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β-Ketoadipate Pathway in *Trichosporon cutaneum* Modified for Methyl-Substituted Metabolites

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***Trichosporon cutaneum*, when grown with *p*-cresol, catalyzed intradiol fission of the benzene nucleus of 4-methylcatechol before the complete catabolism of these two substrates. Steps in their conversion to pyruvate and acetyl coenzyme A were investigated by using cell extracts, and some properties of various new microbial catabolites are also described. These included (−)-2,5-dihydro-3-methyl-5-oxofuran-2-acetic acid (β-methylmuconolactone) and (−)-3-keto-4-methyladipic acid and its coenzyme A ester; the latter was degraded by an enzymatic reaction sequence that included the coenzyme A esters of methylsuccinic, itaconic, and citramalic acids. A notable feature of this sequence is the formation of β-methylmuconolactone which can be readily metabolized, in contrast to the analogous reaction in bacteria that gives the "dead-end" compound γ-methylmuconolactone; this compound cannot be enzymatically degraded and so renders the β-ketoadipate pathway unavailable for methyl-substituted bacterial sources of carbon that are catabolized by way of 4-methylcatechol.**

Cresols are found in petroleum and coal tar (15) and are present in some insect defense secretions (17); they are also formed during fecal metabolism of tyrosine (41). In the case of bacteria, two separate and distinct catabolic routes have been described for *p*-cresol (4-methylphenol); both of them involve *meta*-fission reactions. One of these pathways, which is used by some *Pseudomonas* spp., is initiated by oxidizing the methyl group of the cresol to a corresponding carboxyl group (13). This oxidation occurs in three steps, in the first of which oxygen derived from water, rather than from dioxygen, is incorporated to give 4-hydroxybenzyl alcohol (22). The second and third steps are successive dehydrogenations that give 4-hydroxybenzoic acid as the final product, from which protocatechuate is formed by hydroxylation. The *meta*-fission of protocatechuate (12) arising from *p*-cresol was one of the first dioxygenations in this category to be described (S. Trippett, S. Dagley, and D. A. Stopher, Biochem. J., **76**:9, 1960). By contrast, other *Pseudomonas* spp. degrade *p*-cresol by a different pathway. The methyl group of the substrate remains intact and appears in the structure of propionaldehyde which is formed, along with pyruvate, when 4-methylcatechol is catabolized by *meta*-fission reactions (6).

ortho-Fission reactions are rarely used by bacteria growing at the expense of methyl-substituted phenols and benzoates. This is not always due to tight substrate specificities of the early enzymes of the reaction sequence. For example, although the rates of oxidation of 4-methyl- and 3-methylcatechols by catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus* are only 18 and 12%, respectively, of the rate for catechol (31), the enzyme from *Pseudomonas arvilla* oxidizes catechol and 4-methylcatechol at about the same rate (24), and both methylcatechols are good substrates for the *Brevibacterium fuscum* dioxygenase (26). In such cases, degradation is not complete because a later reaction in the sequence is blocked. Thus, the enzyme that catalyzes the lactonization of *cis,cis*-muconate may still function with a methyl substituent, whereas the delactonizing enzyme may not: a methyl-substituted lactone then accumulates. This was shown for *P. desmolyticum* (8), which converts 4-methylcatechol into (+)-2,5-dihydro-2-methyl-5-oxofuran-2-acetic acid (γ-methylmuconolactone; compound B, Fig. 1) which is not metabolized further. This lactone is also formed when 4-methylcatechol is oxidizied by a *Pseudomonas* strain able to grow with 3-chlorobenzoate (23).

The only procaryote that has been clearly shown to use *ortho*-fission enzymes for degrading 4-methylcatechol to completion appears to be *Gordona rubra* (D. J. Miller and R. B. Cain, Proc. Soc. Gen. Microbiol. **6**:18, 1968); but when cell extracts are used, as distinct from intact cells, a lactone accumulates which is not degraded further under the experimental conditions employed. R. B. Cain, who kindly provided us with a sample, has assigned the β-methylmuconolactone structure (compound A, Fig. 1) (personal communication). In this investigation we show that the compound is a substrate for cell extracts of *Trichosporon cutaneum*. This soil yeast eucaryote uses *m*- or *p*-cresol readily as a major carbon source, and we present evidence that the seven carbon atoms of *p*-cresol are converted into two molecules of acetyl coenzyme A (acetyl-CoA) and one of pyruvate by a novel *ortho*-fission degradative route (Fig. 2).

MATERIALS AND METHODS

Organism and cell extracts. The organism used, *T. cutaneum* ATCC 58094 was grown as described earlier (3, 39) with sources of carbon at the following concentrations: 0.02% cresols supplemented with additional cresol in mid-exponential phase, 0.03% phenol or catechols, 0.05% glucose or organic acids. Larger batches of cells required for enzyme purification were grown with vigorous forced aeration in a 100-liter fermentor (4). Cells were broken either in a Hughes bacterial press (39) or, when more than 50 g of cells were used, in a Bead Beater (Biospec Products, Bartlesville, Okla.)

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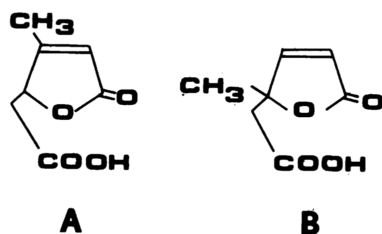

FIG. 1. Structures of methyl-substituted muconolactones. A, 2,5-Dihydro-3-methyl-5-oxofuran-2-acetic acid (β-methylmuconolactone). B, 2,5-Dihydro-2-methyl-5-oxofuran-2-acetic acid (γ-methylmuconolactone).

containing 0.5-mm glass beads. The composition of the buffer used for preparing a particular cell extract depended upon the nature of the experiment and is given in the text.

Analytical methods. Procedures for mass spectrometry and measurements of O_2 consumption using the oxygen electrode were as previously described (3). Carbon dioxide was measured by Warburg manometry with air as the gas phase; β-ketoacids were determined by catalytic decarboxylation with 4-aminoantipyrene (3). After purification, carboxylic acids were estimated by measuring the formation of 2-nitrophenyl-hydrazides; when using this reaction it was necessary, in the case of methylsuccinic and citramalic acids, to extend the incubation period from 30 to 60 min at 40°C to obtain linear standard curves. The pentabromoacetone method was used to determine citric acid (40). The method of Walker (44), in which β-ketoacids are coupled with diazotized *p*-nitroaniline, was used to determine 3-keto-4-methyladipic acid (γ-methyl-β-ketoadipic acid; compound VI, Fig. 2). The coupled product had λ_{max} at 445 nm, and the calibration curve, which was linear to at least 170 nmol, was prepared from a solution of 3-keto-4-methyladipate standardized in turn by catalytic decarboxylation. The presence of CoA slightly lowers measured absorbances; this effect is abolished by adding $HgCl_2$ (2).

