Microbial Degradation of Chlorobenzilate (Ethyl 4,4′-Dichlorobenzilate) and Chloropropylate (Isopropyl 4,4′-Dichlorobenzilate)

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As a result of an extensive examination of over 300 microbial cultures, it was concluded that the acaricides, Chlorobenzilate and Chloropropylate, were best metabolized by a yeast, *Rhodotorula gracilis* (Rennerfelt), to several metabolites in a mineral medium supplemented by sucrose. Metabolites, resulting from the degradation of either acaricide, were identified as 4,4′-dichlorobenzilic acid and 4,4′-dichlorobenzophenone. The probable steps of the degradation pathway are Chlorobenzilate or Chloropropylate → 4,4′-dichlorobenzilic acid → 4,4′-dichlorobenzophenone, although some other intermediate metabolites might exist. Chlorobenzilate, *i.e.*, ethyl ester of 4,4′-dichlorobenzilic acid, was more sensitive to hydrolysis of carboxylesterases than Chloropropylate, *i.e.*, the isopropyl ester of this acid. Some of the parent compounds always remained in the culture media at the end of the eight-week incubation period, no matter how small the initially incorporated amounts.

Microorganisms are known to utilize compounds as either essential substrates for normal metabolism or as non-essential energy sources (Stanier *et al.*, 1963). Some microbes can benefit only from specific organic materials, while others can utilize a wide range of compounds. Of interest, several strains of the bacterium, *Pseudomonas putida*, are capable of using even such substrates as naphthalene or phenol (Stanier *et al.*, 1966), both of which could be a skeletal structure of pesticides. While the metabolic fate of DDT (1,1,1 trichloro-2,2-*bis*(*p*-chlorophenyl) ethane) is known, to some extent, in microorganisms (Kallman and Andrews, 1963; Stenersen, 1965; Mendel and Walton, 1966; Wedemeyer, 1967; Braunberg and Beck, 1968; Matsumura and Boush, 1968; Plimmer *et al.*, 1968), little information on the metabolism of other DDT analogs or related compounds exists.

Chlorobenzilate (ethyl 4,4′-dichlorobenzilate) and Chloropropylate (isopropyl 4,4′-dichlorobenzilate) are chlorinated hydrocarbon acaricides classified as diphenyl aliphatics, or DDT relatives. Horn *et al.* (1955) speculated that Chlorobenzilate administered to dogs and rats might be excreted as a hydrolysis product, 4,4′-dichlorobenzilic acid, in urine. Recently Cassidy *et al.* (1968) reported the fate of Acarol (isopropyl 4,4′-dibromobenzilate), the chemical structure of which is closely related to Chlorobenzilate and Chloropropylate, in apples and in soil. Their results showed that 4,4′-dibromobenzilic acid, a hydrolysis product of Acarol was formed, though not in significant amounts, in both leaves and soil 40 days after treatment. Soil samples from under apple trees taken nine months after spraying contained 4,4′-dibromobenzophenone which was speculated to be the further degradation product of 4,4′-dibromobenzilic acid. These experiments, however, did not clarify whether microorganisms were involved in the degradation of these compounds. So far, no information has been available on the metabolism of Chlorobenzilate and Chloropropylate by microorganisms. In the present study, attempts were made to discover microorganisms which were capable of degrading those acaricides, and to identify resulting metabolites.

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MATERIALS AND METHODS

Acaricide Used. Both Chlorobenzilate and Chloropropylate, used in this study, were of analytical grade. In addition, both compounds were also obtained in radioactive form, labeled at the number one and number two carbon atoms.

Screening of Microorganisms for Capacity to Degrade Chloropropylate. Promising microbial isolates from samples of insecticide-treated soil, described by Matsumura and Boush (1967), were used for surveys of degradation activities. In addition, cultures of fungi and bacteria were also secured through the courtesy of M. P. Backus, A. K. Kelman, and E. F. McCoy, Departments of Botany, Plant Pathology, and Bacteriology, respectively, of the University of Wisconsin at Madison.

