NONINDUCTIVE METABOLISM OF ITACONIC ACID BY *PSEUDOMONAS* AND *SALMONELLA* SPECIES

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ABSTRACT

MARTIN, WILLIAM R. (University of Chicago, Chicago, Ill.), FRANK FRIGAN, AND EDNA H. BERGMAN. Noninductive metabolism of itaconic acid by *Pseudomonas* and *Salmonella* species. J. Bacteriol. **82**:905-908. 1961—Ten molds, three yeasts, and 44 bacteria were screened for the noninductive metabolism of itaconic acid by growing in complete media containing traces of C^{14}-labeled itaconic acid. Several *Salmonella* and *Pseudomonas* species incorporated significant quantities of radioactivity into their cellular components. The enzymatic conversion of itaconic acid to citramalic acid was demonstrated in cell-free extracts from both *P. aeruginosa* and *S. chittagong*.

The formation and the further oxidation of itaconic acid by certain species of *Aspergillus* has been known for over 30 years. Itaconic acid metabolism has been studied with liver mitochondria (Adler, Wang, and Lardy, 1957; Wang, Adler, and Lardy, 1961) and with bacteria grown on itaconate as a sole source of carbon and energy (Brightman and Martin, 1961; Gray and Kornberg, 1960). However, to our knowledge, no efforts have been made to determine whether microbial species not known to produce itaconic acid possess constitutive enzyme systems for its metabolism. This paper describes the examination of 57 such microorganisms for the metabolism of itaconic acid.

MATERIALS AND METHODS

The microorganisms used in this study were obtained from the stock culture collections of the Department of Microbiology and the American Meat Institute Foundation, The University of Chicago. The yeasts and molds were maintained on Czapek's agar and grown in the same liquid medium. Bacilli, mycobacteria, and corynebacteria were maintained and grown on veal infusion agar and broth, the micrococci on Trypticase soybean agar and broth, and the lactobacilli on solid and liquid APT medium (Evans and Niven, 1951). Microorganisms belonging to the *Enterobacteriaceae* and *Pseudomonadaceae* were grown and maintained on nutrient broth and nutrient agar.

C^{14}-labeled itaconic acid was obtained biosynthetically by adding C^{14}-methyl-labeled acetate to a culture of *A. terreus* actively producing itaconic acid (Corzo and Tatum, 1953). After a 5-day incubation period, the culture medium was freed of unincorporated acetate by steam distillation and itaconic acid separated from the culture medium by continuous ether extraction. The labeled itaconic acid was purified by development on a Celite column with ether as an eluant (Phares et al., 1952).

DL-Citramalic acid was synthesized by a combination of the methods of Morawski (1875) and Carius (1864). It was purified from traces of mesaconic acid by passage through a Celite column with ether as an eluant. The product, a noncrystalline viscous substance, was chromatographically pure.

Radioactivity determinations were made on infinitely thin samples with a windowless gas-flow Geiger-Muller counter.

RESULTS

Screening experiments. The microorganisms used in this study were initially screened for itaconic acid utilization by incorporating trace amounts of C^{14}-sodium itaconate into their respective growth media, the rationale being that labeled itaconate would be incorporated into the cellular material of microorganisms normally possessing enzymes for its metabolism and that this incorporation could be readily detected by a direct count of the radioactivity in the cells. The level of itaconate used in these experiments was 0.61 μmoles per ml and the specific activity was 275 count/min per μmole. All microorganisms

were grown at 30 C on a reciprocating shaking machine in 300-ml Erlenmeyer flasks containing 25 ml of their respective media. After complete growth had taken place, the bacteria were harvested, washed twice with distilled water, and plated on counting planchets. With molds, the washed mycelia were hydrolyzed for 6 hr in 5 N H_2SO_4 at 15 lb pressure (121 C). The hydrolyzates were neutralized with $Ba(OH)_2$, the $BaSO_4$ was removed by centrifugation, and the supernatants were plated on planchets for counting.

The microorganisms studied were members of the following genera: *Aspergillus*, *Botrytis*, *Pichia*, *Scopulariopsis*, *Chaetomium*, *Penicillium*, *Saccharomyces*, *Micrococcus*, *Gafkya*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Bacillus*, *Corynebacterium*, *Mycobacterium*, *Salmonella*, *Shigella*, *Aerobacter*, *Serratia*, *Escherichia*, *Alcaligenes*, and *Pseudomonas*. Of these, only *Salmonella* and *Pseudomonas* were observed to incorporate significant levels of radioactivity under the conditions described. Table 1 shows the results of further studies with 15 *Salmonella* species. Of these, 10 incorporated measurable quantities of labeled itaconate carbon. The level of incorporated radioactivity in the *Salmonella* species was low, but since some species showed no incorporation of radioactive carbon (0 count/min) it was concluded that the observed incorporation was significant. Table 2 shows the results of studies with three *P. aeruginosa* strains and one *P. fluorescens* strain. Of these, all incorporated from three to seven times more labeled itaconate carbon than did the *Salmonella*.