Chromatography. Dicarboxylic acids were isolated using a Dowex 1 × 8 (formate form; Bio-Rad Laboratories, Richmond, Calif.) anion-exchange column (1.1 by 12 cm) (1). For elution, a linear gradient of 0 to 0.4 N formic acid (100/100 ml) was used with 15 ml of water on top of the column bed to dilute the acid; the flow rate was approximately 0.5 ml/min. Fractions of 2.2 ml were collected and dried in a heated vacuum desiccator containing $CaCl_2$ and solid NaOH (7). Residues in the tubes were dissolved in water, and acids were titrated with 0.1 N NaOH using phenolphthalein as an indicator. Authentic methylsuccinic acid eluted between fractions 50 and 60, citramalic acid between fractions 45 and 52, and itaconic acid between fractions 61 and 68. When enzymatic reaction mixtures were chromatographed, fractions eluting at these positions were dissolved in ether, spotted onto thin-layer silica gel plates, and sprayed with methyl red indicator to detect the acid. Fractions which contained the acid were converted to trimethylsilyl derivatives and then examined by gas chromatography-mass spectrometry.

Some separations were carried out using thin-layer plates of silica gel or cellulose as previously described (3). The following solvents were used and are referred to by their numbers in the text: solvent 1, benzene-methanol-acetic acid (45:4:8); solvent 2, benzene-acetic acid-water (125:72:3); solvent 3, benzene-ethyl acetate-formic acid (5:5:1); and solvent 4, benzene-acetone-formic acid (5:5:1).

High-pressure liquid chromatography was performed on a Beckman 334 apparatus equipped with a Hitachi variable-wavelength UV-vis detector and an Ultrasphere-ODS reverse-phase column (0.46 by 25 cm). When lactones were chromatographed, the solvent was a mixture of 72.5% 0.01 M H_3PO_4 containing 1% 2-propanol and 27.5% methanol (flow rate, 1 ml/min). This flow rate was also used for separating CoA esters in a solvent of 81.5% 0.05 M phosphate buffer (pH 5.3) plus 18.5% methanol.

Chemicals. All commercial preparations of enzymes, coenzymes, and citramalic acid were from Sigma Chemical Co., St. Louis, Mo. Cresols, DL-methylsuccinic, itaconic, mesaconic, and citraconic acids were from Aldrich Chemical Co., Inc., Milwaukee, Wis. *cis,cis*-Muconic acid, (±)-muconolactone, and (±)-β-methyl-muconolactone were gifts from P. J. Chapman, and β-methylmuconolactone of biological origin was a gift of R. B. Cain; we showed that the latter compound was the (−)-isomer. 4-Methylcatechol was crystallized from toluene before use. CoA esters of dicarboxylic acids were synthesized from the anhydride by the method of Simon and Shemin (33). Anhydrides were prepared by refluxing the corresponding acid in redistilled acetic anhydride (43). DL-Methylsuccinic anhydride was crystallized from the reaction mixture, collected, and dried (mp 32 to 33°C). Itaconic anhydride was prepared in a similar manner (mp 66 to 68°C). Citraconic anhydride was distilled from the reaction mixture, and the fraction boiling at 212 to 213°C was collected.

When 3-methyl-*cis,cis*-muconic acid was required, it was generated in situ from 4-methylcatechol using partially purified catechol oxygenase (free of cycloisomerase) and used immediately. Catechol oxygenase was purified from *T. cutaneum*, grown with phenol, by $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-Sephacel (42). Since preparations usually retained some cycloisomerase activity after this procedure, they were either rechromatographed on the same column or refractionated with a narrower salt cut to remove this residual activity before use. In some instances, the catechol oxygenase preparation was also used to generate *cis,cis*-muconic acid in situ from catechol.

Preparation of γ-methylmuconolactone. Previous authors (8, 23) have used different *Pseudomonas* strains for the isolation of this lactone. We used *P. putida* ATCC 12633 because most of the classical studies of the β-ketoadipate pathway in bacteria were performed with this strain, sometimes designated *P. putida* biotype A, strain 90 (37). These studies include the direct proof by Stanier et al. (38) that β-ketoadipate (B. A. Kilby, Biochem. J. **43**:v, 1948) is a catabolite of catechol and protocatechuate and also the later work that established details of the biochemistry (29) and enzymology (36) of the pathway. It was of interest therefore to show that, although extracts of this organism readily degrade the muconolactone, they synthesize but do not degrade γ-methylmuconolactone (compound B, Fig. 1) and can be used to prepare this compound.

P. putida ATCC 12633 was grown in the basal medium of Hareland et al. (18) supplemented with 0.06% benzoic acid and adjusted to pH 7.2. Cells were harvested at an A_{540} of 0.88 and, after being washed with 0.05 M Na^+-K^+ phosphate buffer (pH 7), were stored frozen for up to 36 h before use. Frozen cells (2 g) were broken in a Hughes bacterial press and resuspended in twice their weight of 0.05 M Tris hydrochloride (pH 7.7), containing 10 μM $MgCl_2$, and after the addition of DNase the extract was clarified by centrifugation. This extract (2 ml) was added to a 500-ml Erlenmeyer flask containing 30 ml of 0.05 M 3-(*N*-morpholino)propanesulfonic acid–NaOH buffer (pH 7.0) plus 10 μM $MgCl_2$. The

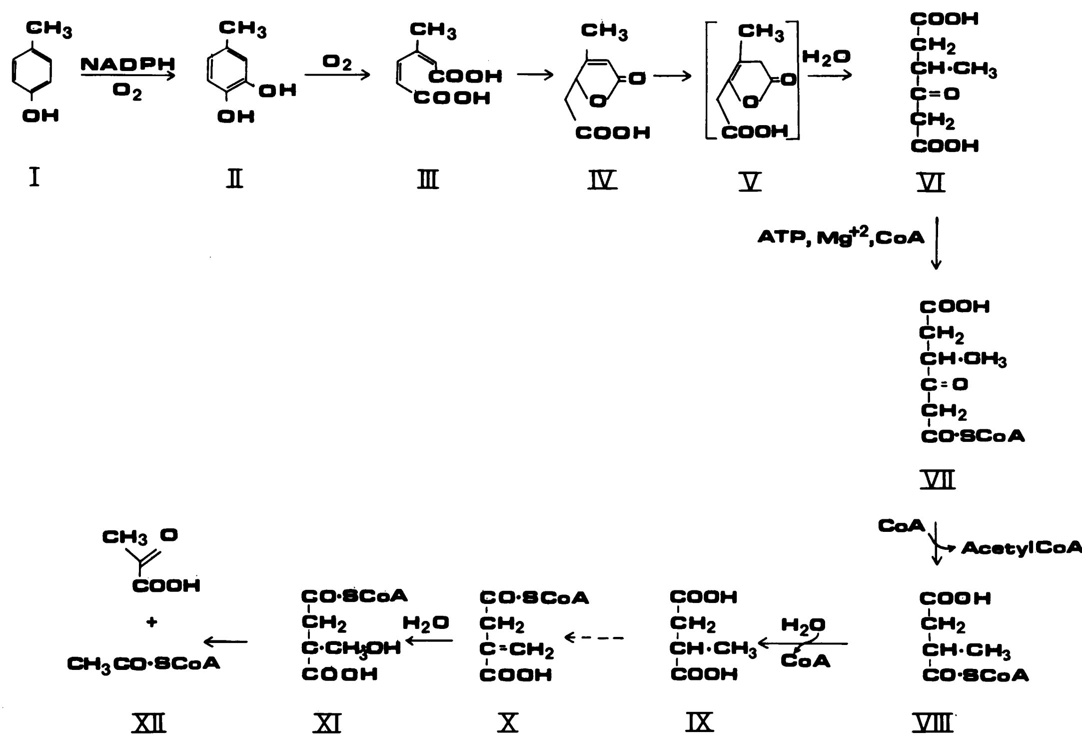

FIG. 2. Reaction sequence for the degradation of *p*-cresol. Metabolites shown include *p*-cresol (I), 4-methylcatechol (II), 3-methyl-*cis,cis*-muconic acid (III), β-methylmuconolactone (IV), 3-keto-4-methyladipic acid (γ-methyl-β-ketoadipic acid; VI), 3-keto-4-methyladipyl-CoA (VII), methylsuccinyl-CoA (VIII), methylsuccinic acid (IX), itaconyl-CoA (X), and citramalyl-CoA (XI).