Testing of Chloropropylate degradation was conducted by incubating each culture in 10 ml of the screening medium, a solution of yeast extract and mannitol, as described by Fred and Waksman (1928) in a screw-capped 20-ml test tube at 30° C. for 48 hours. The culture was then further incubated at 30° C. for eight weeks, without shaking, after the addition of 10 μl. of $10^{-8}M$ C^{14}-Chloropropylate dissolved in acetone. The screw-cap was kept loose so as not to cause anaerobic conditions. The reaction was stopped and the pH lowered to approximately 2.0 by adding 0.5 ml. of 20% trichloroacetic acid to the culture medium. This mixture was immediately extracted three times, each time with 10 ml. of ether. Whenever the separation of the layers became difficult, the whole system was briefly centrifuged. The ether phase was dried over anhydrous sodium sulfate, and further evaporated by a gentle air stream to dryness. The oily residue thus obtained was extracted five times, each time with 5 ml. of hexane. The hexane phase was concentrated by a gentle air stream to 0.1 ml. For the separation of Chloropropylate and its metabolites, if any, thin-layer chromatography was used. A 20-μl. portion of the concentrated material was spotted on a No. 6060 Eastman 20- × 20-cm. (Rochester, N. Y.) chromatographic sheet 2 cm. from the edge. The sheet was then developed in a solvent mixture of hexane, ethanol, and acetic acid (17:2:1 by volume) until 15 cm. from the origin. The resulting chromatogram was air dried and autoradiogrammed on Kodak medical X-ray film of high contrast for four weeks.

Control experiments (acaricides minus microorganisms) established that the above treatments of extraction and thin-layer chromatography produced 1.5 and 3.4% of breakdown products from Chlorobenzilate and Chloropropylate, respectively. To demonstrate the presence of water-soluble metabolites, 0.5 ml. of aqueous phase, after ether extraction, was added to 10 ml. of scintillation liquid, and radioactivity was measured by a liquid scintillation spectrometer. A total of 359 cultures was screened. The actual number of species involved was probably much less, because a number of cultures, unless obtained from culture collections of known species and strains, were no doubt similar.

Identification of Metabolites of Chlorobenzilate and Chloropropylate. A yeast, *Rhodotorula gracilis* (Rennerfelt), was selected for further studies based on the screening results. The methods for determining the possible microbial degradation of Chlorobenzilate and Chloropropylate were as follows: from a nutrient agar slant of *R. gracilis*, to which 2 ml. of sterile distilled water was added, 2 ml. of cell suspension was transferred to a 2-l. Erlenmeyer flask containing 500 ml. of the basal medium (NH_4NO_3, 5.0 g.; K_2HPO_4, 2.5 g.; $MgSO_4 \cdot 7H_2O$, 1.0 g.; $FeCl_3 \cdot 6H_2O$, 0.2 g.; and distilled water, 1 liter) (after Shimahara and Yamashita, 1967) plus 1% sucrose and 100 mg. of Chlorobenzilate or Chloropropylate fortified with $10^{-6}M$ of the appropriate radioactive compound. The material thus described was incubated at 30° C. on a gyratory water-bath shaker at 100 cycles per minute for two weeks. At the end of this incubation period, 25 ml. of 20% trichloroacetic acid was added.

The mixture was then immediately extracted three times, each time with 600 ml. of ether. The ether phase was dried for 12 hours over anhydrous sodium sulfate, and then evaporated under reduced pressure to dryness by using a rotary evaporator at 30° C. An oily residue was thus obtained. A 0.05-ml. portion of residue was extracted with hexane as previously described and this phase was evaporated to 0.1 ml. using the previously described procedure. All of the concentrated materials were spotted on a silica gel GF plate (0.5 mm. thick). The plate was then developed 15 cm. from the origin in a previously described solvent mixture. The chromatogram obtained was air dried and autoradiogrammed for one month. The radioactive spots of the silica gel absorbent, as judged from the X-ray film, were scraped from the plate with a metal blade and their radioactivities determined using a Tri-Carb liquid scintillation counter Model 314E (Packard Instrument Co., Downers Grove, Ill.).