TABLE 1. *C^{14}-Itaconic acid* uptake by Salmonella species*

Organism†	Count/min of total culture
Salmonella paratyphi A	4
S. paratyphi B	2
S. minnesota	54
S. enteritidis	39
S. typhimurium	53
S. chittagong	76
S. newington	32
S. oregon	28
S. pullorum	0
S. london	24
S. typhosa	0
S. amatum	16
S. newport	32
S. binza	3
S. give	29

* The specific activity of itaconic acid was 275 count/min per μmole.

† All were 24-hr cultures in nutrient broth, pH 7.0, containing 0.6 μmole per ml of C^{14} itaconic acid.

TABLE 2. *C^{14}-Itaconic acid* uptake by Pseudomonas species*

Organism†	Count/min of total culture
Pseudomonas fluorescens	173
P. aeruginosa 139	528
P. aeruginosa 153	599
P. aeruginosa 172	460

* The specific activity of itaconate was 275 count/min per μmole.

† All were 24-hr cultures in nutrient broth, pH 7.0, containing 0.6 μmole itaconate per ml.

Ether solubility of cellular radioactivity. The observed incorporation of labeled itaconate carbon by the *Salmonella* and *Pseudomonas* species may have been due to intracellular trapping of small amounts of the acid rather than to its metabolism. To test this possibility, *S. minnesota*, *S. chittagong*, *P. aeruginosa*, and *P. fluorescens* were each grown in 100-ml lots for 24 hr. For this experiment, labeled itaconate was added to a concentration of 1.23 μmoles per ml. Cells from each culture were washed three times with distilled water and hydrolyzed with 5 N H_2SO_4 for 6 hr at 15 lb pressure. Aliquots of the cellular hydrolyzates were plated for counting. Each hydrolyzate was continuously extracted with ether for 20 hr, and the amounts of radioactivity present in the ether and aqueous phases were determined. The results (Table 3) showed that between 85 and 95% of the radioactivity was ether-insoluble. Since itaconic acid is quantitatively extracted by ether under these conditions, it may be concluded that the incorporated itaconic acid has been converted to cellular material.

Cell-free enzyme studies. Evidence for the noninductive nature of the observed itaconate metabolism was obtained with cell-free extracts of *S. chittagong* and *P. aeruginosa*. Both organisms were grown in nutrient broth without any added itaconic acid. Washed cells from 5 liters of growth medium were suspended in 20 ml deionized distilled water, containing 0.02% reduced glutathione, and ruptured with a French pressure

cell at a pressure of 15,000 lb/in^2. Cell debris and unbroken cells were removed by centrifugation at 1,700 × *g*. The faintly turbid supernatant was diluted to 40 ml with distilled water and centrifuged at 100,000 × *g* for 45 min. The clear supernatant fraction obtained by this last procedure was tested for activity against itaconate.

All enzyme reactions were carried out under an atmosphere of nitrogen at 31 C. The components of the reaction mixture were present in the following amounts: sodium itaconate, 10 μmoles; enzyme fraction, 1.5 ml; 0.06 M phosphate buffer, pH 6.8, 0.2 ml; and distilled water to 2.0 ml. After incubation with the substrate for 2 hr, the contents of the reaction vessel were analyzed for the appearance of new nonvolatile acids by ascending paper chromatography.

In the preparation derived from *P. aeruginosa* cells, an additional acid with an R_F identical to that of authentic citramalic acid was formed when itaconate was added to the reaction mixture. No change was observed when itaconate was added to a boiled enzyme control. By this method of analysis, no new product could be demonstrated when itaconate was incubated with the cell-free extracts obtained from *S. chittagong* cells.

An enzymatic conversion of itaconic acid to citramalic acid could be demonstrated with extracts from both *Salmonella* and *Pseudomonas* cells when C^{14}-labeled sodium itaconate was used as a substrate. In these experiments, larger amounts of enzyme and milligram amounts of substrate were incubated in the same proportions.

TABLE 3. *Ether solubility of radioactivity incorporated from itaconic acid**

Organism†	Total radioactivity incorporated per culture	Ether-soluble radioactivity	
	count/min	*count/min*	*% of total*
S. minnesota	4,947	590	11.92
S. chittagong	5,517	781	14.15
P. fluorescens	36,761	1,861	5.06
P. aeruginosa	51,084	3,769	7.38

* The specific activity of the itaconate was 275 count/min per μmole.

† All were 100 ml 24-hr cultures in nutrient broth, pH 7.0, containing 1.23 μmole itaconate per ml.

TABLE 4. *Production of C^{14}-citramalic acid from C^{14}-itaconate* by cell-free extracts of Salmonella chittagong and Pseudomonas aeruginosa*

Source of enzyme	Specific activity of citramalic acid†
	(count/min per μmole)
Pseudomonas aeruginosa	1,850
Salmonella chittagong	80

* The specific activity of itaconate was 8,635 count/min per μmole.

† Ten mg (7.23 μmole) carrier citramalate was added at end of 2 hr reaction.

After 2 hr, the mixture was acidified with H_2SO_4 and 10 mg carrier citramalic acid added.