mixture was well stirred magnetically, and 20-mg quantities of recrystallized 4-methylcatechol were added at 0, 18, 45, 65, and 85 min. The disappearance of substrate was monitored by mixing portions of the reaction mixture with alkaline $FeSO_4$, and when the purple color had almost faded completely fresh substrate was added. At 120 min, 0.5 ml of crude extract and 25 mg of 4-methylcatechol were added. A final addition of 4-methylcatechol was made at 140 min. Throughout the experiment, the pH was maintained by the periodic addition of 5 N NaOH. In all, 150 mg of 4-methylcatechol was metabolized.

After a total reaction time of 4 h, the reaction mixture was cooled on ice and acidified to pH <2 by the addition of 5 N HCl. The resulting precipitate was discarded after centrifugation. Solid $(NH_4)_2SO_4$ was then added to give 80% saturation, the resulting precipitate was removed by centrifugation, and the supernatant was extracted with four 55-ml portions of diethyl ether. These extracts were combined and dried over $MgSO_4$, and the ether was removed under reduced pressure. The yellowish oil that remained was induced to crystallize by scratching the side of the vessel with a glass rod, and the resulting white solid was redissolved in a minimal amount of ethyl acetate. Upon addition of petroleum ether (bp 60 to 70°C) to give slight cloudiness, the product crystallized at −15°C as long white needles (yield, 66 mg; 38%; mp 49 to 51°C). Gas chromatography-mass spectrometry showed that when derivatized, one trimethylsilyl group was taken up, giving a molecular ion at 228.2 *m/e* and a base peak at 169.1 *m/e*. An underivatized sample, examined by direct probe, showed a molecular ion at 156.2 *m/e*, a base peak at 97.1 *m/e*, and a peak at 141 *m/e*. These peaks are characteristic of γ-methylmuconolactone (23). The proton nuclear magnetic resonance spectrum (in deuteroacetone) was identical to that described by Knackmuss et al. (23) for γ-methylmuconolactone and did not reveal the presence of any impurities.

Enzyme assays. Aromatic hydroxylase activity was assayed by measuring either the disappearance of NADPH at 340 nm or the O_2 uptake in the oxygen electrode; dioxygenases were also assayed by measuring O_2 consumption. Cycloisomerase activity was measured by monitoring the disappearance of *cis,cis*-muconic acid at 260 nm or 3-methyl-*cis,cis*-muconic acid at 255 nm. The values for the molar extinction coefficients used in this work were those of Dorn and Knackmuss (14). Reactions were carried out in 1 ml of 0.05 M 3-(*N*-morpholino)propanesulfonic acid–NaOH buffer (pH 7), typically using 120 nmol of muconic acid.

Delactonizing activity was determined by monitoring the decrease in A_{230} for (±)-muconolactone (34). When β-methylmuconolactone was used as substrate, activity was measured by monitoring the appearance of 3-keto-4-methyladipic acid (γ-methyl-β-ketoadipic acid) by the method of Walker (44). Typically, assay mixtures contained 1.6 ml of 0.1 M Tris-SO_4 buffer (pH 8.0) and 1.5 μmol of β-methylmuconolactone. Portions were discharged at various times into trichloroacetic acid, and protein was removed by centrifugation before the assay was performed. Disappearance of β-methylmuconolactone was also monitored in some instances by deproteinizing portions of the reaction mixtures with methanol-H_3PO_4 and, after centrifugation, analyzing the supernatant by high-pressure liquid chromatography.

Methylsuccinyl-CoA hydrolase was monitored by measuring the disappearance of substrate at 230 nm. Cuvettes contained 1 ml of 0.05 M Na^+-K^+ phosphate buffer (pH 7), approximately 0.15 μmol of methylsuccinyl-CoA, and 140 μg of extract protein. The molar extinction coefficient of the substrate was assumed to be the same as that of succinyl-

CoA (4,500 M^{-1} cm^{-1}) (35) when concentration changes were calculated from changes in A_{230}.

RESULTS AND DISCUSSION

Oxidation of compounds by whole cells. In previous metabolic studies, strains of *T. cutaneum* were grown at the expense of phenol (27) and *m*- or *p*-cresol (20). Our strain grew with any one of these compounds as sole source of carbon but did not grow with *o*-cresol, *p*-toluic acid, or dimethylphenols. Some of the postulated metabolites of *p*-cresol such as β-methylmuconolactone (IV, Fig. 2) and methylsuccinic acid (IX) served as single-carbon sources, whereas itaconic, citraconic, or mesaconic acids did not. Cells were grown with phenol, *p*-cresol, succinate, and compounds IV and IX, and rates of oxidation were measured for phenol, cresols, catechols, and 4-hydroxybenzoic, protocatechuic, and methylsuccinic acids (Table 1). The fact that growth with *p*-cresol conferred the ability to oxidize 4-methylcatechol but not protocatechuate or 4-hydroxybenzoate is in agreement with the scheme of Fig. 2 in which the methyl substituent of the cresol is not oxidized before degradation. However, caution must be exercised when making deductions from experimental results obtained with washed cell suspensions of *T. cutaneum*. Thus, Table 1 shows that growth with phenol also induces the ability to oxidize 4-methylcatechol, just as growth of this strain with salicylate induces oxidation of metabolites of the tryptophan catabolic pathway which does not include salicylate itself (4). Washed suspensions of cells grown with methylsuccinic acid oxidized 4-methylcatechol (and 3-methylcatechol) but exhibited an unexpected loss of ability to oxidize their growth substrate (Table 1). In view of these ambiguities, the following experiments were conducted with crude cell extracts, rather than with intact cells, of *T. cutaneum* grown with *p*-cresol.