For clean-up of the remaining concentrated material, a 2.5-cm. diameter chromatographic column was prepared by packing 10 g. of 100-mesh silicic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) slurry in cyclohexane. The residue dissolved in 10 ml. of cyclohexane was quantitatively transferred to the column. An additional 60 ml. of 14-to-1 cyclohexane–ether was added and allowed to pass through the column, making a total of 70 ml. of eluate collected. The elution was continued using 50 ml. of 9-to-1 cyclohexane–ether and 70 ml. of 3-to-1 cyclohexane–ether, respectively. Each of the three fraction eluates collected was evaporated to dryness under a gentle air stream. The residues were transferred, singly, with hexane to three 15-ml. centrifuge tubes. The solvent was again evaporated to dryness with the aid of a gentle air stream, and 0.2 ml. of ether was added to each residue sample.

Each fraction was assayed for Chlorobenzilate or Chloropropylate metabolites. The metabolites were further purified on preparative thin-layer chromatography using silica gel GF plates. The concentrated fractions were spotted at the origin as a thin band across the plates. Following the 15-cm. development of the plates with 17-to-2-to-1 hexane–ethanol–acetic acid and evaporation of the solvent by cool air, the plates were examined under an ultraviolet lamp at 2537 Å and the dark UV absorbing bands were marked. Silica gel of each dark band was collected using a vacuum zone collector (Desage-Brinkmann) and extracted with 25 ml. of ether using a Soxhlet extraction apparatus. The ether extract was then concentrated to 0.5 ml. under a gentle air stream and spotted on another thin-layer plate, which was developed in a solvent mixture of 3-to-1 cyclohexane–ether for a distance of 15 cm. from the origin. At the edge of the same plate, a mixture of 4,4′-dichlorobenzilic acid, 4,4′-dichlorobenzophenone and Chlorobenzilate or Chloropropylate was spotted as references. After detection of the band positions, each band was collected and extracted with ether as previously described. The ether extract was again spotted on another thin-layer plate which was developed with a mobile phase of cyclohexane–ether (14-to-1). The resulting chromatogram was subjected to autoradiography with the previously described X-ray film for 3 days. The portion of silica gel that corresponded to the radioactive spot was collected and extracted in the same manner. Crystallization of the metabolites was attempted by dissolving the residue in a 0.5-ml. pear-shaped boiling flask. One milliliter of hexane was added to this solution, and the solvent was evaporated under a gentle air stream until crystals appeared.

RESULTS AND DISCUSSION

Selection of Microorganisms. Figure 1 presents the results of the metabolism of Chloropropylate by promising microorganisms after an eight week incubation at 30° C. The R_f value for the parent compound was 0.80. The spots with R_f values of 0.27 and 0.90 probably indicated 4,4′-dichlorobenzilic acid and 4,4′-dichlorobenzophenone, respectively, since the R_f values for these spots matched with those of authentic reference compounds which were chromatogrammed together. The identities of the other spots are unknown. Since insignificant or no levels of radioactivity remained in the aqueous phase after ether extraction, studies on water-soluble metabolites were not conducted. Cultures No. 5 and 33 were isolated from soil samples screened from Shell Chemical Corporation, near Denver, Colo.; cultures No. 26 and No. 56 were from an orchard near Fredericksburg, Ohio (Matsumura and Boush, 1967); and cultures No. 71 and No. 389 were secured through the courtesy of E. F. McCoy and M. P. Backus, University of Wisconsin, Madison, Wis., respectively. Culture No. 71 is a yeast, *Rhodotorula gracilis* and No. 389 is a fungus, *Penicillium agyptiacum*. Culture No. 33 belongs to the bacterial genus *Pseudomonas*, but remaining cultures have not yet been identified. This particular *Pseudomonas* species (No. 33) is known to degrade dieldrin, a most persistent insecticide, to various metabolites (Matsumura and Boush, 1967; Matsumura *et al.*, 1968). It would seem that this microorganism may possess considerable flexibility in degrading various pesticides. Culture No. 33 grew much more slowly in the basal medium supplemented by 1% sucrose than did the other cultures. Since culture Nos. 5, 26, and 56 have not yet been identified, and No. 389, *P. aegyptiacum*, did not appear to be especially active in the degradation of Chloropropylate, Culture No. 71, *R. gracilis*, was chosen for further studies.

