Metabolism of Endothall by Aquatic Microorganisms

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[^{14}C]Endothall [7-oxabicyclo(2.2.1)heptane-2,3-dicarboxylic acid] labeled in the oxabicyclo ring was readily degraded by microorganisms in lake water and hydrosoil through a pathway involving splitting of the ring. An *Arthrobacter* species isolated from hydrosoil by the enrichment method was able to utilize endothall as the sole source of carbon and energy for its growth. The ^{14}C from endothall was incorporated into cellular amino acids, proteins, nucleic acids, and lipids and was also released as $^{14}CO_2$. After short-term incubation of the *Arthrobacter* with [^{14}C]endothall, ^{14}C was incorporated into citric, aspartic, and glutamic acids and some unidentified compounds. [^{14}C]Glutamic acid accounted for a large proportion of the total ^{14}C incorporated into the alcohol-soluble cell fraction. Treatment with 2×10^{-2} *M* monofluoroacetate caused a severalfold accumulation of [^{14}C]citric acid, but only partially inhibited the incorporation of ^{14}C into glutamic acid. It appears, therefore, that the ^{14}C from ^{14}C-ring-labeled endothall is incorporated into glutamic acid *via* the tricarboxylic acid cycle and an alternate, unknown pathway.

In recent years there has been an increased use of chemical methods to control aquatic weeds. Among the major concerns associated with the use of herbicides for controlling aquatic weeds are the potential toxicity of the herbicide and its metabolites to the aquatic fauna and flora and their possible adverse effect on man through his drinking water supplies. Evaluation of the safety of an herbicide used in the aquatic environment requires a knowledge of its fate. Among the various factors which determine the fate of a pesticide, microbial transformation is one of the most important.

The herbicide endothall [7-oxabicyclo(2.2.1)heptane-2,3-dicarboxylic acid] has been found to be particularly effective against certain submersed aquatic weeds (Frank *et al.*, 1961; Walker, 1963). Presently, very little is known about the metabolism of this herbicide by aquatic microorganisms; however, a few workers have investigated its persistence in soil. Montgomery and Freed (1964) observed that, under conditions favoring high microbiological activity, nearly all of the endothall applied to the soil was degraded in a period of 7–10 days. The findings of Comes *et al.* (1961) indicated that the dissipation of endothall was greater in moist soil than in air-dry soil and that the rate of inactivation of the herbicide was more rapid at 20–30° than at lower temperatures. Horowitz (1966) also reported that endothall persisted considerably longer in dry soil than in moist soil. These findings suggest that the breakdown of endothall in soil is mainly a microbiological process since conditions favoring the activity of microorganisms enhanced the degradation of this compound. Jensen (1944) isolated strains of *Arthrobacter* from soil which were able to decompose endothall. Although these findings indicate that soil microorganisms can degrade endothall, no information is available on the products resulting from its metabolism. This investigation was undertaken to study the degradation of endothall by a mixed population of microorganisms present in the aquatic environment and to elucidate the metabolism of the herbicide by a pure culture of an aquatic microorganism.

MATERIALS AND METHODS

Chemicals. [^{14}C]Endothall labeled in positions two and three of the oxabicyclo ring, with a specific activity of 11.8 μCi per mg, and unlabeled endothall, analytical grade, were furnished by the Pennwalt Corporation. The purity of the labeled endothall was greater than 98%, as determined by paper and thin-layer chromatography.

