The Utilization of Itaconate by *Pseudomonas* sp.

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Although many micro-organisms are known to grow on itaconate [$\text{CH}_2\text{:C}(\text{CO}_2^-)\cdot\text{CH}_2\cdot\text{CO}_2^-$] as their sole source of carbon, the pathway whereby this substrate is catabolized by obligatorily aerobic bacteria, to provide both energy and the precursors of cell materials, is not established. Brightman & Martin (1961) suggested that, in *Pseudomonas* sp., this branched-chain dicarboxylic acid, and its isomer mesaconate [$\text{CH}_3\cdot\text{C}(\text{CO}_2^-)\cdot\text{CH}\cdot\text{CO}_2^-$], were converted into straight-chain molecules by a process of intramolecular rearrangement which was possibly akin to that involved in the formation of mesaconate from glutamate by the anaerobe *Clostridium tetanomorphum* (Wachsmann, 1956; Barker, Wilson & Munch-Petersen, 1957; Munch-Petersen & Barker, 1958). An alternative mode of utilization of itaconate, involving its activation to itaconyl-coenzyme A followed by hydration and cleavage to acetyl-coenzyme A and pyruvate, was suggested by studies on itaconate oxidation in liver mitochondria (Adler, Wang & Lardy, 1957; Wang, Adler & Lardy, 1961).

It is the purpose of the present paper to describe a route of itaconate catabolism in *Pseudomonas* B₂aba which is similar to that occurring in liver mitochondria. Portions of this work have been published in a preliminary form (Cooper & Kornberg, 1962*a*, *b*).

MATERIALS AND METHODS

Maintenance and growth of organisms. The original culture of *Pseudomonas* B₂aba was a gift from Dr J. Lascelles. The organism was maintained by frequent subculture on Oxoid nutrient-agar slopes.

For experimental use, cells from the nutrient-agar slopes were used to inoculate 700 ml. of medium containing: sodium-potassium phosphate buffer, pH 7.1 (12.5 mM); NH_4Cl (12.5 mM); disodium itaconate (25 mM); 210 mg. of Difco yeast extract; and salts (7 mg. of $\text{CaCl}_2\cdot 6\text{H}_2\text{O}$, 14 mg. of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 11 mg. of $\text{MnSO}_4\cdot 4\text{H}_2\text{O}$, 0.7 mg. of $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 1 mg. of $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 1.2 mg. of $\text{CoSO}_4\cdot 7\text{H}_2\text{O}$ and 1.2 mg. of $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$). The medium was shaken in a 2 l. conical flask at 30° in a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The bacteria were harvested during the logarithmic phase of growth (at cell densities of 0.25–0.35 mg. dry wt./ml.) by centrifuging at 1500*g* for 5 min. at 2°. For studies on the oxidation of substrates, the packed cells were washed with 1.1% (w/v) KCl and suspended in 1.1% (w/v) KCl. For the preparation of extracts, the cells were washed with,

and resuspended in, 10 mM-sodium-potassium phosphate buffer, pH 7.0.

Preparation of cell extracts. Suspensions of freshly grown *Pseudomonas* B₂aba were subjected for 2–4 min. at 0° to the output of a MSE ultrasonic disintegrator, operating at 1.5A. The suspensions were centrifuged at 24 000*g* for 15 min. at 0°: the supernatant solutions thus obtained were used for experimental purposes.

Determination of protein. Soluble protein was generally measured by the biuret method as modified by Gornall, Bardawill & David (1949). The protein content of dilute solutions, such as those obtained during chromatography on DEAE-cellulose columns, was estimated by the method of Warburg & Christian (1941).

Separation of the enzymes acting on itaconate. Step 1: preparation of ultrasonic extracts. Extracts were prepared either from freshly grown cells or, for large-scale isolation procedures, from cells that had been stored at –15°. Concentrated suspensions of such cells (containing 80 mg. dry wt./ml. of 10 mM-sodium-potassium phosphate buffer, pH 7.0) were exposed for 10 min. to the output of the ultrasonic disintegrator. The resultant suspensions were centrifuged at 30 000*g* for 30 min. at 0–2°. The precipitate was discarded. All further operations were carried out at 0–3°.

Step 2: treatment with protamine sulphate. To the supernatant solution from step 1 was added 2% (w/v) protamine sulphate solution (1 mg. of protamine sulphate for every 10 mg. of soluble protein) slowly and with stirring. After 20 min., the precipitate was removed by centrifuging at 30 000*g* for 15 min. and discarded.

Step 3: fractionation with ammonium sulphate. To each 100 ml. of supernatant solution was added 17.6 g. of solid $(\text{NH}_4)_2\text{SO}_4$ (30% saturation). After 30 min., the precipitate was centrifuged down at 12 000*g* for 10 min. and discarded. To each 100 ml. of the supernatant solution was added 19.8 g. of solid $(\text{NH}_4)_2\text{SO}_4$ (60% saturation) and, after 30 min., the precipitate was collected by centrifugation: this precipitate was dissolved in a small volume of 5 mM-sodium-potassium phosphate buffer, pH 7.0, and was dialysed against several changes of this buffer overnight.