Conversion of *p*-cresol (I) to 4-methylcatechol (II). Hydroxylase activity toward *p*-cresol was exhibited by crude extracts, and two fractions were separated on a column of phenyl Sepharose (32). The fraction specific for *p*-cresol was used in the following experiments. First, assays were performed in the oxygen electrode with 1.5 ml of 0.05 M Na^+-K^+ phosphate buffer (pH 7.4), 550 nmol of NADPH, 200 nmol of *p*-cresol, and 0.1 mg of catalase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Reactions were initiated by the addition of approximately 50 μg of the partially purified *p*-cresol hydroxylase. Four separate determinations showed that 1.09 ± 0.03 mol of O_2 per mol of *p*-cresol were consumed. This ratio was higher when catalase was not included in the mixture. Next, the four reaction mixtures were combined with a fifth and, after acidification with 5 N H_2SO_4, were extracted with three 4-ml portions of ethyl acetate. These extracts were combined, dried over anhydrous Na_2SO_4, and evaporated to dryness over a stream of nitrogen, and the residue was chromatographed on silica gel thin-layer plates (solvent 1) and on cellulose thin-layer plates (solvent 2). A single compound was detected which cochromatographed with authentic 4-methylcatechol. A control mixture, which had been acidified before the addition of enzyme and carried through the procedure, did not contain 4-methylcatechol. These experiments were repeated with cells grown with *m*-cresol, which was also converted to 4-methylcatechol.

TABLE 1. Rates of oxidation by washed cells

Compound oxidized	Oxygen uptake (nmol of O_2/min per mg)[a] by cells grown on:				
	Phenol	*p*-Cresol	β-Methyl-muconolactone	Methyl-succinic acid	Succinic acid
Phenol	23.7	16.0	3.1	8.2	1.5
p-Cresol	3.3	18.7	3.2	8.6	<1
m-Cresol	<1	17.3	2.4	9.9	<1
o-Cresol	<1	<1	<1	<1	<1
Catechol	12.2	13.3	4.7	16.3	1.5
4-Methylcatechol	8.2	16.7	9.8	11.5	<1
3-Methylcatechol	2.6	8.7	4.7	6.4	<1
4-Hydroxybenzoic acid	<1	<1	<1	<1	1.5
Protocatechuic acid	<1	<1	<1	<1	<1
Methylsuccinic acid	<1	<1	<1	<1	<1

[a] Values are given as nanomoles of O_2 per minute per milligram of cells (wet weight), after subtracting an endogenous rate of approximately 5 nmol of O_2/min per mg.

Conversion of 4-methylcatechol (II) to (−)-3-keto-4-methyladipic acid (VI). Addition of crude extract to a solution of 4-methylcatechol gave an almost stoichiometric yield of β-keto acid, assayed by decarboxylation with 4-aminoantipyrene. The product was isolated by the following procedure using duplicate 500-ml Erlenmeyer flasks, each containing 36 ml of 0.1 M Tris hydrochloride buffer (pH 7.2), 103 mg of protein in a crude extract prepared in the same buffer, and 15 mg of 4-methylcatechol. Each mixture was stirred magnetically, and disappearance of 4-methylcatechol was monitored by observing the purple color given when alkaline ferrous sulfate was added to small portions removed from the reaction at various intervals of time. When the color became faint, 15 mg of 4-methylcatechol in 1 ml of buffer was added and the testing was resumed. Additions were made at 14, 28, 43, 55, 69, 80, and 90 min, and periodic additions of 2 N NaOH were also required to maintain the pH at 7.0 to 7.3. In 1.5 h, a total amount of 120 mg of 4-methylcatechol was added. The mixture was stirred for 40 min after the final addition of 4-methylcatechol had been used up; the pH was then adjusted to 8.0 with 2 N NaOH, and stirring was continued for an additional 90 min, at which time 5 ml of 70% perchloric acid was added, and the precipitated protein was removed by centrifugation. The pH was then adjusted to 7.5 with solid KOH, the resulting potassium perchlorate precipitate was removed by centrifugation, the pellet was washed with a little water, and the supernatant was added to that of the previous step. The volume was then reduced to about 15 ml under vacuum at a temperature that did not exceed 40°C. A precipitate formed and was removed by centrifugation, the pellet was washed with water, and the volume made up to 25 ml. The pH of this solution was then brought to approximately 1.5 with concentrated HCl, and solid $(NH_4)_2SO_4$ was added to give 75% saturation at room temperature. This solution was clarified by centrifugation and then extracted with five 60-ml portions of diethyl ether. The combined extracts were dried over $MgSO_4$ and, after concentration to 25 ml on a rotatory evaporator, were treated with activated charcoal, filtered, and evaporated under a stream of air to leave a yellowish oil. The following procedure gave crystals of two types, and evidence is presented below that these were different forms of the same compound, 3-keto-4-methyladipic acid.

The oil was dried under vacuum for 1 h, when a few crystals formed. Crystallization was completed by standing

at −15°C to give 225 mg of a yellowish solid melting at 65 to 70°C. These crystals were redissolved in a minimal amount of diethyl ether, and then a little petroleum ether (bp 60 to 70°C) was added to produce incipient cloudiness. Crystallization was allowed to occur at −15°C overnight. The resulting product was white and melted at 72 to 75°C, with some crystals melting at 85°C. The total crop was then recrystallized, first from ether-petroleum ether at −15° and again at 4°C. Crystals were washed with a small quantity of ice-cold ether and dried in a vacuum dessicator. Two distinct crystal forms were observed: large clusters of flat crystals and small, rounded clumps. The large crystals melted at 74 to 75°C, whereas the small, rounded forms melted at 89 to 91°C, both with decomposition. It should be noted that oxaloacetic acid, also a dibasic β-keto acid, exists in crystalline forms with a range of melting points (5).

Characterization of (−)-3-keto-4-methyladipic acid (VI). The two crystalline forms were combined for all experiments except the determination of infrared spectra. The product behaved as a β-ketoacid: it formed an acidic 2,4-dinitrophenylhydrazone and gave a positive Walker reaction (44), and upon decarboxylation with 4-aminoantipyrene, 94 to 96% of the carbon dioxide expected from 3-keto-4-methyladipic acid was evolved. A value of −25.9° for $[\alpha]_D^{26}$ was calculated from the optical rotation of a solution containing 4.9 mg/ml. Since this is a newly discovered metabolite, the following properties are also recorded. In phosphate buffer (pH 7.2), the UV spectrum showed a small peak at 280 nm with strong absorption below 210 nm; in ether, a large, broad peak at 240 nm was observed. Each crystalline form was made into a KBr pellet, and separate infrared spectra were obtained. These were in agreement with the assigned structure and were essentially identical except that a medium-strong absorption at 835 cm^{-1} in the large crystal form was shifted to 930 cm^{-1} for the small, round crystal form.

The proton nuclear magnetic resonance spectrum of the compound in nitromethane-d_3 was also consistent with the structure of 3-keto-4-methyladipic acid. Its principal features (δ values being relative to trimethylsilane) were as follows. A doublet at 1.22 δ was assigned to the protons of the methyl-group substituent, split by the C-4 proton; this proton was assigned a signal at 3.13 δ that showed a complex splitting pattern as expected. The protons of the C-5 methylene group would be expected to have different chemical shifts because of the adjacent asymmetric center, and signals were observed at 2.52 and 2.80 δ, which were assigned to these protons. Each signal was split with J_{AB} = 17.27 Hz, a typical coupling constant for geminal protons, and each was also split by the proton at C-4 with coupling constants of 8.62 and 5.19 Hz. A quartet at 3.73 δ was assigned to the protons on C-2. This feature was abolished by heating the sample to 100°C for 20 min and resulted in the simultaneous appearance of an intense singlet at 2.18 δ. From this it is evident that when the β-ketoacid was decarboxylated by heat treatment, the C-2 methylene group was converted to a methyl group adjacent to the carbonyl group, the protons of which exhibit chemical shifts of about 2.10 δ (43). Further, since the protons of the original methylene group are acidic, we found that the quartet at 3.73 δ was abolished by adding deuterium oxide, when proton exchange occurred.