Metabolism of [^{14}C]Endothall in Lake Water and Hydrosoil. The degradation of [^{14}C]endothall in water and bottom sediment was determined by monitoring $^{14}CO_2$ evolution. [^{14}C]Endothall was added at a concentration of 10 ppm to a 100-ml suspension of water and hydrosoil (10 g of hydrosoil + 90 ml of water) collected from Otisco Lake in New York State. The soil suspension was incubated with endothall for 30 days at 25°, and $^{14}CO_2$ evolution from the soil suspension was measured in a closed system through which CO_2-free air was circulated. The CO_2 present in the incoming air was removed by passing it through 1 *N* NaOH. The air was then passed through sterile distilled water before passing it through the soil suspension. The $^{14}CO_2$ evolved from the soil was collected in a CO_2 trapping solution consisting of monoethanolamine and 2-methoxymethanol (1:2 v/v) (Metcalf *et al.*, 1967). Soil suspension sterilized by autoclaving for 30 min and by subsequent addition of 0.1% sodium azide was maintained as a control. The solution was removed and replaced every other day during the period of the experiment. The metabolic $^{14}CO_2$ was determined by adding 1-ml aliquots of trapping solution to 15 ml of scintillation solution and counting it for radioactivity in a Nuclear Chicago liquid scintillation counter.

Isolation of Aquatic Microorganisms. A population of aquatic microorganisms effective in the degradation of endothall was developed by an enrichment culture technique. Isolates were obtained from enrichment cultures which were inoculated with a 1-ml suspension of lake water and hydrosoil and incubated at 25° on a rotary shaker. The enrichment medium contained 1.0 g of dipotassium salt of endothall, 0.4 g of KH_2PO_4, 1.6 g of K_2HPO_4, 0.5 g of NH_4NO_3, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.25 g of $FeCl_3 \cdot 6H_2O$ per liter of distilled water. The pH of the medium was adjusted to 7.0. After an incubation period of 10 days, 1 ml of contents from the inoculated flask was withdrawn and transferred to a flask containing fresh enrichment medium. This procedure was repeated to enrich for endothall-utilizing organisms. A dilution plate method was used for the final isolation of microorganisms from the enrichment culture solutions. The isolates were identified according to the scheme of Skerman (1967).

Culture Methods. Cultures of an *Arthrobacter* sp. were maintained on agar slants consisting of the enrichment medium and 2% agar. For growth in liquid medium, the cells were routinely cultured in the mineral medium described above, which contained 1000 ppm of endothall. The medium was inoculated with cell suspension in the logarithmic phase of growth and the cultures were incubated on a rotary shaker at 25°. Growth was determined by measuring the absorbance at 600 nm. A calibration curve relating absorbance at 600 nm and the dry weight of the bacteria in cell suspension were used to determine the dry weight of the cells.

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The studies on the metabolism of endothall by *Arthrobacter* were done with endothall-grown cells which were harvested by centrifugation after 18 hr of incubation (log phase), washed twice with phosphate buffer, and suspended in a fresh mineral medium to obtain an optical density of 1.0 at 600 nm (0.25 mg dry weight of cells/ml of cell suspension). These cells were incubated with [^{14}C]endothall at 25°.

Metabolism of Endothall by *Arthrobacter*. Twenty-milliliter aliquots of cell suspension were placed in 50-ml Erlenmeyer flasks. [^{14}C]Endothall mixed with nonradioactive endothall was added to the cell suspension at concentrations ranging from 25 to 1000 ppm and the flasks were placed on a rotary shaker. At various intervals after the addition of endothall, aliquots of cell suspension were removed and filtered through a Millipore filter. The filtrate was counted for radioactivity in a liquid scintillation counter. The utilization of endothall by the cells was determined by following the disappearance of ^{14}C from the medium. The rate of endothall utilization was calculated from the linear portion of the curve.

For studying the metabolism of endothall by *Arthrobacter*, [^{14}C]endothall was added to the cell suspension at a concentration of 25 ppm. $^{14}CO_2$ evolution from the culture was measured periodically for 4 hr in a closed system, as described above. One-milliliter aliquots of the cell suspension were removed from the incubation flask at appropriate intervals, filtered through a Millipore filter, and washed. For determining the amount of ^{14}C in the cells, the filter was transferred to a liquid scintillation vial and counted. An aliquot of the filtrate was counted to determine the amount of ^{14}C remaining in the medium. To determine the distribution of ^{14}C among various cellular constituents, the cell suspension was centrifuged and the cells were fractionated according to the procedure of Roberts *et al.* (1955).

