THE BACTERIAL OXIDATION OF PHENYLACETIC ACID

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The bacterial oxidation of aromatic compounds closely related in chemical structure may proceed by different pathways. Thus, while the main features of the breakdown of mandelic acid are established, those of phenylacetate are not, except insofar as it is known to follow a different route (Stanier, 1950). The divergencies may possibly indicate a difference in the mode of ring fission; and since it is established that mandelic acid and certain other aromatic compounds give rise to β-ketoadipic acid (Kilby, 1948, 1951; Stanier, 1950), we have investigated the production of keto acids in phenylacetate metabolism. At the same time we have attempted to determine whether the reactions of the tricarboxylic acid cycle play a part in these oxidations, and whether there is any evidence under favorable conditions for an abridged cycle as postulated by Barron, Ardao, and Hearon (1950) for *Corynebacterium creatinovorans* and termed by them the "dicarboxylic acid cycle".

Pyruvic acid accumulates in aerated cultures of *Aerobacter aerogenes* growing on glucose or various dicarboxylic acids only when its rate of production exceeds the demands of logarithmic growth, while shortly after cell division ceases its presence may no longer be detected (Dagley *et al.*, 1951). Since conditions in a growing or fully grown culture may not favor detection of metabolic intermediates, we have followed keto acid production by nonproliferating suspensions aerated in media from which the source of nitrogen has been omitted. Under such conditions the concentration of keto acids in the medium rises initially, attains a maximum, and then declines eventually to zero when the rate of decomposition exceeds the rate of formation. In studies of keto acid production, therefore, it is essential to conduct kinetic investigations over a period of time defined by the position of the concentration maximum.

EXPERIMENTAL METHODS

The organism used throughout this work was the *Vibrio* O1 originally isolated by Happold and Key (1932). This organism is capable of utilizing as sole metabolites various phenols, substituted benzoic acids, and phenolic amino acids (Evans, 1947).

The basal medium was as follows: Carbon source 0.01 M, $(NH_4)_2SO_4$ 1 g, K_2HPO_4 0.5 g, NaCl 5 g, $MgCl_2$ 0.5 g, $CaCl_2$ 0.1 g, $CuSO_4 \cdot 5H_2O$ 10 mg, $ZnSO_4 \cdot 7H_2O$ 8 mg, $FeCl_3$ 10 mg, $MnSO_4$ 3 mg per liter of glass distilled water. The pH was adjusted to 7.2; the additions of $MgCl_2$ and $CaCl_2$ were made aseptically after autoclaving; cells were trained to metabolize the various carbon sources by at least 3 serial subcultures in the growth medium aerated by a gentle stream of sterile air at 30 C. Batches of fully grown cultures could then be harvested

by centrifugation after overnight growth and cell suspension in M/15 phosphate buffer (pH 7.0) prepared after washing twice with distilled water.

Production of keto acids was determined as follows: the cells from 1 liter of a fully-grown culture were harvested, washed, resuspended in 1 ml of phosphate buffer, and added to 100 ml of aerated basal medium from which $(NH_4)_2SO_4$ was omitted. In all experiments the concentration of the carbon source was 0.027 M. At suitable intervals, samples were withdrawn and rapidly centrifuged giving clear supernatants for the estimation of keto acids by the method of Friedemann

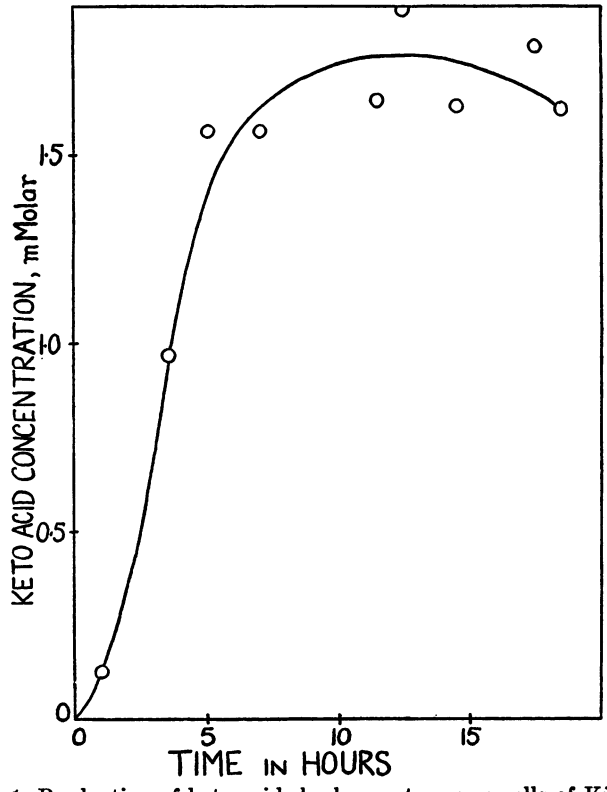

Figure 1. Production of keto acids by benzoate-grown cells of *Vibrio* O1.

