively with water, and the <sup>14</sup>C-labeled material was subsequently eluted with 30 ml of 1 M NaOH (98% recovery). The fraction containing <sup>14</sup>C was acidified with 3.2 M H<sub>2</sub>SO<sub>4</sub>, and the sample (30 ml) was extracted with two 40-ml portions of diethyl ether (96% recovery). The <sup>14</sup>C product was back extracted from the solvent into a 1% (vol/vol) ammonia solution (95% recovery). Samples (1 ml) of the ammoniacal solution were acidified with H<sub>2</sub>SO<sub>4</sub> and subjected to analysis by high-pressure chromatography and

gas-liquid-phase chromatography as described above. The <sup>14</sup>C-containing product of <sup>14</sup>C-labeled paraquat degradation was found to cochromatograph with an acetic acid standard on both analytical systems. No other substances were found by these methods.

No evidence was found for the presence of any <sup>14</sup>C-labeled heterocyclic ring structures after the degradation of ring-labeled paraquat despite extensive analysis of the culture cell-free supernatant by a variety of TLC techniques (see above). By using a solvent system (TLC system iii above) previously found by other workers (22, 26, 27) to be optimal for the analysis of paraquat and its analogs, no discrete areas of <sup>14</sup>C-containing products could be discerned by the autoradiographical techniques described above.

Spheroplasts of *L. starkeyi* prepared as described above were incubated for 48 h at 30°C in paraquat-defined minimal medium containing 0.1 M sorbitol and 5 mg of lyophilized β-1,3-glucanase per ml with either ring-<sup>14</sup>C- or *methyl*-<sup>14</sup>C-labeled paraquat. Analysis of culture <sup>14</sup>C, paraquat, and glucose showed that *L. starkeyi* spheroplasts were incapable of metabolizing paraquat. The addition of metabolic cofactors such as flavin adenine dinucleotide, flavin mononucleotide, NAD<sup>+</sup>, NADP<sup>+</sup>, NADH, NADPH, and ATP had no effect on paraquat degradation by spheroplasts (data not shown).

Incubation of cell wall preparations of *L. starkeyi* as described above was equally ineffective in transforming paraquat whether in the presence or absence of added cofactors.

## DISCUSSION

The degradation of paraquat by *L. starkeyi* has been reported by others (1, 2). In one such study, no degradation products other than <sup>14</sup>CO<sub>2</sub> were identified from the catabolism of ring-<sup>14</sup>C-labeled paraquat (2). Anderson and Drew (1) were equally unable to demonstrate the presence of paraquat degradation products other than CO<sub>2</sub>. In the latter study, *L. starkeyi* was shown to be capable of degrading paraquat over a wide range of pHs and temperatures, but the degradation occurred only after a protracted lag period. The

lag period could be reduced by inoculating medium with cells previously exposed to paraquat. These authors also showed that *L. starkeyi* was incapable of growth under anaerobic conditions and that degradation of paraquat could not occur in the absence of a suitable carbon source. The results obtained in this study support these findings.

The majority of other studies on the in vitro degradation of paraquat have proved contradictory and inconclusive. In those cases in which degradation was said to have occurred (determined colorimetrically), degradation rates were low and variable. The *N*-methyl betaine of isonicotinic acid, 4-carboxy-1-methyl pyridinium ion, has been reported as having arisen from paraquat degradation together with the demethylated paraquat analog 1-methyl-4-bipyridylum chloride (8). The *N*-methyl betaine has also been reported as being a product of paraquat degradation by *Streptomyces* sp. and *Nocardia* sp. (19). However, the analytical methods used in many of the above reports have subsequently been shown to be problematical (22, 26, 27) and may have led to misinterpretation of results. Furthermore, the *N*-methyl betaine of isonicotinic acid has been shown to be the major photolytic degradation product of paraquat (23).

The changes in culture parameters after the degradation of ring-<sup>14</sup>C-labeled paraquat by *L. starkeyi* described in this study (Fig. 2) are in broad agreement with those obtained by Anderson and Drew (1). By using a standard procedure for the isolation of carboxylic acids (described above), it has been shown in this study that the accumulated <sup>14</sup>C-labeled product of paraquat degradation is acetic acid. It is apparent that only unmodified cells of *L. starkeyi* can degrade paraquat. Removal of the cell wall destroys this ability, as does disruption of the cell membrane, evidenced by the inability of cell extracts to degrade the herbicide.

Paraquat has been shown to be actively accumulated in mammalian lung tissue by an energy-dependent process (21). It is probable that the active transport of paraquat into cells of *L. starkeyi* is mediated by a similar energy-dependent mechanism, as the yeast cells are incapable of degrading paraquat in the absence of a suitable energy source such as glucose. The cell membrane of *Escherichia coli* is impermeable to paraquat in its oxidized state (13, 14). If the same is true of *L. starkeyi*, the molecule must be reduced to cross the cell membrane. Pyridine (10, 29) and *N*-methyl pyridinium iodide (17, 18) may also require a reductive step in their catabolism. The instability of the reduced paraquat molecule in the presence of oxygen may indicate that a cell wall- or membrane-associated site of cation reduction requires protection from oxygen and may only function correctly in the intact cell. Disruption of the integrity of the system would lead to the observed loss in degradative ability.

When methyl-<sup>14</sup>C-labeled paraquat was degraded by *L. starkeyi*, >80% of the <sup>14</sup>C content of the original culture was lost as <sup>14</sup>CO<sub>2</sub>. This <sup>14</sup>CO<sub>2</sub> either may originate directly from a simple demethylation of paraquat to leave 1-methyl-4-bipyridylum ion or 4,4'-dipyridyl or may be derived from a rapidly metabolized leaving group, such as methylamine, which may be cleaved from the parent molecule. No evidence for the production of a demethylated heterocyclic ring structure could be found. In addition, *L. starkeyi* cannot utilize 4,4'-bipyridylum ion as a carbon source (data not shown).

The degradation of ring-<sup>14</sup>C-labeled paraquat results in the accumulation of <sup>14</sup>C-labeled acetic acid in the extracellular medium. The paraquat carbon skeleton thus appears to be degraded into C<sub>2</sub> fragments, there being no evidence for the accumulation of larger fragments. Assuming [<sup>14</sup>C]acetic acid

is not resynthesized from C<sub>1</sub> fragments, a mechanism for the breakdown of paraquat may be postulated. After demethylation or removal of a methylamine group from the molecule, the remaining carbon skeleton is progressively degraded via a number of short-lived intermediates to acetic acid. The <sup>14</sup>C-labeled acetic acid thus formed may be further degraded to <sup>14</sup>CO<sub>2</sub> via general oxidative metabolic pathways.

No evidence for the accumulation of methylamine was found. However, as the culture was severely nitrogen limited and methylamine is a readily utilizable nitrogen source, it is likely that any methylamine produced would be too rapidly metabolized to exist in sufficiently high concentrations to be determined by the methods described. This would be especially true if ring cleavage of the parent molecule and concomitant methylamine production were the rate-limiting steps in paraquat degradation.

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