Short-Term Products of Endothall Assimilation. In these studies, the cells were suspended in 0.002 *M* phosphate buffer (pH 7.0). Ten-milliliter aliquots of cell suspension were placed in 50-ml Erlenmeyer flasks. [^{14}C]Endothall was added to the cultures at a concentration of 100 ppm to obtain relatively high ^{14}C-labeling in the initial products. At various intervals after the addition of endothall, 20 ml of boiling absolute methanol was added to each flask, which was then placed in a hot water bath for 15 min. The methanol extract was centrifuged and the supernatant was removed. The pellet was extracted twice with 50% methanol. For separation of the products of endothall assimilation, the pooled extract was concentrated and an appropriate aliquot was chromatographed two-dimensionally on Whatman No. 1 filter paper. The chromatograms were developed in the first direction with phenol–acetic acid–water with EDTA and in the second with butanol–propionic acid–water (Pedersen *et al.*, 1966). Location of the labeled metabolites on the chromatograms was detected by autoradiography. The radioactive compounds were identified by cochromatography with authentic compounds. The radioactivity of each spot was determined by cutting it out and counting it in the liquid scintillation counter.

Endothall Assimilation in the Presence of Monofluoroacetate. The cells were treated with 2×10^{-2} *M* monofluoroacetate for 30 min prior to incubation with endothall. The fluoroacetate-treated cells and untreated controls were incubated with 100 ppm of [^{14}C]endothall for 15 min. The cells were then centrifuged at 3° and the supernatant was discarded. After washing the pellet with 10 ml of ice-cold 100-ppm nonradioactive endothall solution, the cells were extracted with hot 80% methanol, followed by two extractions with 50% methanol. To determine the total ^{14}C in the extract, an aliquot of the pooled extract was assayed for ^{14}C in a liquid scintillation counter. The ^{14}C remaining in the cell residue was also counted. Aliquots of the cell extract were analyzed for assimilation products by two-dimensional paper chromatography and autoradiography, as described above.

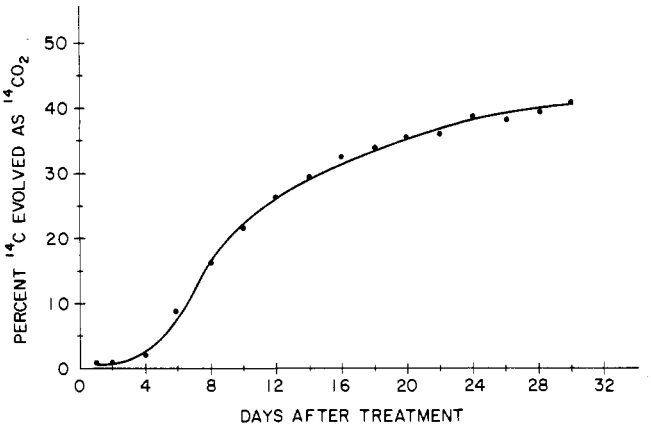

Figure 1. $^{14}CO_2$ evolution from lake water and hydrosoil treated with 10 ppm of [^{14}C]endothall.

RESULTS

Metabolism of Endothall in Lake Water and Hydrosoil. The evolution of $^{14}CO_2$ from lake water and hydrosoil treated with 10 ppm of [^{14}C]endothall is shown in Figure 1. Degradation of endothall began almost immediately after addition of the herbicide; about 25% of the initial ^{14}C added was evolved as $^{14}CO_2$ within 10 days after treatment. Thereafter, the evolution of $^{14}CO_2$ continued at a slower rate and amounted to 40% of the initial ^{14}C after 30 days. $^{14}CO_2$ evolution was not detected from the sterilized soil suspension. These results indicate that aquatic microorganisms readily metabolize endothall.

Assimilation of Endothall by *Arthrobacter*. A species of bacteria capable of utilizing endothall as the sole source of carbon was isolated from hydrosoil by an enrichment culture technique. The species was identified as belonging to the genus *Arthrobacter*. Growth of this species in the presence of various concentrations of endothall as the sole source of carbon was examined. At a concentration of 500 ppm of endothall, the total amount of cell growth equivalent to 3.25 mg dry weight/10-ml cell suspension was produced 18 hr after inoculation. An increase in endothall concentration from 500 to 1000 ppm increased the total growth proportionately without significantly changing the growth rates.

