TCA Metabolism by Soil Microorganisms

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Trichloroacetate (TCA) metabolism by a *Pseudomonas* sp. isolated from soil requires an exogenous supply of yeast extract for optimal liberation of halide ion. Carbon dioxide evolution and distribution of radioactivity into cellular components from TCA-1-^{14}C and TCA-2-^{14}C are not equal. The nonparallel metabolic pathways for the two carbons in TCA would seem to eliminate a free symmetrical intermediate such as oxalic acid as the first product of TCA decomposition. Serine and two additional metabolites were detected on paper chromatograms. Preliminary enzymic studies demonstrated the necessity of certain cofactors for fragmentation of TCA. Deletion of NADPH or CoA had an inhibitory effect on the capacity of the cell free system to liberate $^{14}CO_2$ from TCA-1-^{14}C.

Sodium trichloroacetate (TCA) is a widely used herbicide for the control of certain grasses. Soil microorganisms are known to attack many of the simple chlorinated aliphatic acids (Kearney *et al.*, 1965). Jensen (1957, 1960) isolated a group of *Arthrobacter*-like bacteria that grew feebly in mineral salt–TCA solutions. Metabolism was accompanied with Cl^- release from TCA. Two strains of *Pseudomonas dehalogenans* could be adapted to attack TCA, but mono- and dichloroacetate were attacked more rapidly (Jensen, 1960). Dehalogenation of TCA has been reported by Hirsch and Alexander (1960) with an unidentified *Pseudomonas*, and by Hirsch and Stellmach-Helwig (1961) with another unidentified soil bacterium. Gemmell and Jensen (1964) isolated two strains of soil bacteria (probably systematically related to *Arthrobacter*) that grew feebly in all media examined. TCA and its theoretical dehalogenation product, oxalate, could both serve as carbon sources. Amino acids were stimulators to the dehalogenation process, but glucose was inhibitory.

Although the dehalogenation process has been studied extensively from a microbiological standpoint, the biochemical aspects of TCA metabolism have not been reported. The object of the present paper is to report on metabolism studies with a pure culture of a soil microorganism utilizing sodium TCA labeled with ^{14}C in carbons 1 and 2. Some preliminary results with a crude enzyme system capable of fragmenting TCA will also be described.

EXPERIMENTAL

Growth Conditions and $^{14}CO_2$ Studies. A *Pseudomonas* sp. was isolated from soil solutions containing 1 gram (acid equivalent) per liter of the sodium salt of TCA. Growth was determined by noting an increase in turbidity and the release of halide ion (Iwasaki *et al.*, 1952). Cells were routinely cultured in 1 liter of nutrient solution with the same composition as previously described (Kearney, 1965), containing 1 gram per liter of TCA. Trichloroacetic acid was neutralized to pH 7.0 with concd. K_2HPO_4 before addition to sterilized nutrient solution. The TCA-nutrient solution was then filtered aseptically into a sterile flask. Cells were cultured for 6 to 8 days on a shaker at 25° to 30° C. The pH at harvest was 7.0. For $^{14}CO_2$ evolution studies, 1 liter of cells was harvested by centrifugation, resuspended in 10 ml. (absorbance = 0.19), and then incubated for various intervals with ^{14}C-TCA. Labeled $^{14}CO_2$ was trapped in standard alkali (5 ml. of 0.1*M* NaOH). Saturated $BaCl_2$ (2 ml.) was added to the trapping solution, and the precipitate collected on filter paper. The precipitated $Ba^{14}CO_3$ was washed with 2- to 5-ml. aliquots of hot distilled water and 10 ml. of ethanol. All samples were corrected for background, self absorption, and dilution.

Reagents and Substrates. TCA-1-^{14}C (spec. act. 6.4 mc. per mmole) and TCA-2-^{14}C (spec. act. 6.7 mc. per mmole) were obtained from Nuclear-Chicago Corp. 3-Phosphoglyceric acid, phosphoserine and the enzyme alkali phosphatase were from California Biochemical Co.

RESULTS

Growth of the *Pseudomonas* sp. with TCA as the sole carbon source was extremely feeble. Metabolism of TCA as measured by Cl^- liberation was materially enhanced by the addition of small amounts of exogenous carbon. Figure 1 shows Cl^- liberation as a function of time in a solution containing various amounts of yeast extract. As a consequence of the enhanced dehalogenation stimulated by the addition of 0.5 gram per liter of yeast extract, all future cultures were supplemented with the external carbon source.