Confirmation of the structure of compound VI was obtained by gas chromatography-mass spectrometry. The spectrum showed a parent ion of 130 *m/e*, a principal ion of 43 *m/e*, and other peaks consistent with the structure of methyllevulinic acid, the decarboxylation product of compound VI. The gas chromatogram of the trimethylsilyl derivative of compound VI gave rise to two peaks, but the mass spectra gave the same parent ion for each fraction, having a mass corresponding to the derivatization of compound VI by three trimethylsilyl groups. Further, the fragmentation patterns were the same for each fraction, except for a few slight differences in relative intensities. It is suggested that the two peaks observed in the gas chromatogram were given by derivatives of two enol forms of compound VI, possibly *cis*- and *trans*-isomers.

Identification of metabolites formed in the conversion of 4-methylcatechol (II) to (−)-3-keto-4-methyladipic acid (VI). Figure 3 shows the spectral changes resulting from the action of crude extract of *p*-cresol-grown cells upon 4-methylcatechol. A peak at 280 nm due to 4-methylcatechol disappeared and was replaced by another at 255 nm; the spectrum of the new intermediate was that of 3-methyl-*cis*,*cis*-muconic acid (III, Fig. 2) (14). The muconic acid was then converted isosbestically into a compound that was not degraded further and, like the two lactones of 3-methylmuconic acid (Fig. 1), absorbed light maximally at 210 nm. The compound was isolated and characterized by the following procedure, making use of catechol 1,2-dioxygenase and muconate cycloisomerase, purified as described elsewhere (32).

4-Methylcatechol (0.45 μmol) in 2.5 ml of 0.05 M phosphate buffer (pH 7) was incubated in a cuvette with 0.39 U of catechol 1,2-dioxygenase, and the increase in A_{260} due to 3-methylmuconate formation was monitored. When the reaction was complete, 20 μg of muconate cycloisomerase (purified from *p*-cresol-grown cells) was added, and the conversion of the muconate to its lactone was monitored to completion. The reaction mixture was then cooled on ice and passed through a PM-10 ultrafiltration membrane (Amicon Corp., Lexington, Mass.) to remove protein. The filtrate was acidified with a few drops of 5 N phosphoric acid and examined by high-pressure liquid chromatography, using authentic samples of β- and γ-methylmuconolactones as standards. These were well resolved between 4 and 5 min after the start of a run. The peak for the isolated compound coincided with that for β-methylmuconolactone (IV, Fig. 2); no γ-isomer was detected in the sample.

The accumulation of lactone in the experiment (Fig. 3) was due in part to the need to use dilute extracts to avoid interference with spectrophotometric measurements. A higher concentration of extract was used, at a pH more favorable to lactone degradation, in the following experiment designed to determine which of the enantiomers of compound IV (Fig. 2) is the metabolic intermediate. Two reaction mixtures, each containing 17.3 mg of crude cell extract protein in 2.5 ml of 0.05 M Tris-SO_4 (pH 9.0), were incubated with 16 μmol of either (±)- or (−)-β-methylmuconolactone. Portions (200 μl) were withdrawn at intervals of time and discharged into 50 μl of 10% trichloroacetic acid. The precipitate was removed by centrifugation, and 25 μl of the clear solution was taken for analysis of 3-keto-4-methyladipic acid by the colorimetric method (44). It was found that (−)-β-methylmuconolactone was degraded completely, whereas one-half of the racemic mixture was metabolized (Fig. 4). Analysis by high-pressure liquid chromatography showed that the lactone was not hydrolyzed under these conditions when enzyme was omitted. The rate of the enzymatic reaction decreased with pH below 8.0, and the reaction was strongly inhibited when phosphate buffer was used.

Our experiments provided no information concerning the immediate precursor of compound VI. In Fig. 2 an enol-lactone is suggested by analogy with the known chemical

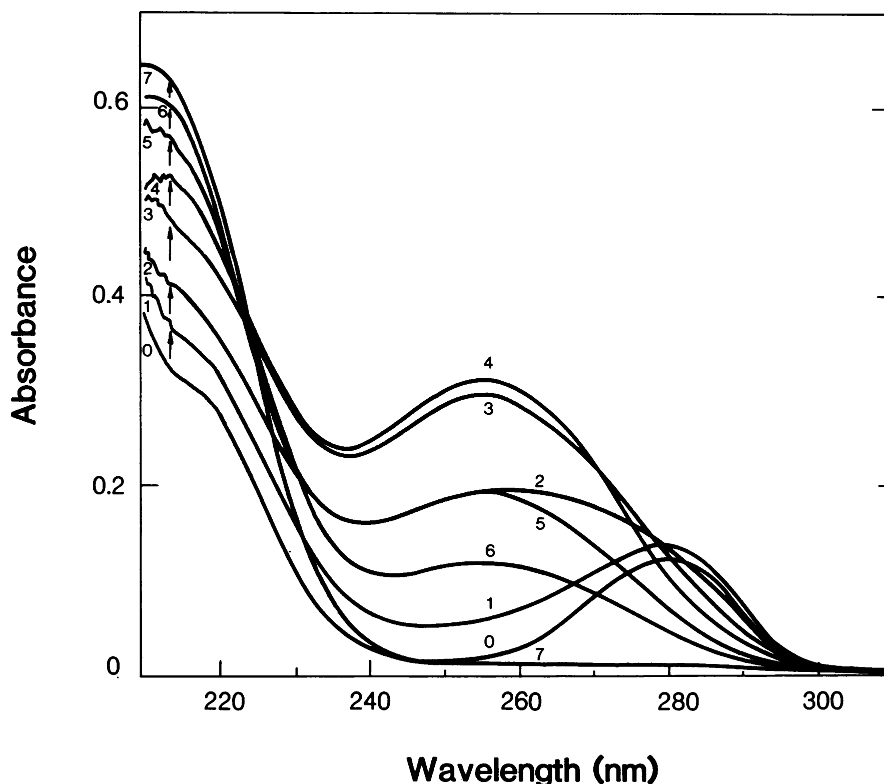

FIG. 3. Spectral changes resulting from the addition of cell extract to a solution of 4-methylcatechol. The cuvette contained 50 nmol of 4-methyl-catechol in 1 ml of 0.05 M Tris-H_2SO_4 buffer (pH 7.4). Spectra were scanned initially (scan 0), immediately after adding extract (scan 1), and at 4-min intervals thereafter (scans 2 through 7). The spectrum showed no significant change by 23 min after scan 7.

structure of the precursor of β-ketoadipic acid (29); if formed, it may remain enzyme bound.