Metabolism of Chlorobenzilate and Chloropropylate. Figure 2 shows the results of metabolism of C^{14}-labeled Chloro-

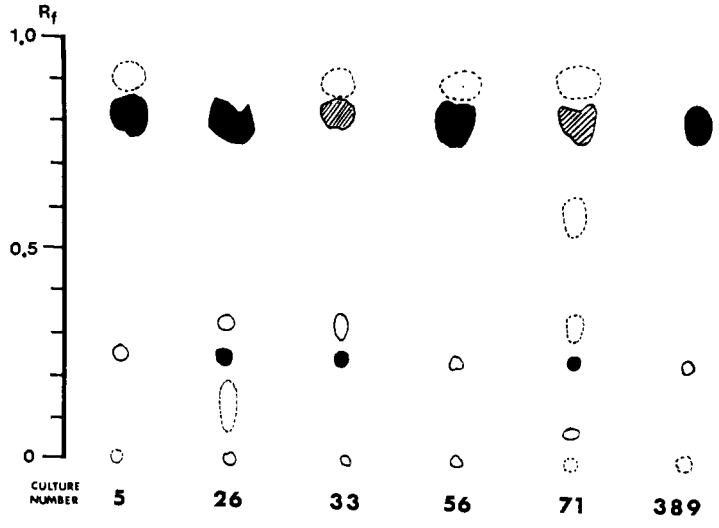

Figure 1. Autoradiographic presentation of thin-layer chromatograms of C^{14}-Chloropropylate and its metabolites produced by cultures of certain microorganisms

Spots showing strongest radioactivity are represented by black; medium radioactivity, shaded; weak radioactivity, open circles with solid line; and the weakest, open circles with dotted line

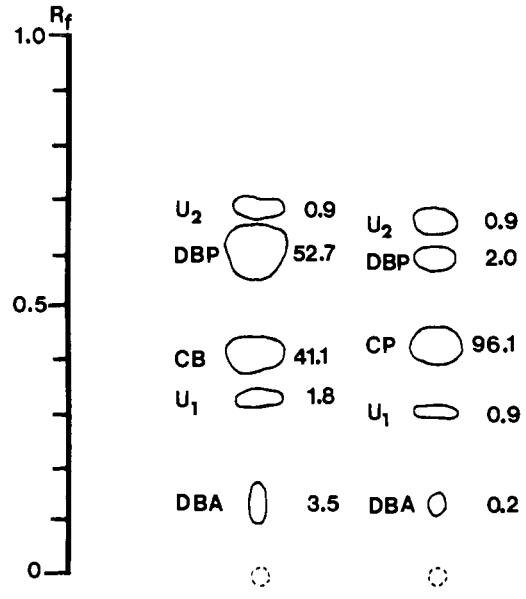

Figure 2. Metabolism of Chlorobenzilate and Chloropropylate by *Rhodotorula gracilis* incubated in basal medium supplemented by 1% sucrose as carbon source

CB: Chlorobenzilate; CP: Chloropropylate; DBA: 4,4′:-dichlorobenzilic acid; DBP: 4,4′-dichlorobenzophenone; and U-1 and U-2: unknown metabolites. Numerical figures at the right side of each compound represent percentages of radioactivity in that particular spot