Step 4: column chromatography. A batch of 4 g. of DEAE-cellulose (Whatman DE 50) was suspended in 200 ml. of 5 mM-sodium-potassium phosphate buffer, pH 7.0, and the pH of the suspension was adjusted to 7.0 by the addition of 2*N*-HCl. The resin was washed several times with the 5 mM-sodium-potassium phosphate buffer, pH 7.0, and, as a thick slurry, was poured into a chromatographic column (2 cm. × 38 cm.) in which the resin was allowed to pack under a pressure of 5 lb./in.² applied from a nitrogen cylinder. The resin was equilibrated with 5 mM-sodium-potassium phosphate buffer, pH 7.0, and the dialysed material was applied to the column (160 mg. of protein was added for each 1 g. of resin). The column was developed

initially with 5 mM-sodium-potassium phosphate buffer, pH 7.0, and subsequently with a linear gradient of KCl. This gradient was obtained by allowing 250 ml. of a mixture of 400 mM-KCl and 5 mM-sodium-potassium phosphate buffer, pH 7.0, to flow, with constant stirring, into a vessel containing 250 ml. of 5 mM-sodium-potassium phosphate buffer, pH 7.0. The flow rate was 10–15 ml./hr. and 5 ml. fractions were collected.

Spectrophotometric procedures. All spectrophotometric assays were performed at $20 \pm 1^\circ$ in silica cells (1.5 ml. vol., 1 cm. light-path) with an Optica model CF-4 recording spectrophotometer.

Preparation of coenzyme A derivatives. Succinyl-CoA was prepared by the method of Simon & Shemin (1953). Itaconyl-CoA was prepared from itaconic anhydride and CoA by a similar procedure. Yields of up to 21% of the active isomer were obtained (based on the quantities of pyruvate formed). The itaconic anhydride was prepared by the method of Anschütz & Petri (1880) and was twice recrystallized from acetic acid (m.p. 68° , uncorr.).

Preparation of DL-citramalic acid. An initial sample of DL-citramalic acid was given by Professor Sir Hans Krebs, F.R.S. Later samples were prepared by the method of Michael & Tissot (1952). High yields of crystalline material were obtained (m.p. 115° , mixed m.p. 115° , uncorr.). DL-[1- ^{14} C]Citramalic acid was prepared from Na ^{14} CN by essentially the same method. In this case the DL-[1- ^{14} C]-citramalic acid was isolated by elution with 0.5N-HCl from a Dowex 1 (X 8; OH $^-$ form) column.

Identification of hydroxamates. Hydroxamic acids were analysed by high-voltage paper-electrophoresis at 6 kv and 70 ma for 60 min. in 0.2M-acetic acid-pyridine buffer, pH 4. The hydroxamates of itaconic acid, succinic acid and acetic acid readily separated in this system; they were detected by spraying with the concentrated FeCl $_3$ reagent of Stadtman & Barker (1950).

Other chemicals. Na ^{14} CN was purchased from The Radiochemical Centre, Amersham, Bucks. Reduced and oxidized nicotinamide nucleotides, crystalline lactate dehydrogenase, CoA, oxaloacetic acid, sodium pyruvate and α -oxoglutaric acid were purchased from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany; itaconic acid, citraconic acid, L-malic acid and protamine sulphate ('exherring') were from L. Light and Co. Ltd., Colnbrook, Bucks.; ATP was from Schwarz BioResearch Inc., Mount Vernon, N.Y., U.S.A.; mesaconic acid was from H.M. Chemical Co., Santa Monica, Calif., U.S.A.; glyoxylic acid was from British Drug Houses Ltd., Poole, Dorset. All other reagents were of the highest purity commercially available.

RESULTS

Oxidation of substrates. Washed suspensions of itaconate-grown *Pseudomonas B₂aba* readily oxidized itaconate, mesaconate, DL-citramalate, succinate, L-malate and α -oxoglutarate (Table 1); the oxidation of pyruvate, acetate and citraconate occurred at a slower rate. For itaconate, the quantities of oxygen consumed (corrected for the endogenous respiration) were 44% of those required for complete oxidation; the observed Q_{O_2} of 94 μ l. of oxygen absorbed/mg. dry wt./hr. hence cor-

Table 1. *Oxidation of substrates by washed suspensions of Pseudomonas B₂aba*

The main compartments of Warburg manometer flasks contained, in 2.5 ml., 5 μ moles of sodium-potassium phosphate buffer, pH 7.1, and washed cells (5–6 mg. dry wt.). The centre wells contained 400 μ moles of KOH. Substrates (20 μ moles of acetate and pyruvate, 10 μ moles of others) were added from the side arms after thermal equilibration at 30° .

Substrate added	Q_{O_2} (μ l. of O $_2$ absorbed/mg. dry wt./hr.)			
	Itaconate-grown cells		Succinate-grown cells	
	Observed	Corrected for endogenous respiration	Observed	Corrected for endogenous respiration
None	39	0	40	0
Itaconate	133	94	40	0
DL-Citramalate	133	94	40	0
Mesaconate	133	94	40	0
Citraconate	89	50	40	0
Acetate	99	60	109	69
Pyruvate	59	20	59	19
α -Oxo-glutarate	130	91	100	60
L-Malate	135	96	161	121
Succinate	172	133	204	164

responds to the utilization of 2.1 μ moles of itaconate/mg. dry wt. of cells/hr. That both D- and L-citramalate were oxidized by the itaconate-grown cells was shown by using DL-[1- ^{14} C]citramalate as substrate. At least 93% of the radioactive material was utilized.