When a cell suspension of *Arthrobacter* was incubated with endothall, it was observed that the herbicide was rapidly removed from the solution. Figure 2 shows the rate of endothall assimilation by *Arthrobacter* at various herbicide concentrations. The rate of assimilation increased with an increase in endothall concentration up to 1000 ppm. The rate was a direct function of the endothall concentration only in the concentration range of 0 to 250 ppm. At endothall concentrations above 250 ppm, the rate of assimilation did not increase in proportion to the increase in the concentration of endothall in the medium.

Metabolism of Endothall by *Arthrobacter*. The metabolism of [^{14}C]endothall by *Arthrobacter* was determined by collecting the $^{14}CO_2$ evolved by the culture and by identifying the ^{14}C metabolites present in the cell and the culture solution. Figure 3 shows the distribution of ^{14}C among the culture solution, the cells, and the $^{14}CO_2$ evolved at various times after incubation of the cell suspension with 25 ppm of [^{14}C]endothall. After 30 min, approximately 83% of the endothall in the culture solution had been removed. Thereafter, there was a gradual decline in the amount of ^{14}C in the external solution, and after 45 min the amount of ^{14}C in the medium remained almost constant. The amount of ^{14}C in the cells reached

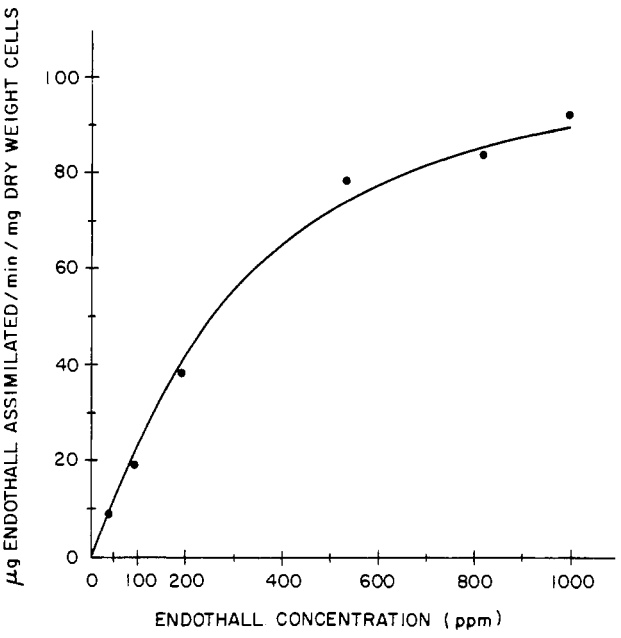

Figure 2. Rate of endothall assimilation by *Arthrobacter* as a function of endothall concentration.

its maximum value 45 min after incubation. In a separate experiment, the cells were harvested by centrifugation after 45 min of incubation with [^{14}C]endothall and extracted with 80% methanol, and the extract was analyzed by two-dimensional paper chromatography. The results showed that almost all of the ^{14}C in the extract was present in the form of endothall metabolites. This indicates that the herbicide was rapidly metabolized upon being taken up by the cell and consequently the cells were able to remove a large portion of it from the medium.

The levels of ^{14}C in the cells gradually decreased after 45 min; this was accompanied by an increase in the evolution of metabolic $^{14}CO_2$. Of the ^{14}C which could be accounted for after incubating the cells for 4 hr with