Coenzyme requirements for degradation of (−)-3-keto-4-methyladipic acid (VI). A crude cell extract, prepared in 0.1 M Na^+-K^+ phosphate buffer (pH 7.4), was treated with Dowex resin to remove endogenous CoA (9). 3-Keto-4-methyladipate was then degraded rapidly only after additions were made of CoA and ATP; Mg^{2+} ions were also required for maximal activity (Fig. 5). Under these conditions a small amount of the keto acid remained, but later experiments showed that the substrate disappeared completely when the amount of ATP in the reaction mixture was increased. It appeared, therefore, that the keto acid was converted into its CoA ester, and this suggestion was supported by the detection of the transient appearance of a species absorbing light at 305 nm during the course of the reaction. A number of β-ketoacyl–CoA esters, including β-ketoadipyl-CoA (21), exhibit strong light absorption at 305 nm, ascribed to the enol form when complexed with Mg^{2+}. This β-ketoacyl-activating system evidently differs from the bacterial activation of β-ketoadipate (21) insofar as succinyl-CoA was ineffective, participation of ATP being obligatory for *T. cutaneum*.

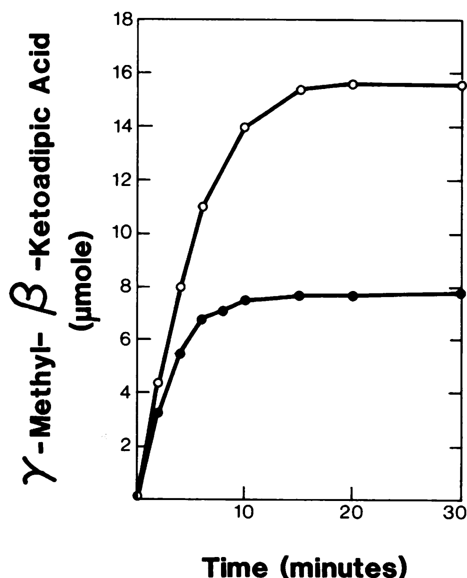

FIG. 4. Conversion of (±)-β-methylmuconolactone (●) and (−)-β-methylmuconolactone (○) to 3-keto-4-methyladipic acid (γ-methyl-β-ketoadipic acid). Reaction mixtures contained 16 μmol of lactone.

Thioesterification of either of the carboxyl groups of 3-keto-4-methyladipic acid could occur in principle. Structure VII (Fig. 2) is assigned on the basis of experiments, to be described below, which show that equimolar amounts of acetyl-CoA and methylsuccinyl-CoA arise by thiolytic fission. The biologically formed substrate meets the structural requirements for this reaction, as shown in Fig. 2.

Metabolism of 3-keto-4-methyladipyl-CoA (VI). In bacteria, β-ketoadipyl-CoA undergoes thiolytic fission with CoA to give acetyl-CoA and succinyl-CoA (21). On the other hand, it has been claimed (30) that the eucaryote *Neurospora crassa* degrades β-ketoadipic acid by an alternative route that involves CoA but is hydrolytic rather than thiolytic; we

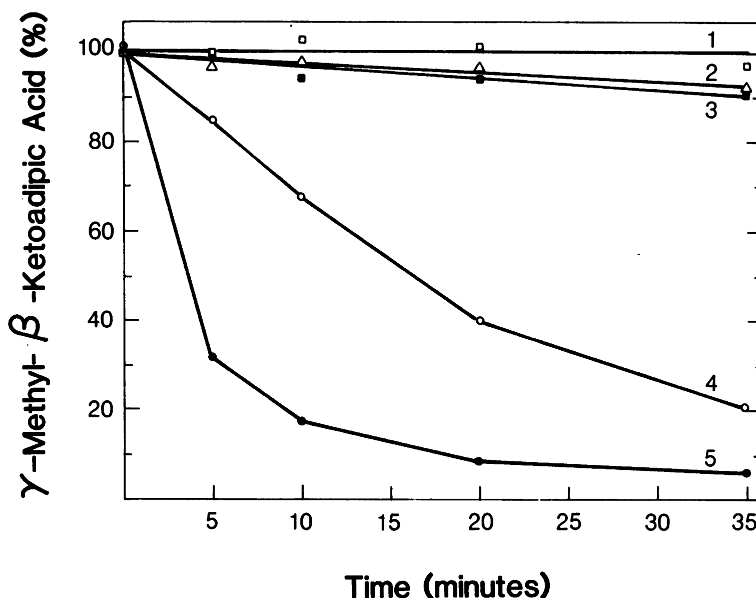

FIG. 5. Dependence of 3-keto-4-methyladipic acid (γ-methyl-β-ketoadipic acid) degradation upon ATP, CoA, and Mg^{2+} ions. Reaction mixtures contained 0.84 mg of cell extract protein, 0.89 μmol of CoA, 0.84 μmol of ATP, and 0.59 μmol of the β-ketoacid in 1 ml of 0.05 M Tris-H_2SO_4 buffer (pH 8) containing 5 mM $MgCl_2$. Curves: 5, complete reaction mixture; 1, using boiled extract; 2, minus ATP; 3, minus CoA; 4, minus Mg^{2+}.

present evidence below that unesterified methylsuccinic acid (IX, Fig. 2) is a catabolite of the eucaryote *T. cutaneum*. To decide conclusively whether 3-keto-4-methyladipyl-CoA undergoes thiolysis or hydrolysis, it was necessary to separate the activating enzyme that forms the CoA ester from the enzyme that catalyzes its cleavage and to separate both of them from an enzyme present in crude extracts that hydrolyzes methylsuccinyl-CoA to give the free acid. We describe below (i) the separation of these enzymes, (ii) the evidence for thiolytic fission of 3-keto-4-methyladipyl-CoA, and (iii) the products of thiolytic fission.

(i) Separation of enzymes. An extract was prepared in 6 ml of 0.025 M morpholinoethanesulfonic acid–NaOH buffer (pH 6.0) from 4.5 g of cells broken in the Hughes press, and after centrifugation the supernatant solution was allowed to stand for 45 min on ice and then recentrifuged. The extract (79 mg of protein) was applied to a carboxymethyl-cellulose column (1.0 by 10.5 cm) previously equilibrated with the same buffer. The column was washed with buffer until 13 2.2-ml fractions had been collected. Stepwise elution was then carried out, first with 33 ml of 0.025 M Tris-SO_4 buffer (pH 7.2) containing 0.065 or 0.07 M KCl and then with 33 ml of 0.025 M Tris-SO_4 (pH 7.2) containing 0.11 M KCl. An elution profile is shown in Fig. 6. The enzyme catalyzing synthesis of 3-keto-4-methyladipyl-CoA (synthetase) eluted in the initial wash with pH 6 buffer; however, the synthetase was not free from the other two enzymes, and further treatment was required. The cleavage enzyme for 3-keto-4-methyladipyl–CoA was washed from the column by 0.11 M KCl and was free from contaminating activities; the hydrolase for methylsuccinyl-CoA was eluted mainly in the 0.07 M KCl wash. All but traces of hydrolase and cleavage enzyme were removed from the synthetase preparation by a second application to another carboxymethyl-cellulose column, followed as before by elution with pH 6 buffer. A fraction of the eluate precipitating between 35 and 75% saturation with $(NH_4)_2SO_4$ was then collected, and the purified synthetase was dissolved in 1.5 ml of 0.05 M Tris-SO_4 buffer (pH 8.3).