Washed suspensions of succinate-grown *Pseudomonas B₂aba* oxidized succinate and L-malate rapidly; α -oxoglutarate and acetate were oxidized less rapidly. Pyruvate was oxidized slowly, and itaconate, DL-citramalate, mesaconate and citraconate were oxidized at a negligible rate (Table 1).

These results suggest that itaconate-grown cells contain enzymes that catalyse the conversion of itaconate and related compounds into intermediates of the tricarboxylic acid cycle, and that such enzymes are largely absent from cells grown on succinate.

Effect of arsenite on oxidations. The oxidation of pyruvate and of α -oxoglutarate by itaconate-grown cells was completely inhibited by 2 mM-sodium arsenite: this also abolished itaconate oxidation and led to the accumulation of a material that reacted with 2,4-dinitrophenylhydrazine to form a yellow 2,4-dinitrophenylhydrazone. Analysis of this derivative by paper chromatography (El Hawary & Thompson, 1953) and measurement of its absorption spectrum in alkaline solution showed it to be identical with the derivative formed from authentic sodium pyruvate. Under these conditions, 1.1 μ moles of pyruvate were formed in

90 min. from 10 μ moles of itaconate, which suggested that pyruvate was an intermediate in the catabolism of itaconate.

Formation of pyruvate from itaconate by cell-free extracts. Ultrasonic extracts of itaconate-grown cells did not show any oxygen uptake when incubated for 2 hr. with itaconate either in the absence or in the presence of Mg^{2+} ions, CoA and ATP. However, in the presence of Mg^{2+} ions, CoA and ATP, pyruvate (identified as its 2,4-dinitrophenylhydrazone) was formed from itaconate: little or no pyruvate was formed when itaconate, CoA or ATP was omitted (Table 2).

Although such extracts also did not show any oxygen uptake when incubated with pyruvate, ATP, Mg^{2+} ions and CoA, it was conceivable that pyruvate was not the first oxo compound formed from itaconate. This was tested by incubating cell-free extracts with semicarbazide plus itaconate and necessary cofactors. At the end of the incubation, the semicarbazones were decomposed with 6*N*-hydrochloric acid and 2,4-dinitrophenylhydrazine, and the 2,4-dinitrophenylhydrazones formed were extracted and chromatographed (Collins & Kornberg, 1960). Again, the derivative of pyruvate was the only 2,4-dinitrophenylhydrazone present and, though traces were formed in the absence of added CoA, the accumulation of significant quantities depended on the presence of itaconate, ATP and CoA. This confirmed that pyruvate was the first oxo compound formed from itaconate, and indicated that its formation involved a CoA derivative.

Measurement of itaconate activation. The activation of itaconate, which had been indicated as a necessary step in the formation of pyruvate, was demonstrated by the formation of a hydroxamic acid when ultrasonic extracts of itaconate-grown cells were incubated with hydroxylamine, itaconate, Mg^{2+} ions, CoA and ATP. The dependence of hydroxamate formation on the presence of all these components of the incubation system is shown in Table 3. Approx. 2 μ moles of hydroxamic acid were formed/mg. of protein/hr. in the complete system. Isolation of the hydroxamates formed, by the method of Stadtman & Barker (1950), and analysis by paper chromatography or high-voltage paper electrophoresis, revealed only one hydroxamic acid to be present, which corresponded in position to authentic acethydroxamic acid. However, when samples from the incubation mixtures were analysed directly by paper electrophoresis, without prior fractionation with ethanol, an hydroxamate corresponding to the major (slow) spot derived from authentic itaconylhydroxamic acid was also found to be present. Both hydroxamates were absent from systems from which the necessary cofactors had been omitted. This difference in the results obtained in the two methods

of analysis may be ascribed to the low solubility of itaconylhydroxamic acid in ethanol.

Spectrophotometric assay of pyruvate formation. The formation of pyruvate from itaconate was measured at pH 8.0 by following the decrease in extinction at 340 $m\mu$ concomitant with the oxidation of $NADH_2$ in the presence of an excess of lactate dehydrogenase (EC 1.1.1.27) or, at pH 6.8, in the assay system of Dixon & Kornberg (1959) by measuring the increase in extinction at 315 $m\mu$ concomitant with the formation of pyruvate phenylhydrazone. Only 50–100 μ g. of protein was needed for these assays, and complete dependence

Table 2. *Pyruvate formation from itaconate by cell-free extracts*

The main compartments of Warburg manometer flasks contained, in 2.5 ml., 120 μ moles of tris-HCl buffer, pH 8.0, 10 μ moles of $MgCl_2$ and 1 ml. of an ultrasonic extract of itaconate-grown *Pseudomonas B*_{aba} (containing 8 mg. of soluble protein and 10 μ moles of sodium phosphate buffer, pH 7.5). The side arms contained 0.5 μ mole of CoA, 10 μ moles of ATP and 10 μ moles of itaconate. (In one case, 0.2 μ mole of CoA was added.) The centre wells contained 400 μ moles of KOH. After thermal equilibration at 30°, the contents of the side arms were tipped into the main compartment. After 2 hr., samples (0.20 ml.) were removed from each cup and analysed for oxo compounds by the method of Friedemann & Haugen (1943).