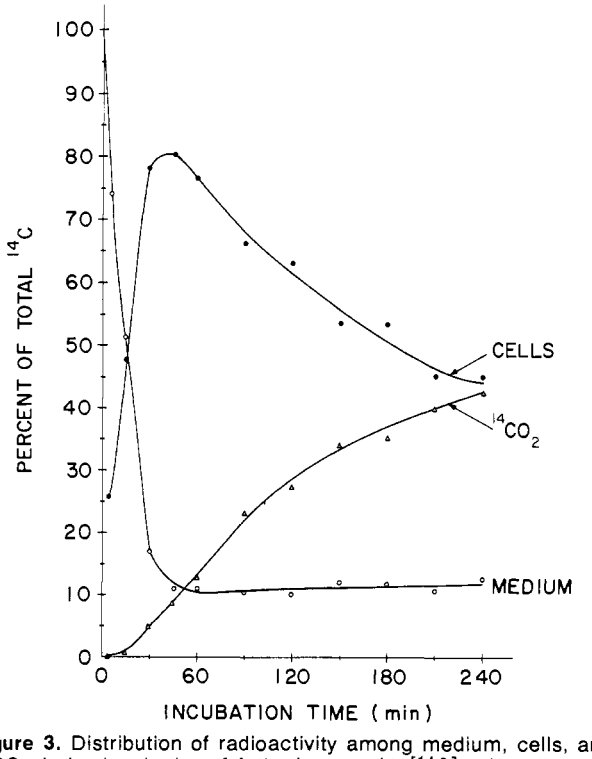

Figure 3. Distribution of radioactivity among medium, cells, and $^{14}CO_2$ during incubation of *Arthrobacter* with [^{14}C]endothall.

Table I. Distribution of ^{14}C in Certain Cellular Components of *Arthrobacter* Incubated with [^{14}C]Endothall

Incubation time, min	Percent ^{14}C incorporated into: Metabolic intermediates	Lipids	Nucleic acids	Residual proteins
15	60.2	7.7	14.4	17.8
30	51.2	9.2	18.4	21.2
45	40.3	11.7	20.8	27.2

[^{14}C]endothall, about 45% was assimilated by the cells, 12% was present in the medium, and about 42% was evolved as $^{14}CO_2$. The ^{14}C remaining in the medium is believed to be due to unused endothall, traces of ^{14}C impurities, and some dissolved $^{14}CO_2$.

Since endothall was readily assimilated by *Arthrobacter*, it was of interest to determine the distribution of the assimilated carbon among the various cellular fractions. For this purpose the cells were subjected to Roberts' fractionation after being incubated with [^{14}C]endothall for different periods. After 15 min of [^{14}C]endothall incubation, a major proportion of the ^{14}C assimilated by the cells was in the form of metabolic intermediates (Table I). With time, the proportion of ^{14}C incorporated into transient intermediates decreased, while the ^{14}C incorporated into lipids, nucleic acid, and the protein fraction increased.

Analysis of the culture medium after 30 min of endothall assimilation indicated that all the ^{14}C in the medium was present in the form of endothall and its impurities. The amount of ^{14}C impurities in the medium did not change, indicating that they were not metabolized by the cells.

Short-Term Products of [^{14}C]Endothall Assimilation. In an attempt to determine the pathway for endothall metabolism, short-term kinetic studies on the assimilation of [^{14}C]endothall were carried out. The relative distribution of ^{14}C among the labeled products in the aqueous methanol-soluble fraction at different times after incubation of *Arthrobacter* with [^{14}C]endothall is shown in Table II. Figure 4 shows a copy of a typical autoradiogram of [^{14}C]endothall and its metabolites. ^{14}C from endothall was rapidly incorporated into citric, aspartic, and glutamic acids. Other early major ^{14}C products were detected on the paper chromatograms in an area generally occupied by phosphate esters (Bassham and Kirk, 1960). In addition, small amounts of ^{14}C appeared in some unknown compounds. The time course of ^{14}C incorporation into various compounds showed that [^{14}C]glutamic acid was a major product in all samples. After 30 sec, about 30% of the ^{14}C incorporated into the soluble fraction was in glutamic acid and the percentage incorporated into this compound increased as [^{14}C]endothall assimilation proceeded. After 15

Table II. Distribution of ^{14}C Among Products Formed from [^{14}C]Endothall in *Arthrobacter*