Metabolism Studies. The 100-fold concentrated cells harvested by centrifugation in the cold were placed in a gas-tight CO_2-trapping system, and aerated for 15 to 30 minutes with CO_2-free air at 30° C. before inoculation into labeled TCA solutions. Labeled solutions of TCA-1-^{14}C and TCA-2-^{14}C were prepared as follows: Each tube contained 3.5 ml. of nutrient solution, 0.4 ml. of phosphate buffer, pH 7.0 (0.1*M*), 0.05 ml. of TCA-^{14}C (0.5 μc.), 0.2 ml. of Na-TCA (20 μmoles), 0.8 ml. of cells and 0.06 ml. of water, to give a final volume of 5 ml. Blanks were prepared by adding 0.8 ml. of nutrient solution instead of cells. CO_2 traps were refilled with CO_2-free base after 15, 30, 45, 60,

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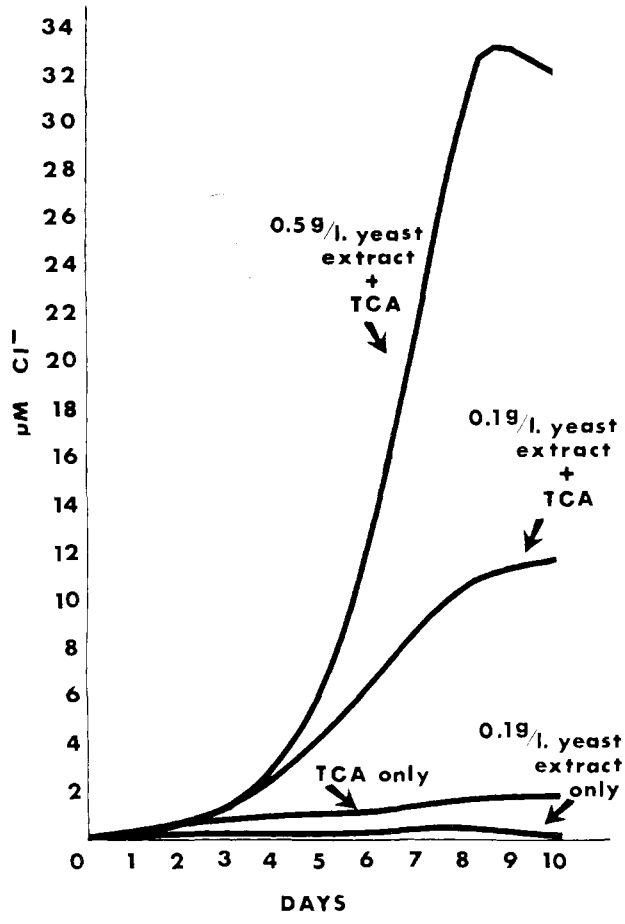

Figure 1. Chloride ion liberation from sodium-TCA by isolated soil microorganism in presence of various concentrations of yeast extract in media

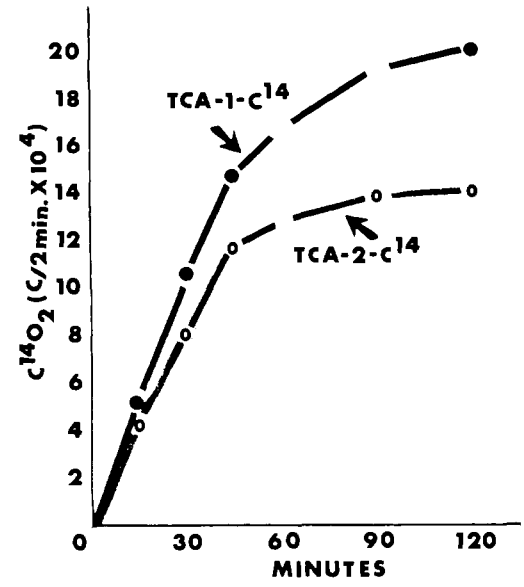

Figure 2. $^{14}CO_2$ evolution from TCA-1-^{14}C and TCA-2-^{14}C in culture solutions of isolated soil microorganism during 2-hour metabolism study

90, and 120 minutes. Figure 2 shows $^{14}CO_2$ evolution from TCA-1-^{14}C and TCA-2-^{14}C during a 2-hour experiment. Labeled $^{14}CO_2$ was evolved from both labeled forms of TCA. $^{14}CO_2$ production was more extensive from TCA-1-^{14}C than from TCA-2-^{14}C. This experiment was replicated twice and repeated on four separate occasions. In each experiment, the rate of $^{14}CO_2$ from TCA-1-^{14}C always exceeded that from TCA-2-^{14}C. The possibility of this difference being due to mixed cultures of microorganisms in the inoculation was studied. Reisolated cultures produced identical results. Differences in specific activities were examined and found to be within 4% of each other. Results would indicate, therefore, that the difference between rates of CO_2 production from carbons 1 and 2 is real.