System	Pyruvate formed (μ moles)
Complete	1.26
Complete but with only 0.2 μ mole of CoA	0.88
Mg^{2+} ions omitted	1.09
CoA omitted	0.14
ATP omitted	0.04
Itaconate omitted	0.02

Table 3. *Activation of itaconate*

The complete system contained 50 μ moles of tris-HCl buffer, pH 7.4, 50 μ moles of itaconate, 5 μ moles of ATP, 0.2 μ mole of CoA, 8 μ moles of GSH, 5 μ moles of $MgCl_2$, 200 μ moles of freshly neutralized hydroxylamine hydrochloride, 0.2 ml. of ultrasonic extract of itaconate-grown *Pseudomonas B*_{aba} (containing 2 mg. of soluble protein) and water to 1.0 ml. The reaction was stopped after 30 min. at 30° by the addition of 2 ml. of the $FeCl_3$ reagent of Lipmann & Tuttle (1945). Precipitated protein was removed by centrifugation and the hydroxamate colour was measured at 540 $m\mu$ against a blank from which itaconate had been omitted.

System	Hydroxamate formed (μ moles)
Complete	1.88
Mg^{2+} ions omitted	0.42
CoA omitted	0.26
ATP omitted	0.14
GSH omitted	1.01

on Mg^{2+} ions, CoA and ATP for pyruvate formation from itaconate was readily demonstrated: results obtained with the former assay procedure are illustrated in Fig. 1. In the phenylhydrazine assay system the rate of pyruvate formation was $3.1 \mu\text{moles/mg. of protein/hr.}$ and, in the lactate dehydrogenase-NADH₂ assay system it was $3.9 \mu\text{moles/mg. of protein/hr.}$; in both types of assay an initial lag was observed before the maximum rate was achieved.

Synthetic itaconyl-CoA could replace itaconate, CoA and ATP (but not Mg^{2+} ions) and decrease the lag observed in these spectrophotometric assay systems. Even when only $0.02 \text{ mM-itaconyl-CoA}$ was used, pyruvate was formed at a rate of $8.4 \mu\text{moles/mg. of protein/hr.}$ in the lactate dehydrogenase-NADH₂ assay procedure, and at a rate of $4.3 \mu\text{moles/mg. of protein/hr.}$ in the phenylhydrazine assay system.

Dependence of pyruvate formation on added co-enzyme A. The procedure used for demonstrating the formation of pyruvate by crude extracts of *Pseudomonas B₂aba* when incubated for 2 hr. with Mg^{2+} ions, CoA, ATP and itaconate (Table 2) did

Fig. 1. Pyruvate formation from itaconate catalysed by extracts of itaconate-grown *Pseudomonas B₂aba*. The complete system contained, in 1.0 ml. , $80 \mu\text{moles}$ of tris-HCl buffer, pH 8.0, $2 \mu\text{moles}$ of $MgCl_2$, $1 \mu\text{g.}$ of crystalline lactate dehydrogenase, approx. $0.1 \mu\text{mole}$ of NADH₂, itaconate-grown cell extract (containing $50\text{--}100 \mu\text{g.}$ of soluble protein), $5 \mu\text{moles}$ of itaconate, $0.2 \mu\text{mole}$ of CoA and $5 \mu\text{moles}$ of ATP. Curve A: $MgCl_2$ or CoA or ATP omitted initially from the complete system and added at the point marked with an arrow. Curve B: reaction started by the addition of itaconate at the point marked with an arrow. Curve C: itaconate, CoA and ATP omitted and replaced by approx. $80 \mu\text{moles}$ of synthetic itaconyl-CoA, added at the point marked by the arrow. The graphs were taken from a tracing made with an Optica recording spectrophotometer.

not permit of the demonstration that CoA was required in stoichiometric amounts, but this was readily demonstrated spectrophotometrically. Over the range tested ($0\text{--}35 \mu\text{moles}$ of CoA), the quantities of pyruvate formed, calculated from the observed decreases in extinction at $340 \text{ m}\mu$ with appropriate corrections for the slight endogenous removal of NADH₂, were proportional to the quantities of CoA added.

Spectrophotometric measurement of acetyl-co-enzyme A formation. The formation of acetyl-CoA from itaconate, CoA, ATP and Mg^{2+} ions was demonstrated as the reduction of NAD in the presence of L-malate, malate dehydrogenase (EC 1.1.1.37) and citrate synthase (EC 4.1.3.7): the rate of formation of NADH₂ concomitant with the oxidation of L-malate was a measure of the rate of formation of citrate and hence of the formation of acetyl-CoA (Ochoa, 1955). At pH 9.2, the maximum rate of NADH₂ formation in this system was $1.9 \mu\text{moles/mg. of protein/hr.}$ and occurred after an initial lag. The formation of NADH₂ was completely dependent on CoA, ATP, Mg^{2+} ions, L-malate and itaconate. Synthetic itaconyl-CoA replaced CoA, ATP and itaconate: although only $0.02 \text{ mM-itaconyl-CoA}$ was used, NADH₂ was formed at $2.2 \mu\text{moles/mg. of protein/hr.}$, and the lag period was shortened (Fig. 2).