and Haugen (1943). A calibration curve was prepared with freshly distilled pyruvic acid using a Spekker photoelectric absorptiometer with Ilford filters (Neutral H508 and Green OG1).

For paper chromatography of 2:4-dinitrophenylhydrazones of keto acids the method of Cavallini *et al.* (1949) was employed, using as solvent a mixture of butanol, ethanol, and ammonium carbonate buffer in the ratio of 80:22:38 by volume.

Oxygen uptakes were measured with the conventional Warburg apparatus. All experiments were conducted at 30 C in an atmosphere of air using 1.0 ml of cell suspension in a final volume of 3.0 ml; 0.6 ml of 0.01 M substrate was added from the side arm, and KOH was used in the center well to absorb carbon dioxide.

RESULTS

Cells trained to metabolize benzoic acid. Figure 1 shows the total accumulation of keto acids from benzoic acid. A washed suspension of cells trained to metabolize this substrate was aerated with basal medium from which the source of nitrogen for growth had been omitted.

The nature of the keto acids produced was investigated by means of paper chromatography. Ten ml samples were withdrawn at intervals from media in which keto acid production was being followed. After centrifugation, 2:4-dinitrophenylhydrazine reagent (Friedemann and Haugen, 1943) was added and after standing for half an hour at 25 C the solution was extracted with ethyl acetate in a separating funnel. The 2:4-dinitrophenylhydrazones were re-extracted with 10 per cent Na_2CO_3 solution, acidified, and finally extracted with the minimum amount of ethyl acetate. These extracts gave bright yellow spots on chromatograms, and their passage could be followed visually. Two widely separated spots were observed, one in a position indicating pyruvic acid in the original medium and the other, a slower-moving spot, indicating a dicarboxylic keto acid. The latter spot was then eluted with 10 per cent Na_2CO_3 solution, acidified, and re-extracted with a small quantity of ethyl acetate, and the resulting 2:4-dinitrophenylhydrazone solution boiled for a few minutes and chromatographed. After this treatment, eluates arising from samples taken during aeration of the original medium in the first 5 hours no longer produced the dicarboxylic keto acid spot which was replaced completely by a fast-moving spot corresponding to levulinic acid. This behavior is to be expected on the assumption that the original dicarboxylic keto acid was β-ketoadipic acid, formed in the accepted pathway for the microbial oxidation of benzoic acid, since its 2:4-dinitrophenylhydrazone rapidly decomposes in boiling ethyl acetate solution to give the derivative of levulinic acid.

For a sample taken after 6 hours, and for all subsequent samples, two spots were also given, but the position of the dicarboxylic keto acid derivative remained unaltered after the boiling treatment previously described. A second dicarboxylic acid, therefore, had taken the place of β-ketoadipic acid in the metabolizing fluid, and the following evidence was obtained that the new compound was α-ketoglutaric acid: (1) the 2:4-dinitrophenylhydrazone of α-ketoglutaric acid was not decomposed when boiled in ethyl acetate solution; (2) its Rf value, 0.20, was the same as that of the derivative of the dicarboxylic acid from the metabolizing fluids; (3) possible confusion with the 2:4-dinitrophenylhydrazone of oxalacetic acid, which runs only a little faster on the chromatogram, was eliminated either by maintaining samples of the metabolizing fluids at 100 C for 15 minutes in 0.5 N HCl or by treating them with aniline citrate before addition of the 2:4-dinitrophenylhydrazine reagent: neither method eliminated the spot of Rf 0.20; (4) the absorption spectrum was shown to be identical with that of the 2:4-dinitrophenylhydrazone of α-ketoglutaric acid. Spots were eluted from the chromatogram with 10 per cent Na_2CO_3 solution, the color developed by addition of an equal volume of 1.5 N NaOH, and the absorption curve obtained using a Beckman spectrophotometer. Comparison was made against a standard

curve established for α-ketoglutarate of comparable concentration. These curves are shown in figure 2.