A second experiment was initiated to determine the distribution of the labeled carbons between various cellular components. The $^{14}CO_2$ evolution pattern closely followed that noted in Figure 2. After termination of the CO_2 study, labeled cells were collected by centrifugation and fractionated into transient intermediate, lipid, nucleic acid, and protein fractions, as described by Roberts *et al.* (1957). More than twice the amount of activity in cellular materials was contributed by TCA-2-^{14}C than by TCA-1-^{14}C (108,540 *vs.* 39,820 counts per 2 minutes, respectively). Labeled carbon from TCA-2-^{14}C was found primarily in the protein fraction (54%), the remaining activity being divided between the lipid fraction (25%), the nucleic acid fraction (12%), and the transient intermediate fraction (9%).

Products of Metabolism. To determine the identity and distribution of various labeled metabolites from TCA produced by bacterial cells, solutions were prepared to contain 0.2 ml. of cells, 0.1 ml. of TCA (0.5 μc.), 0.1 ml. of buffer, pH 7.0, and 0.6 ml. of nutrient solution, to give a total volume of 1 ml. After incubation for different time intervals, 5 ml. of warm acetone was added to the culture tubes, which were then held in a warm water bath for approximately 15 minutes. Cellular debris was removed by centrifugation and samples were reduced in volume for chromatography on Whatman No. 1 filter paper. Paper chromatograms were developed in the first direction with phenol-water and in the second with butanol–propionic acid–water (Benson *et al.*, 1950). Chromatograms were exposed to "no screen" x-ray film for two weeks, and activity in each spot was then determined with a hand counter. Generally, TCA and three metabolites were detected on chromatograms of samples taken during the first 45 minutes (Figure 3). An early major metabolite (unknown I) was detected on the chromatogram just below the red dye marker. Several amino acids, notably serine, are known to move in this vicinity with authentic samples in the same solvent systems (Benson *et al.*, 1950).

Unknown I was subsequently identified as the amino acid, serine. Identity was confirmed by cochromatography in three solvent systems with authentic serine on cellulose-coated thin-layer plates. Two additional spots (unknowns II and III) were detected on paper chromatograms in an area generally occupied by phosphate esters (Figure 3). However, they could not be identified as suspected products of serine metabolism, including phosphoserine or 3-phosphoglyceric acid. Since these two latter metabolites tended to accumulate after the disappearance of serine, no further attempts were made to identify them. The per cent distribution of radioactivity in serine and the two unknowns is shown

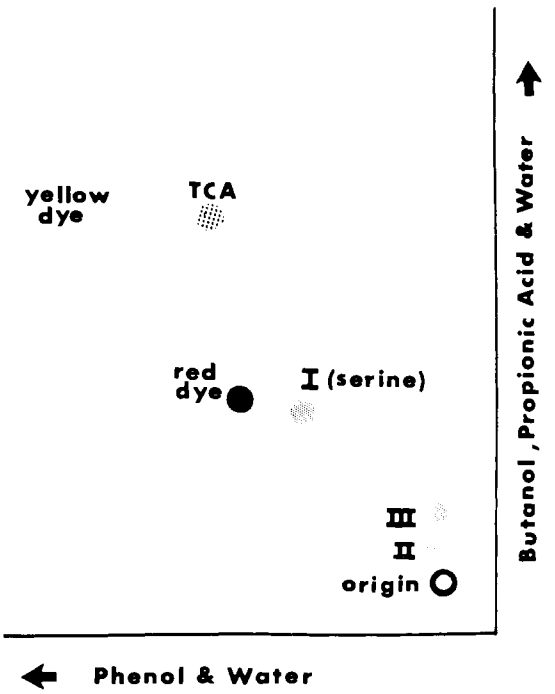

Figure 3. Distribution of ^{14}C from TCA-2-^{14}C into various metabolites on two-dimensional paper chromatogram developed first in phenol–water and in the second direction by butanol–propionic acid–water (4:1:5)