Fig. 2. Acetyl-CoA formation from itaconate catalysed by extracts of itaconate-grown *Pseudomonas B₂aba*. The complete system contained, in 1.0 ml. , $80 \mu\text{moles}$ of tris-HCl buffer, pH 9.2, $2 \mu\text{moles}$ of $MgCl_2$, $0.15 \mu\text{mole}$ of NAD, $5 \mu\text{moles}$ of L-malate, itaconate-grown cell extract (containing $50\text{--}100 \mu\text{g.}$ of soluble protein), $5 \mu\text{moles}$ of itaconate, $0.2 \mu\text{mole}$ of CoA and $5 \mu\text{moles}$ of ATP. Curve A: $MgCl_2$ or CoA or ATP omitted initially from the complete system and added at the point marked by an arrow. Curve B: reaction started by the addition of itaconate at the point marked with an arrow. Curve C: L-malate omitted from the cuvette initially and added at the point marked by the arrow. Curve D: itaconate, CoA and ATP omitted and approx. $70 \mu\text{moles}$ of synthetic itaconyl-CoA added at the point marked by the arrow. The graphs were taken from a tracing made with an Optica recording spectrophotometer.

Stoichiometry of pyruvate and acetyl-coenzyme A formation from itaconyl-coenzyme A. The amounts of pyruvate and of acetyl-CoA formed from itaconyl-CoA were proportional to the amounts of itaconyl-CoA added (Table 4). Since the itaconyl-CoA preparation used absorbed light strongly at 232 m μ , it was not possible to use more than two, rather low, concentrations of this substrate for measurements of the quantities of acetyl-CoA formed. However, the results obtained indicate that itaconyl-CoA gave rise to acetyl-CoA and pyruvate in equimolar amounts. This reaction is unlikely to be a direct cleavage of itaconyl-CoA as such a cleavage would yield acetyl-CoA and acrylate, and acrylate did not give rise to pyruvate when substituted for itaconyl-CoA. The reaction thus probably involves a prior hydration of itaconyl-CoA to citramalyl-CoA (cf. Wang *et al.* 1961), as is also indicated by the observation that washed suspensions of *Pseudomonas B₂* grown on itaconate readily oxidized citramalate, whereas washed suspensions of the organism grown on succinate did not (Table 1).

Formation of pyruvate and acetyl-coenzyme A from citramalate. When citramalate was substituted for itaconate in the spectrophotometric assay systems (which contained ATP, Mg²⁺ ions and CoA), neither the formation of acetyl-CoA nor that of pyruvate was detected. The addition of acetate to the reaction mixture had no effect, but, when succinate was included, pyruvate and acetyl-CoA were

formed (after a lag) at rates of 6.8 and 2.2 μ moles/mg. of protein/hr. respectively: this lag was considerably shortened by incubating the reaction system with succinate before the addition of citramalate. Again, no pyruvate or acetyl-CoA formation from citramalate, incubated with succinate, was observed when enzyme, ATP, Mg²⁺ ions or CoA was omitted.

The requirement for succinate, CoA and ATP could be replaced by synthetic succinyl-CoA, when pyruvate was formed at 8.1 μ moles/mg. of protein/hr. It is presumed that succinyl-CoA and citramalate react to give citramalyl-CoA and that this substance is cleaved to pyruvate and acetyl-CoA. The origin of this pyruvate was established with the use of DL-[1-¹⁴C]citramalate: when this labelled substrate was incubated with succinyl-CoA and bacterial extract, a radioactive oxo compound was produced, the 2,4-dinitrophenylhydrazone of which was indistinguishable from that of authentic pyruvate. No such compound was formed from DL-[¹⁴C]citramalate when succinyl-CoA was replaced by succinate in the incubation system. Pyruvate formation was also not detected when acetyl-CoA, or acetyl-CoA plus succinate, was incubated with citramalate, Mg²⁺ ions and extract, which indicated both the specific nature of the CoA donor for the (presumed) formation of citramalyl-CoA and the absence of any enzyme catalysing a possible formation of succinyl-CoA from acetyl-CoA and succinate.

Since (unlike itaconate) citramalate does not appreciably absorb light at 232 m μ , and succinyl-CoA could be prepared in high yield, the production of larger quantities of acetyl-CoA could be demonstrated from citramalate than had been possible with itaconate. By using the assay systems described in Table 4, but with itaconyl-CoA replaced by 57 μ m-moles of succinyl-CoA (assayed as succinylhydroxamate) and 5 μ moles of citramalate, the formation of 44.3 μ m-moles of acetyl-CoA was shown to be accompanied by the formation of 48.5 μ m-moles of pyruvate. This finding established the stoichiometric role of succinyl-CoA in the formation of citramalyl-CoA, and that this material gave rise to equimolar amounts of pyruvate and acetyl-CoA.

Fractionation of extracts of itaconate-grown cells. The results described above suggested that the transformation of itaconyl-CoA into pyruvate and acetyl-CoA involves its hydration to citramalyl-CoA, which is subsequently cleaved. At least two enzymes might be suspected to be involved in this overall reaction.