(ii) Requirements of the cleavage reaction for CoA. Figure 7 shows that the disappearance of 3-keto-4-methyladipyl-CoA from an enzymatic reaction mixture was dependent upon the addition of CoA. In this experiment the substrate was generated by adding synthetase to a solution containing approximately equimolar amounts of CoA and 3-keto-4-

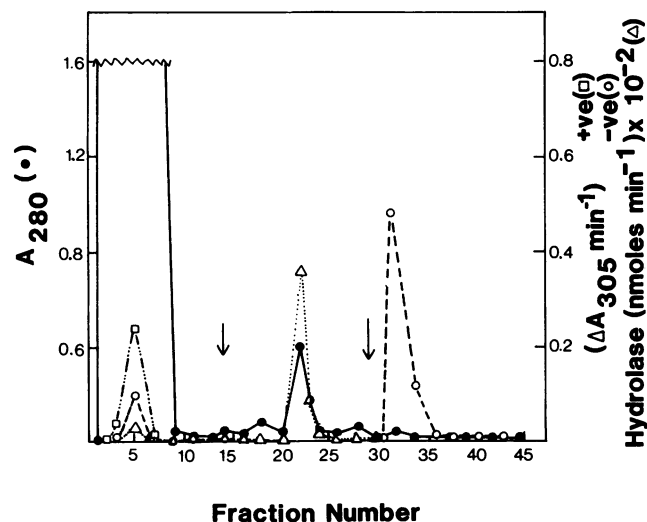

FIG. 6. Carboxymethyl-cellulose chromatography of enzymes involved in metabolism of 3-keto-4-methyladipic acid (VI, Fig. 2). Elution profiles are shown for 3-keto-4-methyladipyl-CoA synthetase (□), cleavage enzyme (○), methylsuccinyl-CoA hydrolase (Δ), and total protein (●). The column was washed with 0.025 M morpholinoethanesulfonic acid–NaOH buffer (pH 6.0) and eluted with buffer containing 0.07 M KCl (first arrow) and 0.11 M KCl (second arrow). Activities are expressed as reaction rates per assay volume of 10 μl (hydrolase) or 25 μl (synthetase and cleavage enzyme).

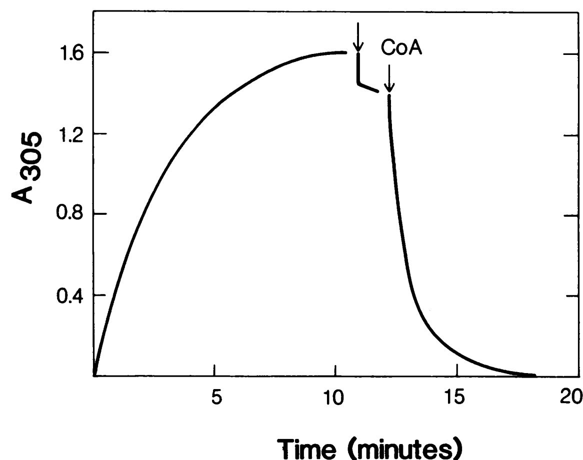

FIG. 7. Dependence of the 3-keto-4-methyladipyl-CoA (VII, Fig. 2) cleavage reaction upon the addition of CoA. Partially purified synthetase was used to accumulate the CoA ester which was monitored from its absorption at 305 nm. Additions of cleavage enzyme and CoA were then shown to be necessary for the rapid degradation of the ester. The reaction mixture contained, initially, 0.34 mg of synthetase, 140 nmol of CoA, 490 nmol of ATP, and 120 nmol of 3-keto-4-methyladipic acid (VI). First arrow, addition of 50 μl of cleavage enzyme; second arrow, addition of 200 nmol of CoA.

methyladipic acid at pH 9 with excess ATP present; the reaction was monitored from changes in light absorption at 305 nm due to the CoA ester. In another experiment, equimolar amounts of ATP and CoA were present with a twofold excess of 3-keto-4-methyladipic acid. After absorption at 305 nm had reached a maximum, more CoA was added but no change in absorption was observed until the addition of cleavage enzyme resulted in the rapid disappearance of the species absorbing at 305 nm. This experiment shows that synthesis, but not degradation, of 3-keto-4-methyladipyl–CoA is ATP dependent.

(iii) Products of thiolytic fission. Products of the CoA-dependent cleavage of 3-keto-4-methyladipyl-CoA were analyzed by high-pressure liquid chromatography essentially as described by Corkey et al. (11). The reaction mixture contained 1 ml of 0.1 M Tris-SO_4 (pH 8), 20 mM KCl, and 1 mM $MgCl_2$ (a low concentration of Mg^{2+} was used to avoid precipitation of magnesium phosphate in the column, which contained phosphate buffer). To this mixture were added 360 nmol of 3-keto-4-methyladipic acid, 360 nmol of CoA, and 1.4 mmol of ATP. The reaction was started by adding sufficient synthetase to produce an increase in A_{305} that ceased after approximately 5 min. Cleavage enzyme (100 μl) and 600 nmol of CoA were then added to the cuvette; A_{305} was reduced virtually to zero after an additional 5 min. The reaction mixture was then deproteinized immediately, neutralized to pH 5 (11), and analyzed. A control mixture containing the same reactants was acidified with 150 μl of 14% perchloric acid before enzyme was added. The high-pressure liquid chromatogram showed two peaks not present in the control with retention times of 9.3 and 7.8 min corresponding, respectively, to authentic acetyl-CoA and methylsuccinyl-CoA. The peak area for methylsuccinyl-CoA was 85% of that for acetyl-CoA; therefore, assuming similar extinction coefficients at the monitoring wavelength (260 nm), it appears that the two metabolites were formed in approximately equal amounts.

Methylsuccinic acid was also isolated from a reaction mixture with 3-keto-4-methyladipic acid as the substrate. The reaction was initiated by adding 3 ml of crude cell extract (88 mg of protein) to 11 ml of 0.1 M Tris hydrochloride buffer (pH 8.2)–10 mM $MgCl_2$–20 mM KCl containing, in addition, 34 μmol of 3-keto-4-methyladipic acid, 50 μmol of ATP, and 41 μmol of CoA (previously dissolved in 1 ml of Tris buffer). After incubation for 90 min, an additional 10 μmol of ATP and 11 mg of crude extract protein were added, and 30 min later the reaction mixture was deproteinized with perchloric acid and centrifuged, and the pH was brought to 12 with solid KOH to hydrolyze CoA esters. After standing for 90 min, the reaction mixture was adjusted to pH 8 with H_2SO_4, and potassium perchlorate was removed by centrifugation. The supernatant was chromatographed on Dowex 1×8 resin, and the fractions collected were dried in vacuo. Fractions eluting at the approximate position of authentic methylsuccinic acid were combined and analyzed by gas chromatography-mass spectrometry after conversion to the trimethylsilyl derivative. The fragmentation patterns for authentic and enzymatically produced samples were essentially identical.