Cells trained to metabolize phenylacetic acid. Production of keto acids from phenylacetate by phenylacetate-grown cells was followed by the methods previously described. The curves obtained differed from figure 1 in showing a lag period of approximately 9 hours before significant keto acid accumulation commenced while the maximum concentration eventually attained was only 300 μM approximately as against 1,800 μM shown in figure 1. The lag in keto acid production may be related to the observation that cells inoculated into complete phenylacetate medium exhibit lag before growth even though they have been previously

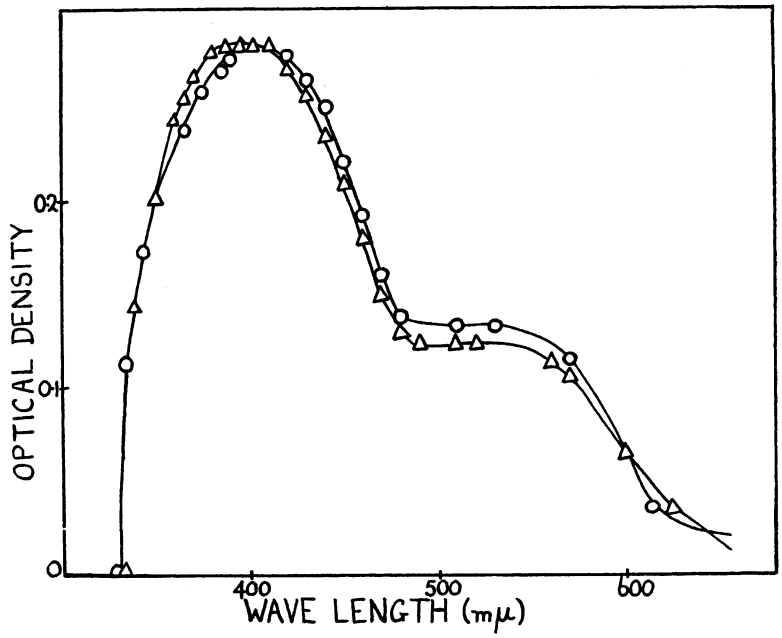

Figure 2. Absorption spectra of: ○ eluate from chromatogram; △ 2:4-dinitrophenylhydrazone of α-ketoglutaric acid.

trained to metabolize phenylacetate. Analysis of the keto acids produced from phenylacetate showed a mixture of pyruvate and α-ketoglutarate, but attempts to show β-ketoadipate were negative for the whole period of keto acid production. Failure to show the presence of a metabolite does not preclude the possibility of its formation, however, since detection depends upon the extent to which the compound is able to accumulate. For an intermediate in any reaction sequence accumulation is governed by the kinetics of formation and decomposition; and the maximum concentration attained may be very small, and possibly below the limits of detection, if the specific reaction rate of decomposition is large compared with that of formation. Nevertheless, the absence of detectable quantities of β-ketoadipate during phenylacetate oxidation may be noted in relation to other differences in metabolism between phenylacetic and other aromatic

acids. Using the technique of simultaneous adaptation (Stanier, 1947, 1950), we have shown that the oxygen uptake curves for cells trained to metabolize phenylacetate showed a well defined period of adaptation with benzoate as substrate, but not with phenylacetate. Similar experiments with mandelic acid and benzaldehyde confirmed the findings of Stanier (1947) with *Pseudomonas fluorescens* (strain A-3-12) that the pathway established for mandelic and benzoic oxidation is not followed in the case of phenylacetic acid. The divergence in pathways may occur at the point of ring fission; phenylacetate may give rise to α-ketoglutarate directly while for benzoate and other compounds β-ketoadipate may be formed first. Our experiments do show definitely, however, that α-ketoglutarate is an intermediate common to both pathways. Its presence in the metabolizing fluids in turn suggests that the subsequent oxidation follows the tricarboxylic acid cycle pathway. Accordingly, curves showing the oxygen uptake of nonproliferating suspensions of phenylacetate-grown cells metabolizing various compounds in the tricarboxylic acid cycle were obtained and are shown in figure 3. It is seen that the cells are fully adapted to all the members of the cycle investigated, except citric acid.