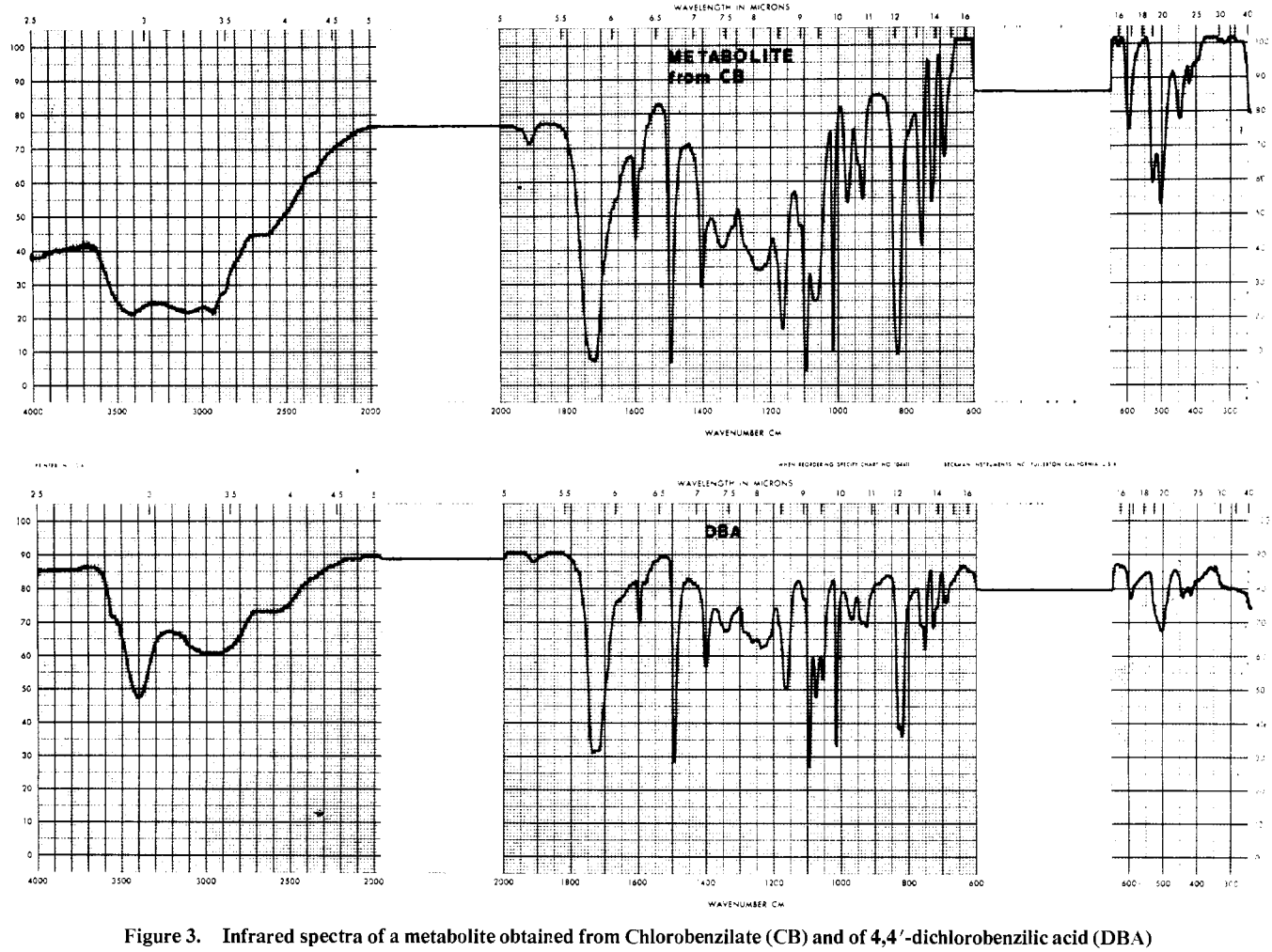

Figure 3. Infrared spectra of a metabolite obtained from Chlorobenzilate (CB) and of 4,4′-dichlorobenzilic acid (DBA)