Ultrasonic extracts of itaconate-grown *Pseudomonas B₂* were prepared and treated with protamine sulphate as described above. Such extracts were fractionated with ammonium sul-

Table 4. *Stoichiometry of pyruvate and acetyl-coenzyme A formation from itaconyl-coenzyme A*

The assay system for pyruvate estimation contained, in 1.0 ml., 85 μ moles of tris-HCl buffer, pH 8.0, 2 μ moles of MgCl₂, 1 μ g. of crystalline lactate dehydrogenase, 0.1 μ mole of NADH₂, itaconate-grown *Pseudomonas B₂* extract (43 μ g. of soluble protein) and itaconyl-CoA as indicated. The oxidation of NADH₂ was measured against a blank which lacked NADH₂. The amounts of pyruvate formed were calculated from the observed decrease in extinction at 340 m μ , with appropriate allowance for the low endogenous removal of NADH₂. The assay system for acetyl-CoA estimation contained, in 0.84 ml., 73 μ moles of tris-HCl buffer, pH 8.0, 2 μ moles of MgCl₂, 0.3 μ mole of oxaloacetate (a) or glyoxylate (b), itaconate-grown *Pseudomonas B₂* extract (43 μ g. of soluble protein) and itaconyl-CoA as indicated. The amounts of acetyl-CoA formed were calculated from the observed decreases in extinction at 232 m μ , measured against a blank which lacked oxaloacetate or glyoxylate.

Vol. of itaconyl-CoA added (ml.)	Pyruvate formed (μ m-moles)	Acetyl-CoA formed (μ m-moles)	
		By assay (a)	By assay (b)
0.01	9.3	8.1	8.6
0.02	18.3	15.3	16.7
0.04	36.5	—	—

phate and the fractions assayed for the ability to form pyruvate from itaconyl-CoA. The fraction obtained between 30 and 40% saturation with ammonium sulphate catalysed pyruvate formation at only a slow rate, and no pyruvate formation was detected when the fraction obtained between 55 and 65% saturation with ammonium sulphate was used. However, when these two fractions were combined, pyruvate was formed rapidly: the observed rate was at least 15 times as fast as that obtained with an equal amount of the 30–40% saturated ammonium sulphate fraction alone. No stimulation of pyruvate formation occurred if either fraction was boiled before addition.

When the 30–40% saturated ammonium sulphate fraction was incubated with itaconyl-CoA and the 55–65% saturated ammonium sulphate fraction was added subsequently, the maximum rate of NADH₂ oxidation was obtained only after a lag period of 1–2 min.: this behaviour was also observed when both fractions were present initially. When, however, the 55–65% saturated ammonium sulphate fraction was preincubated with itaconyl-CoA, the subsequent addition of the 30–40% saturated ammonium sulphate fraction caused a rapid oxidation of NADH₂ without a lag. These results showed that the crude extract could be separated into two fractions effecting the overall conversion of itaconyl-CoA into pyruvate and acetyl-CoA and that the enzyme in the 55–65% saturated ammonium sulphate fraction acted before that in the 30–40% saturated ammonium sulphate fraction. The 55–65% saturated ammonium sulphate fraction was thus tentatively identified as the itaconyl-CoA hydratase and the 30–40% saturated ammonium sulphate fraction as the citramalyl-CoA lyase.

The isolation of these enzymes was initially achieved by chromatography on DEAE-cellulose of cell extracts precipitated between 30 and 60% saturation with ammonium sulphate (see the Materials and Methods section). The two separate enzyme fractions obtained by ammonium sulphate fractionation at 30–40% saturation and 55–65% saturation, added in excess, were used to test for the appearance of their complementary enzymes (necessary for the overall transformation of itaconyl-CoA) in samples obtained after column chromatography. The elution pattern of protein and the position of the enzymes is shown in Fig. 3. Enzyme *A* corresponds to the 55–65% saturated ammonium sulphate fraction and enzyme *B* to the 30–40% saturated ammonium sulphate fraction. By substituting citramalate plus succinyl-CoA for itaconyl-CoA in the assay system, it was further possible, in the presence of an excess of enzyme *B*, to isolate a third enzyme *C* from the column fractions.

Identification of the enzymes. Samples of enzymes *A*, *B* and *C* were incubated in various combinations with (i) itaconyl-CoA, or (ii) succinyl-CoA plus itaconate, or (iii) succinyl-CoA plus citramalate, and pyruvate formation was measured. Enzymes *A* plus *B* plus *C* catalysed the formation of pyruvate from all three substrates. However, enzymes *A* plus *B* were sufficient to catalyse pyruvate formation from itaconyl-CoA (i), and enzymes *B* plus *C* sufficient to catalyse pyruvate formation from succinyl-CoA plus citramalate (iii) (Table 5). It is therefore probable that enzyme *A* is involved in the hydration of itaconyl-CoA to citramalyl-CoA, enzyme *B* in the cleavage of citramalyl-CoA to pyruvate and acetyl-CoA, and enzyme *C* in the transfer of the CoA moiety from succinyl-CoA (to citramalate). This was supported by the use of

Fig. 3. Elution pattern of enzymes from DEAE-cellulose. The continuous line represents the protein concentration measured as $E_{280\text{ m}\mu}$. Peak *A* (●) is itaconyl-CoA hydratase, peak *B* (▲) is citramalyl-CoA lyase and peak *C* (○) is succinyl-CoA-itaconate CoA-transferase. The conditions are given in the text.