Enzymatic hydrolysis of methylsuccinyl-CoA (VIII). We have described the separation of a hydrolase for methylsuccinyl-CoA that was present in cells grown with *p*-cresol. Since this enzyme also hydrolyzes succinyl-CoA and is active against synthetic methylsuccinyl-CoA (which is a mixture of isomers), its specific role in the catabolism of *p*-cresol may be questioned. However, the enzyme was induced specifically by growth with this substrate; thus, extracts of cells grown with *p*-cresol and phenol gave specific activities of 370 and <3 nmol/min per mg of protein, respectively, and succinate-grown cells showed no detectable activity. The reactions shown in Fig. 2 suggest a reason for the removal of CoA from C-4 of compound VIII, namely, that in the course of subsequent reactions giving rise to compound XI, the carboxyl group at C-4 remains free while that at C-1 is esterified by CoA. An analogy may be drawn with the biodegradative reaction sequence for valine (25), in which CoA is removed from C-1 of 3-hydroxyisobutyryl-CoA by a specifically induced hydrolase (28); the free acid is then decarboxylated, and C-3 of the original substrate becomes part of the thioester group of propionyl-CoA. We were unable to determine which enantiomer was the biological form of methylsuccinic acid; the optically active forms have low specific rotations (about 9° [16]), and it was difficult to isolate the amounts required for reliable polarimetric determinations. It should be noted that, upon dehydrogenation, either enantiomer would be expected to give rise to itaconic acid.

Degradation of itaconyl-CoA (X) to pyruvate and acetyl-CoA. As indicated in Fig. 2, no information was obtained concerning the reaction for which methylsuccinate serves as substrate; for although this compound is used as a carbon source by *T. cutaneum*, it is not oxidized by washed suspensions (Table 1) or by the various fractions we prepared from broken cells supplemented with artificial electron acceptors. Further, extracts of cells grown with either *p*-cresol or methylsuccinate showed only traces of activity against citraconic, mesaconic, or itaconic acids as judged from detectable changes produced in their UV spectra, with or without the addition of CoA and ATP to the reaction mixtures. However, positive results were obtained for a sample of itaconyl-CoA (but not for citraconyl-CoA) chemically synthesized from the acid anhydride.

Itaconyl-CoA (50 μl), prepared from approximately 2.2 mM CoA, was incubated with 62 μg of crude extract protein in 1 ml of 0.05 M Tris-SO_4 (pH 8.0)–10 mM $MgCl_2$; the

absorption spectrum from 300 to 230 nm was scanned throughout the incubation. The initial spectrum showed a peak at 260 nm that decreased to zero at 300 nm and exhibited fairly high A_{230} due to the thioester bond. After the addition of extract, the absorbance between 300 and 290 nm was abolished, and there were significant decreases in absorbance at 260 and 230 nm. When spectral changes ceased, the mixture was assayed for pyruvate; 100 nmol of NADH was added, and A_{340} was measured before and after the addition of 25 U of lactate dehydrogenase. The amount of pyruvate determined corresponded to approximately 15% of the CoA used to synthesize the itaconyl-CoA. This is a yield of active isomer similar to that found by Cooper and Kornberg (10), who used this chemical synthesis in studies of itaconate metabolism in a *Pseudomonas* sp. The identity of pyruvate was confirmed by using 10-fold increases in the amounts of cell extract protein and itaconyl-CoA above those used in the assay. When spectral changes were complete, the mixture was treated with 2,4-dinitrophenylhydrazine. After extraction, a 2,4-dinitrophenylhydrazone was detected which comigrated with the authentic derivative of pyruvate on silica gel thin-layer plates in solvent systems 3 and 4. The compound was not detected in a reaction mixture acidified before the addition of cell extract. The significance of this activity was emphasized by the observation that it was induced when cells were grown on *p*-cresol or methylsuccinate, but not when grown on phenol; amounts of pyruvate formed were, respectively, 14, 16, and <1 nmol.

Stoichiometry of itaconyl-CoA (X) degradation. The overall stoichiometry of the conversion of itaconyl-CoA into pyruvate and acetyl-CoA was difficult to establish since the chemical synthesis of the substrate gave only 15 to 20% of biologically active product. However, it was demonstrated by the following experiment that the yields of pyruvate and acetyl-CoA were equimolar. To itaconyl-CoA (2 ml), prepared from 2.2 mM CoA, was added 2 ml of 0.05 M Tris-SO_4 (pH 8) plus 20 mM $MgCl_2$, 4.5 μmol of sodium malate (as the source of oxaloacetate), 4.5 μmol of NAD^+, 18 U of citrate synthase (Sigma), and 1.8 mg of crude extract protein in 100 μl of phosphate buffer. Cell extracts contained powerful malate dehydrogenase activity. The reaction was followed by measuring the increase in A_{340} due to the formation of NADH, which ceased after approximately 10 min. The mixture was then placed on ice, and 200-μl portions were removed and assayed immediately for pyruvate using lactate dehydrogenase and NADH. Samples (1 ml) were also removed, deproteinized, and assayed for citrate. The average of three determinations showed that 163 ± 5 nmol of pyruvate were formed per ml of reaction mixture, while the parallel determinations in triplicate gave 160 ± 3 nmol of citrate formed per ml. No pyruvate or citrate was detected for the same procedure using boiled extract. The yield of pyruvate based on the amount of CoA used was about 16%, similar to that found when the oxaloacetate-generating system was omitted. It has been suggested by other workers who used these reactions that the yield is low because CoA adds to the double bond of the anhydride during synthesis of the ester (35).

Identification of citramalic acid from itaconyl-CoA (X). A dependence of citramalyl-CoA lyases upon Mg^{2+} has been observed previously (10, 19). When we dialyzed a crude cell extract of *T. cutaneum* against EDTA, the formation of pyruvate from itaconyl-CoA was virtually abolished but was restored by the addition of $MgCl_2$. Spectrophotometric measurements showed that itaconyl-CoA disappeared, and, in the experiment described below, citramalic acid was identified after chemical hydrolysis of thioesters. The cell extract used was prepared in 0.05 M Na^+-K^+ phosphate buffer (pH 7.4) containing 10 mM EDTA; it was dialyzed against 2 liters of the same buffer for 11 h, at which time the buffer was replaced, and dialysis continued for an additional 4 h. No pyruvate was formed when this extract was incubated with itaconyl-CoA. To a solution of itaconyl-CoA, prepared by treating 50 mg of CoA with 10 mg of itaconic anhydride, were added 10 ml of 0.1 M Tris-SO_4 buffer (pH 8) containing 0.2 mM EDTA and then 14 mg of dialyzed extract protein. The reaction was monitored by measuring the disappearance of A_{290}. When this ceased, 30 min later, the pH was adjusted to 12 with 5 N NaOH to hydrolyze CoA esters. After 45 min at room temperature, the reaction mixture was neutralized with 5 N H_2SO_4 and deproteinized by ultrafiltration through a PM-10 membrane (Amicon). The filtrate was then chromatographed on a Dowex column, and fractions containing acid that eluted in the position of citramalic acid were combined, converted to trimethylsilyl derivatives, and analyzed by gas chromatography-mass spectrometry. Three other peaks present in the elutate from the column were also given by a control in which boiled extract was used, but in this case there was no detectable acid in the position of citramalic acid. When the enzymatic sample was examined by gas chromatography-mass spectrometry, a single peak was observed with a fragmentation pattern identical to that of authentic citramalic acid.

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