Similarly, production of keto acids from these compounds by suspensions of phenylacetate-grown cells in aerated media was followed, and the results are shown in figure 5. There was rapid production from malate, and chromatographic analysis coupled with estimations using the specific toluene extraction procedure of Friedemann and Haugen (1943) showed that pyruvic acid was the principal product. From acetate, no keto acids were produced during the course of the experiment, and production from phenylacetate was also small since, as previously stated, there is a considerable lag with this substrate before rapid production commences. In the case of citrate there is an initial lag in keto acid accumulation similar to that found in figure 3 for oxygen uptake. The main keto acid formed from citric acid was shown by the methods previously described to be α-ketoglutaric acid.

Cells trained to metabolize acetic acid. Previous work has suggested that when certain microorganisms are trained to grow on acetate as sole source of carbon and energy, an adjustment of their metabolism occurs which calls into play a Knoop-Thunberg condensation of 2 molecules of acetate to give 1 molecule of succinate. This results in the establishment of a dicarboxylic acid cycle (Barron, Ardao, and Hearon, 1950). Thus, Ajl (1950) has shown that the oxidation of α-ketoglutarate by *Escherichia coli* is modified by growth on acetate, while the ability of *A. aerogenes* to produce pyruvate from acetate as sole substrate is acquired only after training to metabolize acetate (Dagley *et al.*, 1951). Curves for oxygen uptake by acetate-grown cells in the presence of the same substrates previously investigated are shown in figure 4. The most striking difference between phenylacetate- and acetate-trained cells is seen to be the inability of the latter to oxidize α-ketoglutarate without a period of adaptation, and we may conclude that this compound does not lie on the metabolic route for acetate oxidation developed by the cells. For the other compounds, the behavior is seen to be similar for the two

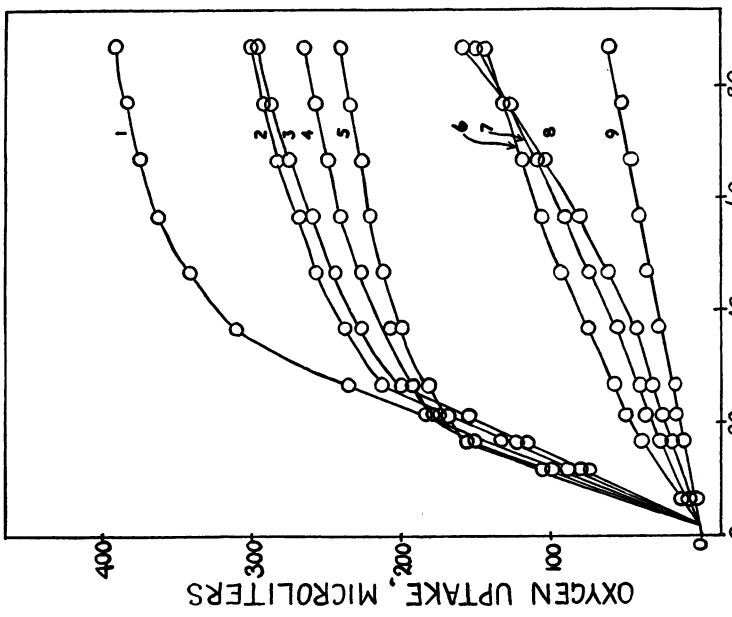

Figure 4. Oxidation of the following organic acids (6 micromoles of each) by *Vibrio* O1 grown on acetate: (1) succinic, (2) fumaric, (3) malic, (4) pyruvic, (5) acetic, (6) citric, (7) α-ketoglutaric, (8) phenylacetic, (9) autorespiration.