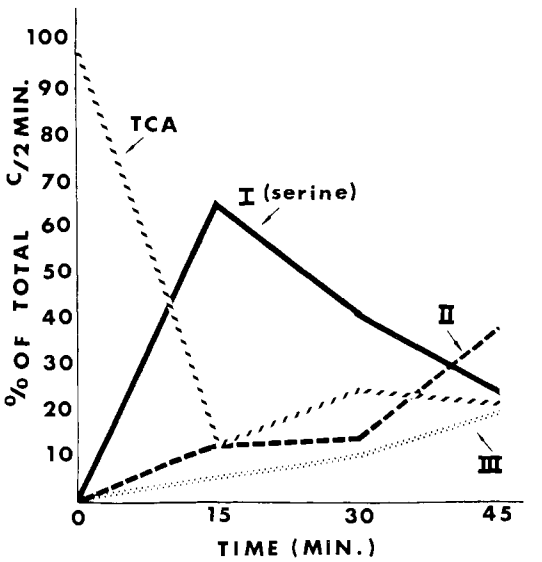

Figure 4. Per cent activity of ^{14}C from TCA-2-^{14}C into serine and two unknowns at various time intervals after addition of labeled TCA

in Figure 4. Other spots were detected on film, but low activity prevented accurate counting to determine per cent distribution.

Enzymatic Studies. To study the initial reactions associated with TCA metabolism in greater detail, some preliminary enzymic studies were conducted with broken cell preparations. Routinely, 2 liters of cells were harvested by centrifugation at 5° C. Harvested cells were washed with ice cold distilled water and resuspended in 20 ml. of 0.4*M* Tris-HCl buffer solution at pH 7.2, containing $5 \times 10^{-4}M$ dithiothreitol. The solubilized cells were next broken in a Raytheon sonic oscillator, Model DF 101, for 15 minutes at 7° C., and the broken cell walls separated by centrifugation at 10,000 × G for 10 minutes.

The assay solution was designed so that any $^{14}CO_2$ evolved from either labeled carbon of TCA or from early metabolites of TCA could be measured. Several cofactors, including coenzyme A (CoA) and adenosine 5′-triphosphate (ATP) plus soluble protein, were added to a gas-tight Warburg vessel, while radioactive TCA (0.5 μc.) plus 1 μmole of unlabeled TCA were added to one side arm of the vessel. Assay solution contained the following: 1 μmole of TCA in 0.1*M* tris buffer (pH 7.2) plus 0.4 μc. of TCA-1-^{14}C or TCA-2-^{14}C in one side arm, and 1 ml. of protein (3 mg. of protein), 10 μmoles of ATP, 1 μmole of CoA, 5 μmoles of glutathione, 5 μmoles of $MgSO_4$ and 0.4 ml. of 0.1*M* tris buffer (pH 7.2). The center well of the vessel contained 0.3 ml. of 20% KOH. The remaining side arm contained saturated $KHSO_4$ (0.5 ml.) and was used to terminate the reaction. The reaction vessel was placed in a water bath at 30° C. and preincubated for approximately 10 minutes. After 45 minutes, the reaction was terminated with acid and the amount of $^{14}CO_2$ determined in barium carbonate precipitates. A total of 13,840 counts per 2 minutes was released from TCA-1-^{14}C as $^{14}CO_2$, as compared to 2020 from TCA-2-^{14}C.

Attempts to purify the enzyme system by $(NH_4)_2SO_4$ precipitation generally led to greatly reduced activity. The essentiality of a number of cofactors, however, was studied in a fraction obtained after elution through Sephadex G-100, previously equilibrated with buffer and dithiothreitol. Table I shows the amount of CO_2 released from TCA-1-^{14}C by the assay mixture minus various cofactors.

DISCUSSION

As a general rule, the chlorinated aliphatic acid herbicides do not persist for extended periods of time in soil when applied at the recommended rates of application. Most of the evidence collected thus far indicates that soil microorganisms play an important part in their detoxification (Kearney *et al.*, 1965). Detailed studies with isolated soil microorganisms indicate that metabolism of the chlorinated aliphatic acids

Table I. Effect of Various Cofactors on Evolution of $^{14}CO_2$ from TCA-1-^{14}C in Crude Enzyme System Eluted through Sephadex G-100. Assays Conducted for 2 Hours at 30° C.