Incubation time, min	Percent ^{14}C incorporated[a]: Citric acid	Glutamic acid	Aspartic acid	Phosphate esters[b]	Alanine	Unidentified
0.5	6.3	28.2	15.7	22.9	ND[c]	24.8
1	3.7	37.2	6.5	31.1	2.0	21.5
2	3.4	46.5	4.3	30.3	1.8	15.5
10	1.8	64.7	2.1	15.6	1.0	15.8
15	1.3	63.2	1.3	16.8	0.6	17.3

[a] Calculated as percentage of radioactivity in ^{14}C metabolites detected on the paper chromatogram. [b] Not positively identified; this area on the paper chromatogram is generally occupied by phosphate esters. [c] Not detectable.

min, [^{14}C]glutamic acid accounted for 63% of the ^{14}C incorporated into the methanol-soluble fraction. ^{14}C was also detected in citric and aspartic acids after 30 sec, but the percentage incorporated into these compounds decreased with time. Alanine did not become labeled until 1 min after the addition of labeled endothall.

The Effect of Monofluoroacetate on [^{14}C]Endothall Assimilation. The assimilation of [^{14}C]endothall by *Arthrobacter* was inhibited by about 23% in the presence of 2×10^{-2} *M* fluoroacetate. Fluoroacetate also produced changes in the relative amounts of ^{14}C-labeled compounds as compared with the controls. Table III shows the effect of fluoroacetate on the distribution of ^{14}C in compounds resulting from [^{14}C]endothall assimilation. The main effect of the inhibitor was to decrease the proportion of [^{14}C]glutamic acid, which was accompanied by an increase in the proportion of [^{14}C]citric acid. Fluoroacetate caused a 40-fold accumulation of labeled citric acid, while the percentage of ^{14}C incorporated in glutamic acid decreased by about 25%. The percentage of [^{14}C]aspartic acid and alanine showed an increase in the presence of fluoroacetate.

Metabolism of Endothall by *Arthrobacter* in the Presence of an Exogenous Carbon and Energy Source. It was noticed that the endothall-adapted cells were able to grow on glucose without a lag, which suggests the possibility that the two compounds were metabolized by the same enzyme system. In order to determine if glucose and endothall compete for the same enzymatic site(s), the metabolism of [^{14}C]endothall by endothall-grown cells was studied in the presence of glucose. As shown in Figure 5, the addition of glucose (0.5 g/l.) to the culture medium did not appreciably affect the evolution of $^{14}CO_2$. These findings seem to indicate that endothall and glucose were not competing for the same enzymatic site(s) and that the cells utilized endothall as the preferential substrate. The small decrease in $^{14}CO_2$ evolution may be attributed to a suppression by glucose of the enzyme(s) responsible for endothall degradation.