Citramalic acid and unreacted itaconic acid were separated from the acidified mixtures by continuous ether extraction for 48 hr, and the citramalic acid was isolated and purified by elution with ether from a Celite column and tested for radioactivity. As a control, the same levels of C^{14}-itaconate and carrier citramalate were separated under identical conditions; the citramalate recovered showed no radioactivity. Citramalic acid from both reaction mixtures was radioactive (Table 4), indicating that both species contain enzymes active in the conversion of itaconic to citramalic acid. Consistent with the whole cell experiments, the *Salmonella* incorporated much less itaconic acid than the *Pseudomonas*.

Citramalic acid obtained from both reactions was chromatographed with three different solvent systems and the location of radioactivity determined with a gas-flow chromatograph scanner. In each case the area of radioactivity was identical to the acid area.

DISCUSSION

Reports in recent years provide evidence for the participation of itaconic acid and its isomer, mesaconic acid, in a number of biological reactions. Arnon et al. (1960) reported the formation of citramalate and itaconate during the photosynthetic assimilation of acetate by *Chromatium*. The enzymatic conversion of itaconate to mesaconate and citramalate, followed by a split of the citramalate to pyruvate and acetate, has been demonstrated by Adler et al. (1957) in mammalian liver extracts, and the synthesis and degradation of glutamic acid has been shown to

occur via citramalic and mesaconic acids in the bacterium *Clostridium tetanomorphum* (Munch-Petersen and Barker, 1958). The formation of itaconic acid from *cis*-aconitic acid (Bently and Thiessen, 1957) and the biological conversion of itaconate to succinate via citramalate (Brightman and Martin, 1960) suggest that itaconate may also act as an intermediate in an alternative respiratory cycle deviating from the Krebs cycle at the level of *cis*-aconitic acid.

The results of this study indicate that several *Salmonella* species and at least two *Pseudomonas* species possess constitutive enzyme systems for itaconic acid metabolism. Other microorganisms included in this study may also possess pathways for itaconate metabolism which were not detected since the screening method used was dependent upon cells being permeable to exogenous itaconic acid.

None of the salmonellae studied grew on itaconate as the sole carbon source. *P. aeruginosa* grew well within 18 hr while *P. fluorescens* required a 48-hr induction period before complete growth occurred in the same medium. At present it is not known whether itaconate acts as an intermediate in an alternative respiratory cycle or as an intermediate in the biosynthesis of specific cellular constituents. The data imply that itaconic acid plays a more restricted role in the metabolism of salmonellae than in that of pseudomonads. It may be concluded that itaconic acid and the related branched chain compounds, citramalic and mesaconic acids, should no longer be considered as metabolic oddities but rather as compounds of potentially broad biological significance.

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LITERATURE CITED

ADLER, J., S. F. WANG, AND H. A. LARDY. 1957. The metabolism of itaconic acid by liver mitochondria. J. Biol. Chem. **229**:865–879.

ARNON, D. I., M. LOSADA, A. V. TREBST, AND S. OGATA. 1960. Bacterial photosynthesis and the assimilation of acetate via citramalic and citric acid. Federation Proc. **19**:328.

BENTLEY, R., AND C. P. THIESSEN. 1957. Biosynthesis of itaconic acid in *Aspergillus terreus*. I. Tracer studies with C^{14}-labelled substrates. J. Biol. Chem. **226**:673–687.

BRIGHTMAN, V., AND W. R. MARTIN. 1961. Pathway for the dissimilation of itaconic and mesaconic acids. J. Bacteriol. **82**: 376—382.

CARIUS, L. 1864. Ueber Citramalsäure und Citraweinsäure. Ann. Chem. Justus Liebigs **129**: 161–168.

CORZO, R. H., AND E. L. TATUM. 1953. Biosynthesis of itaconic acid. Federation Proc. **12**:470.

EVANS, J. B., AND C. F. NIVEN, JR. 1951. Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. J. Bacteriol. **62**:599–603.

GRAY, C. T., AND H. L. KORNBERG. 1960. Enzymatic formation of citramalate from acetylcoenzyme A and pyruvate in *Pseudomonas ovalis* Chester, Catalyzed by "pyruvate transacetase." Biochim. et Biophys. Acta **42**: 371–372.

MORAWAKI, T. 1875. Zur Kenntniss der Oxycitraconsäure und anderer Abkommlinge der Brenzcitronensäuren. J. Prakt. Chem. **119**: 430–470.

MUNCH-PETERSEN, A., AND H. A. BARKER. 1958. The origin of the methyl group in mesaconate formed from glutamate by extracts of *Clostridium tetanomorphum*. J. Biol. Chem. **230**: 649–653.

PHARES, E. F., E. H. MOSBACH, F. W. DENISON, AND S. F. CARSON. 1956. Separation of biosynthetic organic acids by partition chromatography. Anal. Chem. **24**:660–662.

WANG, S. F., J. ADLER, AND H. A. LARDY. 1961. The pathway of itaconate metabolism by liver mitochondria. J. Biol. Chem. **236**:26–30.