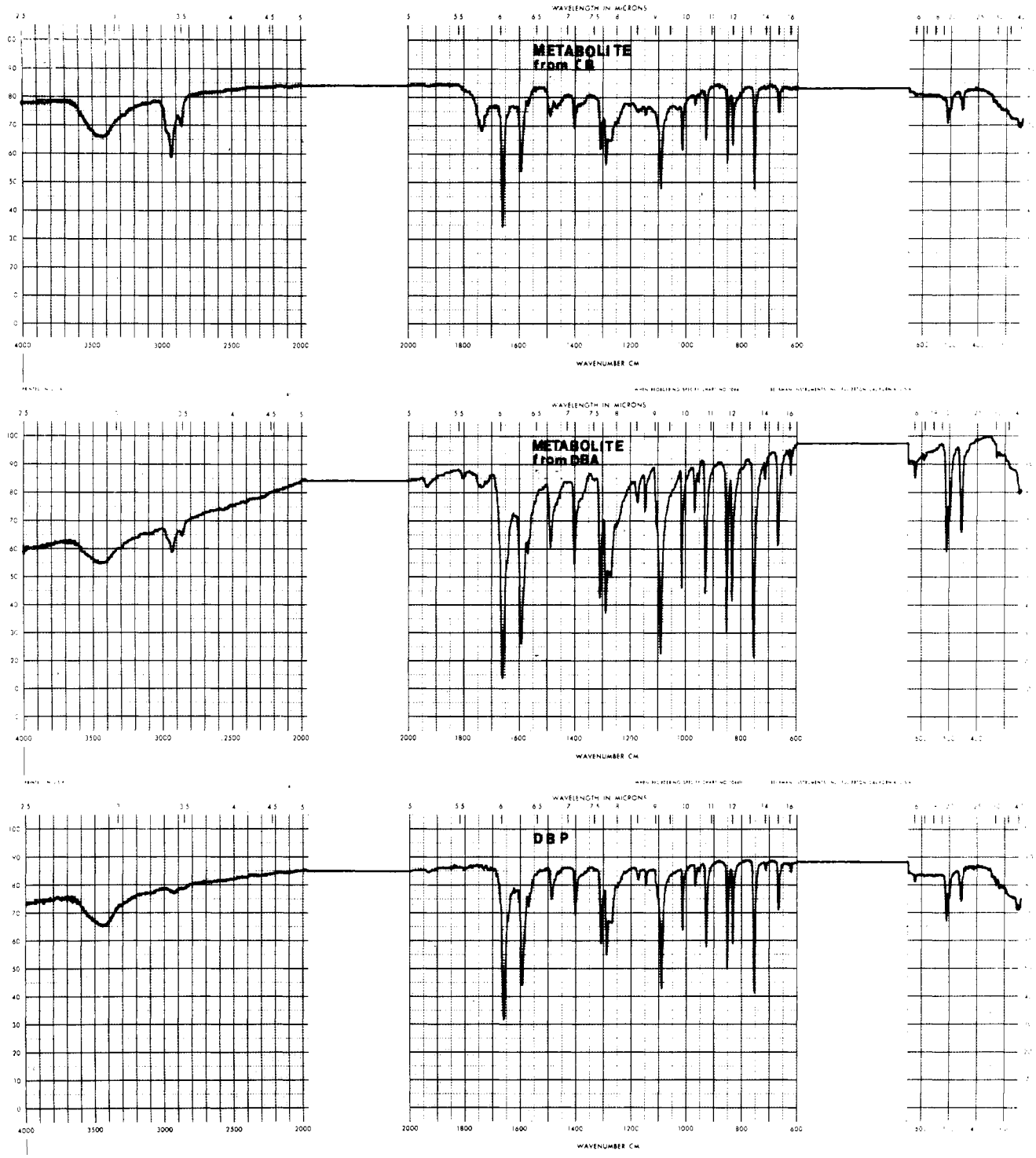

Figure 4. Infrared spectra of metabolites obtained from Chlorobenzilate (CB) and 4,4′-dichlorobenzilic acid (DBA), and of 4,4′-dichlorobenzophenone (DBP)

benzilate and Chloropropylate by *Rhodotorula gracilis* in the previously described basal medium fortified with 1% sucrose. Using comparisons of R_f values of authentic reference compounds, 4,4′-dichlorobenzilic acid and 4,4′-dichlorobenzophenone were tentatively identified as metabolites. The low radioactivity remaining at the origin (Figures 1 and 2) was probably due to an artifact caused by the thin-layer chromatographic treatment, as this fact was always observed even if pure parent compounds were used as the control.

Final identification of some of the metabolites was accomplished by securing melting points and conducting infrared spectroscopy analysis (Figures 3 and 4). However, the amounts of metabolites for U-1 and U-2, as shown in Figure 2, were so small that these methods could not be applied. The melting points observed for the sample and the authentic reference 4,4′-dichlorobenzophenone were 146–148° C. and 147–148° C., respectively, using a micro melting point apparatus. Another metabolite extracted from the silica gel absorbent matched with the R_f value of 4,4′-dichlorobenzilic acid but was not crystallized perfectly so that it was not possible to measure its melting point accurately. The remaining purified compounds were used for securing infrared