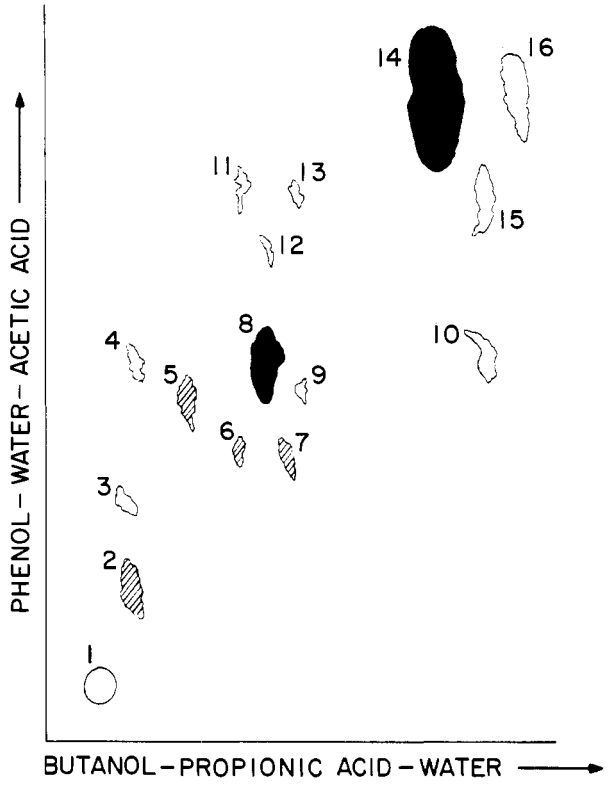

Figure 4. Autoradiographic presentation of a two-dimensional paper chromatogram of [^{14}C]endothall and its metabolites produced by *Arthrobacter*. The numbers in the figure indicate the following compounds: 1, origin; 2, phosphate esters; 6, aspartic acid; 7, citric acid; 8, glutamic acid; 13, alanine; 3, 4, 5, 9, 10, 11, 12, unidentified; 14, endothall; 15, 16, endothall impurities. The black areas indicate highest radioactivity; shaded areas, medium radioactivitv; open areas, weak radioactivity.

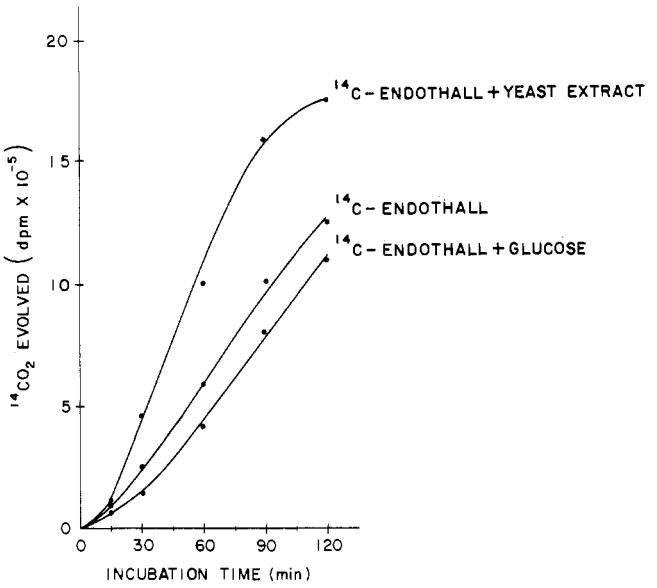

Figure 5. $^{14}CO_2$ evolution by *Arthrobacter* incubated with 25 ppm of [^{14}C]endothall in the presence of glucose or yeast extract.

Since it was observed that endothall was rapidly metabolized by *Arthrobacter* to form different amino acids, it was of interest to determine if the addition of exogenous amino acids in the form of yeast extract would have any influence on the metabolism of endothall. The presence of yeast extract (0.5 g/l.) in the culture medium enhanced the rate of $^{14}CO_2$ evolution from the cells incubated with [^{14}C]endothall (Figure 5). We suggest that in the presence of preformed amino acids, endothall was utilized predominantly for energy-generating reactions, thereby resulting in an increased evolution of $^{14}CO_2$.

DISCUSSION

The results of this investigation clearly indicate that aquatic microorganisms readily degrade endothall. A species of *Arthrobacter* isolated from lake water and sediment was able to utilize endothall as the sole source of carbon and energy for cell growth. Jensen (1964) has reported that an *Arthrobacter* species isolated from soil was also capable of utilizing endothall as the sole source of carbon. Until now the nature of products resulting from endothall degradation by microorganisms was not known. Our studies on the metabolism of [^{14}C]endothall labeled

Table III. Distribution of ^{14}C Among Products Formed from [^{14}C]Endothall by *Arthrobacter* in the Presence of Fluoroacetate

^{14}C product	Percent ^{14}C incorporated[a]	
	Control	Fluoroacetate
Citric acid	0.3	13.5
Glutamic acid	60.1	45.4
Aspartic acid	0.5	1.1
Alanine	0.8	1.3
Phosphate esters[b]	15.5	14.3
Unidentified	23.6	25.7

[a] Calculated as percentage of radioactivity in ^{14}C metabolites detected on the paper chromatogram. [b] Not positively identified; this area on the paper chromatogram is generally occupied by phosphate esters.