Table 5. Formation of pyruvate by purified enzymes

The assay system contained, in 1.0 ml., 80 μ moles of tris-HCl buffer, pH 8.0, 2 μ moles of MgCl₂, 1 μ g. of crystalline lactate dehydrogenase, 0.1 μ mole of NADH₂ and other components as listed below. Pyruvate formation was measured as the rate of decrease of extinction at 340 m μ concomitant with the oxidation of NADH₂. Enzymes *A*, *B* and *C* are defined in the text.

Substrates used	Rate of pyruvate formation (μ m-moles/min.)			
	Enzymes			
	<i>A</i> + <i>B</i>	<i>A</i> + <i>C</i>	<i>B</i> + <i>C</i>	<i>A</i> + <i>B</i> + <i>C</i>
(i) Itaconyl-CoA	10.4	1.3	0.1	10.2
(ii) Succinyl-CoA + itaconate	0	1.2	0.1	9.4
(iii) Succinyl-CoA + citramalate	0	1.3	9.3	9.2

succinyl-CoA plus itaconate (ii) as substrate: no combination of any two of enzymes *A*, *B* and *C* catalysed the overall reaction, and its occurrence required the presence of enzyme *C* (to form the itaconyl-CoA by CoA transfer), enzyme *A* (to hydrate itaconyl-CoA to citramalyl-CoA) and enzyme *B* (to cleave citramalyl-CoA). In accordance with the system of nomenclature suggested by the Commission on Enzymes (1961), the trivial names 'itaconyl-CoA hydratase' may be applied to enzyme *A*, 'citramalyl-CoA lyase' to enzyme *B*, and 'itaconate CoA-transferase' to enzyme *C*. However, as shown in Table 5 (iii), itaconate CoA-transferase can also catalyse the transfer of the CoA moiety to citramalate.

Although the cleavage of citramalyl-CoA is formally similar to the cleavage of citryl-CoA (Srere, 1963), neither purified citramalyl-CoA lyase nor crude cell extracts catalysed the formation of oxaloacetate from citrate in the presence of itaconate CoA-transferase, ATP, succinate and CoA.

An elution pattern for the enzymes similar to that shown in Fig. 3 was obtained when cell extract precipitated by the addition of ammonium sulphate to 75 % saturation (after the customary treatment with protamine sulphate) was applied to DEAE-cellulose columns; 5 mM-sodium-potassium phosphate buffer, containing magnesium chloride (1 mM) and EDTA (1 mM), was used throughout, and a magnesium chloride gradient was used for the elution of protein from the columns. These changes in procedure resulted in considerable improvements in the separation and recovery of enzymes in the extract, and permitted the identification of a fourth enzyme *D*, which was eluted between enzymes *B* and *C* during column chromatography. This enzyme, assayed by pyruvate formation from itaconate plus CoA and ATP, in the presence of an excess of itaconyl-CoA hydratase and citramalyl-CoA lyase, was shown to catalyse the direct activation of itaconate to itaconyl-CoA. That the same enzyme also catalysed the activation of succinate to succinyl-CoA was indicated by the rapid formation of pyruvate when enzyme *D* was incubated with citramalate, succinate, CoA and ATP in the presence of an excess of itaconate CoA-transferase and citramalyl-CoA lyase: no such formation of pyruvate occurred in this system when enzyme *D* was omitted. The conclusion that enzyme *D* was not a specific itaconyl-CoA synthetase but was identical with succinyl-CoA synthetase (EC 6.2.1.4 or EC 6.2.1.5) was further supported by the following observations: (a) the purified enzyme catalysed the formation of hydroxamic acid(s) from itaconate, CoA, ATP and hydroxylamine at two-thirds of the rate observed with succinate as substrate; (b) although the rate of pyruvate formation in the presence of ATP, CoA

and itaconyl-CoA hydratase, citramalyl-CoA lyase, itaconate CoA-transferase and enzyme *D* was only half as rapid when itaconate was the substrate as when citramalate plus succinate were used, itaconate plus succinate formed pyruvate at twice this rate (Table 6); (c) the omission of itaconate CoA-transferase from this assay system did not decrease the rate of pyruvate formation from itaconate, but a decrease of 85 % in the rate was observed when succinate was also added. This indicates that succinate competed with itaconate for the activating enzyme (Table 6). These findings are in agreement with those reported by Adler *et al.* (1957), Wang *et al.* (1961) and Nagai (1963).

Influence of growth substrates on the formation of the enzymes. Extracts of cells grown on itaconate, citramalate, or methylsuccinate contained all four enzymes at high specific activity, but only the itaconyl-CoA synthetase was present in comparably large amounts in extracts of cells grown on succinate, malate, lactate or pyruvate (Table 7). This shows that itaconyl-CoA hydratase, citramalyl-CoA lyase and itaconate CoA-transferase are inducible, whereas (as might be expected from its identity with succinyl-CoA synthetase) the formation of itaconyl-CoA synthetase is not markedly influenced by the nature of the growth substrate. These observations also suggest that the inducible enzymes play a necessary role in the catabolism of itaconate (and of substances closely related to itaconate).

Support for this conclusion is provided by measurements of their differential rates of synthesis during growth, or adaptation to growth, on various substrates.