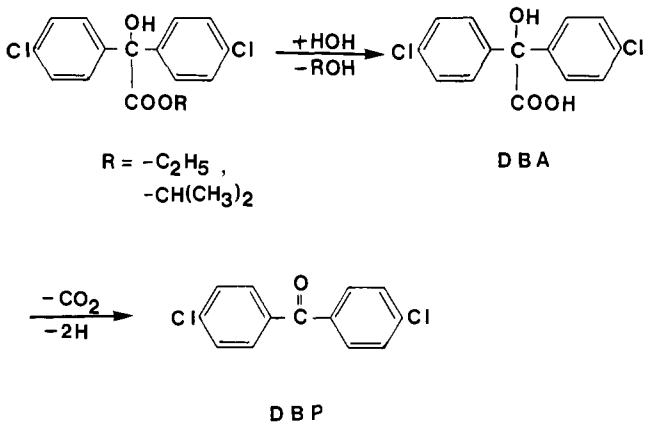

Figure 5. Proposed pathway of Chlorobenzilate and Chloropropylate degradation by *Rhodotorula gracilis*

DBA: 4,4′-dichlorobenzilic acid; DBP: 4,4′-dichlorobenzophenone

spectra. Since the peaks of the two spectra obtained from a metabolite of Chlorobenzilate and from 4,4′-dichlorobenzilic acid were superimposable, this metabolite was considered as 4,4′-dichlorobenzilic acid.

It was postulated that the initial degradation step was the hydrolysis of either Chlorobenzilate or Chloropropylate to 4,4′-dichlorobenzilic acid. And that this acid is further degraded by decarboxylation and dehydrogenation to a probable end product, 4,4′-dichlorobenzophenone (Figure 5). In order to prove the latter step of the degradation pathway, 50 mg. of 4,4′-dichlorobenzilic acid, which was sparingly soluble in water, was incubated with *R. gracilis* in 50 ml. of the basal medium supplemented by 1% sucrose at 30° C. for ten days. After extractions of the culture medium, as previously described, and at the end of incubation and purification, as again described previously, a metabolite was obtained. The R_f value of this compound matched with that of 4,4′-dichlorobenzophenone when thin-layer chromatography was used. The melting point of this compound was 142–148° C. Since the peaks of the three spectra obtained from the metabolites of Chlorobenzilate and 4,4′-dichlorobenzilic acid, and from 4,4′-dichlorobenzophenone were also superimposable, these metabolites were considered as 4,4-dichlorobenzophenone.

The R_f value of another metabolite, obtained from 4,4′-dichlorobenzilic acid as a starting material, matched with that of 4,4′-dichlorobenzhydrol as well as U-1 of Figure 2. In addition, the R_f value of another metabolite, U-2 of Figure 2, chromatographically matched with that of 4,4′-dichlorodiphenyl methane. However, further confirmation of these metabolites was not possible. It appears that with *R. gracilis*, Chlorobenzilate or Chloropropylate is first hydrolyzed to 4,4′-dichlorobenzilic acid through the action of carboxylesterase, which appears to have a higher activity for the ethyl ester (Chlorobenzilate) than for the isopropyl ester (Chloropropylate) as the per cent of remaining Chlorobenzilate was much less than that of Chloropropylate (Figure 2). In other words Chlorobenzilate was apparently degraded to a greater degree by this microorganism than Chloropropylate. Similar results have been obtained with larvae of a mosquito, *Culex tarsalis*, in which the carboxylesterases were more specific for malathion (ethyl ester) than the isopropyl analogue of this compound (Dauterman and Matsumura, 1962).

The 4,4′-dichlorobenzilic acid thus obtained from either Chlorobenzilate or Chloropropylate metabolism is further degraded through the process of decarboxylation and dehydrogenation to a probably final degradation product, 4,4′-dichlorobenzophenone, probably by the activities of enzymes. That 4,4′-dichlorobenzophene is an end product would appear likely, as a high amount of this compound was obtained compared with other metabolites. Neither Chlorobenzilate nor Chloropropylate was metabolized completely regardless of the amount of the compound added to the medium. This incomplete degradation would appear to be due, at least in part, to storage of the apolar compound in fat of the cells, where little enzymatic degradation would occur. It has been shown by Pyke (1958) that *R. gracilis* contains a high amount of fat. The proposed pathway of Chlorobenzilate and Chloropropylate degradation is presented in Figure 5.

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