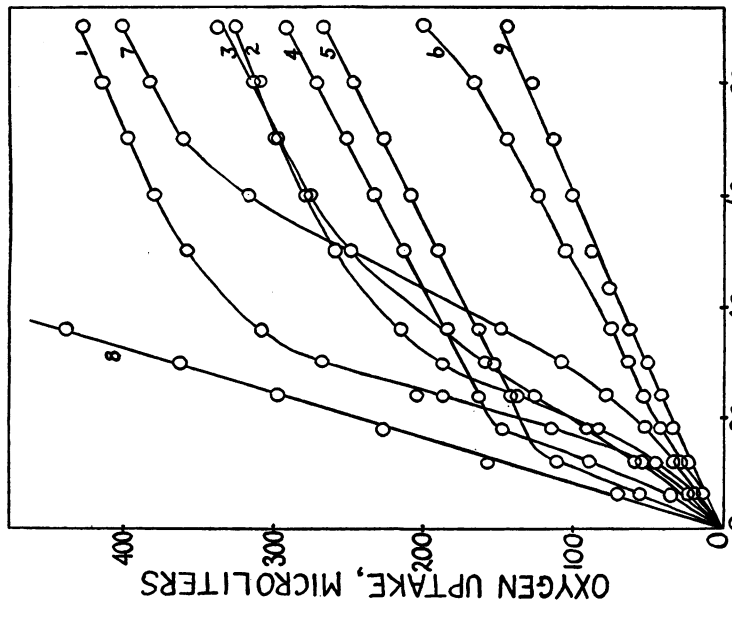

Figure 3. Oxidation of the following organic acids (6 micromoles of each) by *Vibrio* O1 grown on phenylacetate: (1) succinic, (2) fumaric, (3) malic, (4) pyruvic, (5) acetic, (6) citric, (7) α-ketoglutaric, (8) phenylacetic, (9) autorespiration.

types of cells except that phenylacetate-grown cells are adapted to phenylacetate and acetate-grown cells are not.

Keto acid production for acetate-grown cells (figure 6) also contrasts with that for phenylacetate-grown cells in one respect, namely in the relative ease with which they are able to form keto acids from acetate. Thus, the initial rate of accumulation of keto acids was 90 micromoles per liter per hour, whereas none could be detected in acetate media aerated with phenylacetate-grown cells.

Chromatographic analysis coupled with estimations using the specific toluene

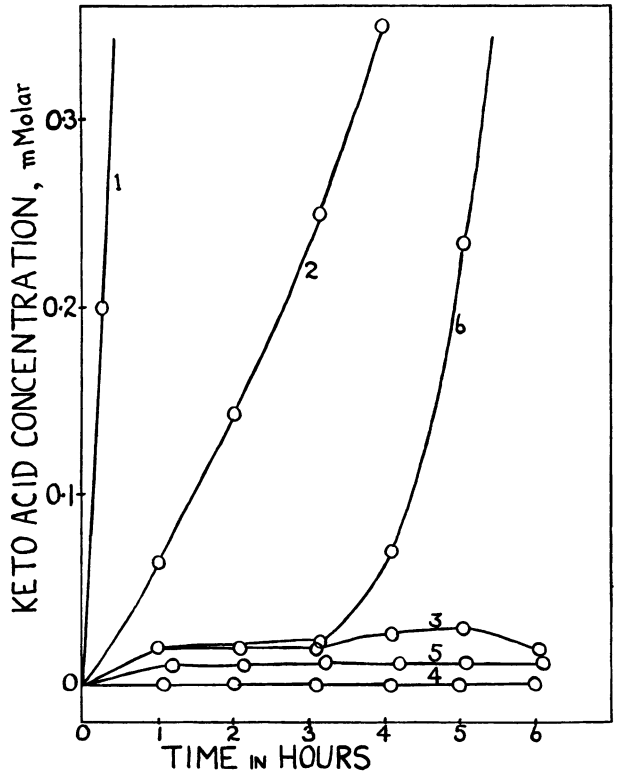

Figure 5. Production of keto acids from the following organic acids by *Vibrio* O1 grown on phenylacetate: (1) malic, (2) fumaric, (3) succinic, (4) acetic, (5) phenylacetic, (6) citric.

extraction procedure showed that the principal keto acid produced from malate and fumarate by acetate-trained cells was pyruvate, but appreciable quantities of α-ketoglutarate could also be detected after two hours' aeration with acetate and succinate. Citrate again gave rise almost entirely to α-ketoglutarate.

DISCUSSION

Experiments both with the present organism and with *Pseudomonas fluorescens* (Stanier, 1947) show that the main pathway of oxidation of phenylacetic acid by microorganisms does not coincide with that established for mandelic and benzoic acids. Our inability to detect β-ketoadipate under conditions for which

it was found in benzoate metabolism suggests a possible difference in mode of ring fission. We have, however, established that α-ketoglutarate is common to both metabolic pathways and suggest that this is the common point of entry to the tricarboxylic acid sequence in the oxidation of aromatic compounds to completion. It is of interest to note that during our estimation and analysis of keto acids produced by *Vibrio* O1 we have found no evidence for the presence of acetoacetic acid.