Assay Solution	Counts per 2 Min., $^{14}CO_2$	% of Complete
Complete[a]	10,620	—
−Mg^{++}	10,810	—
−NADH, −NADPH	690	6.4
−NADH, −NADPH	945	8.8
−TPP	7,625	72
−CoA	5,855	55

[a] Complete assay has the same composition as that listed under *Enzymatic Studies*, plus the cofactors (NADH and NADPH [10 μmoles/assay], and TPP [0.1 μmole/assay]) listed above. NADH = reduced nicotinamide adenine dinucleotide; NADPH = reduced nicotinamide adenine dinucleotide phosphate; TPP = thiamine pyrophosphate.

is usually accompanied by release of Cl^- into the nutrient solution. Figure 1 shows that TCA metabolism follows this same pattern. Unlike 2,2-dichloropropionate metabolism by a species of *Arthrobacter* reported in previous studies (Kaufman, 1964), the *Pseudomonas* sp. isolated from soil exhibits feeble growth on TCA; it must be supplied an exogenous source of carbon before Cl^- release is rapid. Whether energy is needed to activate TCA for metabolism by a constitutive system or whether the activation and initial enzyme must be induced is not yet known.

The initial reactions associated with chlorinated aliphatic acid metabolism involve replacement of the halide atom with a hydroxy group to form the corresponding hydroxy or keto aliphatic acid. Several enzymatic studies have verified this reaction. The enzymatic conversion of 2,2-dichloropropionate (dalapon) to pyruvate (Kearney *et al.*, 1964) and of fluoroacetate to glycolate (Goldman, 1965) have been reported. Resting cells of a species of *Pseudomonad* have been reported to convert 3-bromopropionate to 3-hydroxypropionate (Castro and Bartnicki, 1965).

If hydroxylation is involved in TCA metabolism, then the product of the reaction would be oxalic acid. Oxalate has been suggested as a product of TCA in soil microorganism cultures (Gemmell and Jensen, 1964; Jensen, 1960). Furthermore, oxalate can serve as a carbon source for soil microorganisms adapted to metabolize TCA (Gemmell and Jensen, 1964).

Oxalate catabolism has been demonstrated to proceed by at least three routes (Jakoby and Bhat, 1958) which involve oxidation, decarboxylation, and activation followed by decarboxylation. Oxidation of oxalate yields 2 moles of CO_2 plus hydrogen peroxide, and is found only in mosses and higher plants (Castro and Bartnicki, 1965; Houget *et al.*, 1927, 1928). Direct decarboxylation yields CO_2 and formate, and is found only in white rot, a wood-destroying fungi (Shimazoro and Hayaishi, 1957). A bacterial system, described for a species of *Pseudomonad*, catalyzes the conversion of oxalate to formate and CO_2 in the presence of acetyl CoA and TPP (Jakoby *et al.*, 1956).

If free oxalate is the first product of TCA metabolism, then direct oxidation of 1 mole of oxalate to 2 moles of CO_2 would not be involved in the present system, since large amounts of labeled carbon are incorporated into various cellular components. The appearance of serine as a product of TCA metabolism, however, would be consistent with formate production from oxalate, since formate is known to enter one carbon metabolism and be incorporated into the beta carbon of serine (Rabson *et al.*, 1962). The scheme of reactions shown in Figure 5 could be proposed.

The major objection to the proposed scheme is the inconsistency between free oxalate and the unequal distribution in $^{14}CO_2$ production noted in Figure 2. If TCA is first converted to free oxalate followed by decarboxylation to formate and CO_2, then the rates of

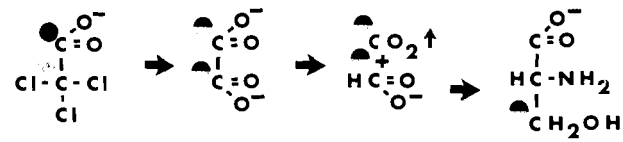

Figure 5. Hypothetical pathway for TCA metabolism into oxalate, formate, CO_2, and serine. Dark dots indicate ^{14}C in carbon 1 of TCA. Light dot represents carbon 2 of TCA

CO_2 production from oxalate, or consequently TCA-1- or TCA-2-^{14}C, should be identical. Oxalate is a symmetrical molecule and enzymatic catalysis should proceed with equal production of CO_2 from either carbon of oxalate, regardless of whether it was derived from TCA-1-^{14}C or TCA-2-^{14}C. The unequal production of CO_2 and the unequal incorporation of radioactivity into various cellular components would speak against the series of reactions delineated in Figure 5.

The enzyme studies, although preliminary observations, suggest that certain cofactors are essential in an isolated cell free system before CO_2 liberation can take place. The inhibition noted by deletion of CoA suggests that activation may be an important part of the process and may lend some support to the TCA-CoA, oxylyl-CoA pathway.

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