in positions two and three of the oxabicyclo ring show that ^{14}C from endothall is rapidly incorporated into cell intermediates like glutamic, aspartic, and citric acids, and alanine, which provide carbon and energy necessary for cell growth. The nature of the ^{14}C products resulting from [^{14}C]endothall assimilation suggests that the herbicide was metabolized through a pathway involving splitting of the oxabicyclo ring. Evolution of $^{14}CO_2$ was also noticed from the cultures incubated with [^{14}C]endothall. The fact that ^{14}C from endothall appeared in the cell intermediates before any noticeable $^{14}CO_2$ evolution was detected seems to indicate that [^{14}C]endothall was first degraded to certain ^{14}C products, which were subsequently metabolized to give off $^{14}CO_2$.

A rapid appearance of ^{14}C in citric acid, an intermediate of the tricarboxylic acid (TCA) cycle, and into aspartic and glutamic acids, the two amino acids most closely associated with the TCA cycle, suggests that carbon from endothall enters the TCA cycle. The failure to detect other intermediates of the cycle could be attributed to a small pool size for these products. The initially higher labeling in aspartic acid than in citric acid suggests that in the earliest stages of incubation the isotope from endothall entered the TCA cycle *via* a route not involving a condensation of acetate and oxaloacetate to form citrate.

[^{14}C]Glutamic acid was a major early product and, irrespective of the period of exposure, the ^{14}C incorporated into glutamic acid exceeded that in any of the TCA cycle intermediates. The initially higher labeling in glutamic acid than in citric acid suggests that the isotope from endothall entered glutamic acid without passing through the stage of citric acid. This hypothesis was supported by the results of our studies on the assimilation of [^{14}C]endothall by *Arthrobacter* in the presence of monofluoroacetate, which is known to inhibit the metabolism of citric acid proceeding *via* the TCA cycle (Elliott and Kalnitsky, 1950; Morrison and Peters, 1954). Kalnitsky and Barron (1947) observed that 2×10^{-3} *M* fluoroacetate completely inhibited the oxidation of acetate in *Corneybacterium* and yeast. If all the glutamic acid in the cells which were assimilating [^{14}C]endothall were derived *via* citric acid, one would expect an almost complete inhibition of ^{14}C incorporation into glutamic acid in the cells treated with a fluoroacetate concentration as high as 2×10^{-2} *M*. Since the percentage of ^{14}C incorporated in glutamic acid was reduced by only about 25% in the presence of fluoroacetate, it appears likely that a pathway not involving the TCA cycle was contributing to the production of glutamic acid from endothall. A partial inhibition of the ^{14}C incorporation in glutamic acid, accompanied by an accumulation of [^{14}C]citric acid in the presence of fluoroacetate, indicates that some glutamic acid may also have been produced from endothall *via* a pathway involving the TCA cycle. The increase in the proportion of [^{14}C]aspartic acid and alanine in the presence of fluoroacetate (Table III) can be explained by assuming that fluoroacetate, by inhibiting the conversion of citric acid, resulted in more ^{14}C being shunted into aspartic acid and alanine.

We conclude from our results that the pathway(s) leading to the formation of glutamic acid directly from endothall plays a significant part in the metabolism of endothall by *Arthrobacter* and that it is unlikely that glutamic acid is derived *via* the TCA cycle exclusively. Although the kinetic analysis did not reveal the pathway by which [^{14}C]endothall or its fragments gave rise to early products of endothall metabolism, we propose on the basis of our findings that a likely initial step in the degradation of endothall is the cleavage of the endoxo bridge to form a cyclohexanone-type compound(s). It has been reported that cyclohexanone and related compounds can be metabolized by microorganisms to produce adipic and valeric acids and other products (Fabre *et al.*, 1959; Imelik, 1948). Similarly, the cyclohexanone-type compound(s) formed from endothall may be degraded *via* ring cleavage to six-or-more-carbon fragments which may yield glutamic acid. Alternately, these compounds may degrade to four-carbon fragments, resulting in the formation of certain TCA cycle intermediates.

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