Recent work with *Corynebacterium creatinovorans* (Barron, Ardao, and Hearon,

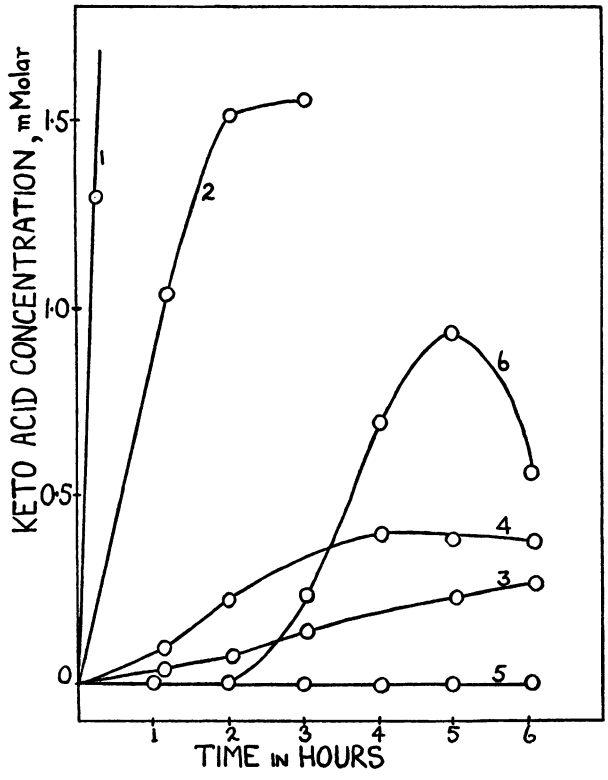

Figure 6. Production of keto acids from the following organic acids by *Vibrio* O1 grown on acetate: (1) malic, (2) fumaric, (3) succinic, (4) acetic, (5) phenylacetic, (6) citric.

1950) and with *E. coli* (Ajl and Kamen, 1951) has suggested that bacteria trained to metabolize acetate may use an abridged dicarboxylic acid cycle coupled to Knoop-Thunberg condensation. While its firm establishment must await further work, in particular the demonstration that cell-free bacterial extracts are able to accomplish the initial oxidative condensation of acetate to succinate, the proposed mechanism may be taken as a basis for the interpretation of our present findings. When *Vibrio* O1 is grown on phenylacetate as sole carbon source, it is able to oxidize acetate readily, but no keto acids are found to accumulate in the metabolizing fluid. Ability to grow on acetate as sole carbon source is apparently related to the development of a mechanism for the production from acetate of

pyruvic acid and other keto acids for growth requirements, and the importance of the postulated dicarboxylic acid cycle may lie in this accomplishment rather than the provision of an additional method of acetate oxidation. Thus, in the case of *A. aerogenes*, the ease with which it grows on a variety of carbon sources is reflected in its ability to produce pyruvic acid from them (Dagley *et al.*, 1951). In the present work we have shown that these changes in metabolism following growth on acetate are accompanied by an inability to oxidize α-ketoglutarate without lag.

Our experiments indicate that the reactions occurring during the oxidation of acetate by *Vibrio* O1 in a nitrogen free medium are not confined entirely to those of the dicarboxylic acid cycle since some α-ketoglutarate is formed in addition to pyruvate. This compound may arise from succinate by carbon dioxide fixation (Ajl and Werkman, 1948), or its presence may indicate the persistence of the tricarboxylic when the dicarboxylic acid cycle has been developed. For citrate-grown *A. aerogenes* oxidizing acetate, Ajl and Wong (1951) have obtained evidence that the two cycles may operate simultaneously. The inability of the *Vibrio* either to oxidize citrate to completion or to convert it to α-ketoglutarate without a lag period may indicate either that this compound does not lie directly on the metabolic route or that permeability considerations prevent its entry into the cell. Our experiments do not permit a firm decision to be made between these two factors.

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SUMMARY

The oxidation of phenylacetic and benzoic acids by *Vibrio* O1 takes place by different metabolic routes, but a section of the tricarboxylic acid cycle is common to both and is entered through α-ketoglutaric acid in both cases. Evidence is presented that when cells are grown on acetic acid as sole carbon source, an abridged cycle may be established. Keto acids do not accumulate when suspensions are aerated with nitrogen free acetate medium unless the cells have been grown on acetic acid as sole carbon source.

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