The high concentrations of the enzymes necessary for the overall formation of pyruvate (and acetyl-CoA) from itaconyl-CoA remained constant

Table 6. *Identity of itaconate-activating enzyme*

The incubation mixtures contained, in 1.0 ml., 80 μ moles of tris-HCl buffer, pH 8.0, 2 μ moles of $MgCl_2$, 1 μ g. of crystalline lactate dehydrogenase, 0.1 μ mole of NADH₂, 5 μ moles of ATP, 0.2 μ mole of CoA, purified itaconyl-CoA synthetase, excess of itaconyl-CoA hydratase, citramalyl-CoA lyase, itaconate CoA-transferase and substrates (5 μ moles of each) as indicated. Itaconate CoA-transferase was omitted where marked with an asterisk.

Substrates	Rate of pyruvate formation (μ m-moles/min.)
Succinate	0
Citramalate	0
Citramalate + succinate	9.2
Itaconate	4.6
Itaconate + succinate	9.2
Itaconate*	5.4
Itaconate + succinate*	0.8

when itaconate-grown cells were used as an inoculum for continued growth on itaconate: the straight line relating the total content of these enzymes in the culture to the increase in cell mass could be extrapolated to pass through the origin and had a slope with a *P* value of 15 (Fig. 4). However, only a slight increase in enzyme content was noted when the itaconate-grown inoculum continued to grow for three generations on succinate as carbon source: under these conditions, the straight line had a slope with a *P* value of 0.8 (Fig. 4), which indicated that the differential rate of synthesis of the enzymes (or of a key enzyme in the sequence) during growth on succinate was less than 6 % of that during growth on itaconate.

Succinate-grown cells continuing to grow on succinate also formed the enzymes catalysing the formation of pyruvate from itaconyl-CoA (or a key enzyme of that sequence) at a negligibly low rate, which was less than 0.03 % of that observed during growth on itaconate. When such succinate-grown cells were inoculated into growth medium containing itaconate as sole carbon source, only slight growth was detected during the first 4 hr., after which the

customarily observed growth rate (mean doubling time 2.6–2.9 hr.) was achieved. During this initial lag period, however, the specific activity of the enzyme(s) necessary for the overall formation of pyruvate from itaconyl-CoA rose rapidly and reached the high level usually noted during growth on itaconate (Fig. 5).

Table 7. Influence of growth substrates on enzyme concentrations in extracts of *Pseudomonas B₂aba*

The basic assay system contained, in 1.0 ml., 80 μ moles of tris-HCl buffer, pH 8.0, 2 μ moles of $MgCl_2$, 1 μ g. of crystalline lactate dehydrogenase and approx. 0.1 μ mole of NADH₂. For the assay of itaconyl-CoA synthetase, the basic system was supplemented with 0.2 μ mole of CoA, 5 μ moles of ATP, 5 μ moles of itaconate, excess of itaconyl-CoA hydratase and excess of citramalyl-CoA lyase. For the assay of itaconate CoA-transferase, the basic system was supplemented with 5 μ moles of DL-citramalate, approx. 90 μ m-moles of succinyl-CoA and excess of citramalyl-CoA lyase. For the assay of itaconyl-CoA hydratase, the basic system was supplemented with approx. 60 μ m-moles of itaconyl-CoA and excess of citramalyl-CoA lyase. For the assay of citramalyl-CoA lyase, the basic system was supplemented with approx. 60 μ m-moles of itaconyl-CoA and excess of itaconyl-CoA hydratase. (In all these procedures, the enzymes added in excess were those purified by column chromatography.) The rates of the reactions are expressed as the quantities of substrates (μ moles) transformed/mg. of soluble protein/hr.

Growth substrate	Itaconyl-CoA synthetase (D)	Itaconate-CoA transferase (C)	Itaconyl-CoA hydratase (A)	Citramalyl-CoA lyase (B)
Itaconate	9.2	14.5	9.2	28.6
Citramalate	11.1	10.0	9.0	16.2
Methylsuccinate	11.6	17.8	11.0	33.6
Succinate	9.1	0.05	0.09	0.55
Malate	7.8	0	0	0.24
Lactate	8.5	0.20	0.91	1.4
Pyruvate	7.6	0.70	1.0	2.8

Fig. 4. Formation of enzymes, catalysing pyruvate production from itaconyl-CoA, by itaconate-grown *Pseudomonas B₂aba* continuing to grow on itaconate (○) or succinate (●) as carbon source. The conditions are given in the text.

Fig. 5. Formation of enzymes, catalysing pyruvate production from itaconyl-CoA, by succinate-grown *Pseudomonas B₂aba* adapting to growth on itaconate. ○, Specific activity; ●, cell density. The conditions are given in the text.

Scheme 1. Suggested pathway for the growth of *Pseudomonas B₂aba* on itaconate as sole carbon source. The letters A–D refer to component enzymes described in the text.

DISCUSSION

The tricarboxylic acid cycle provides both the precursors of cell constituents synthesized during growth and the energy necessary to effect such biosyntheses. This dual role of the cycle implies that reactions must occur which result in the net formation of intermediates of the cycle from the growth substrate, particularly when that substrate is supplied as the sole source of carbon and energy.

Although the route of formation of itaconic acid (which is known to be produced in large quantities by moulds such as *Aspergillus terreus*) has been shown to involve the decarboxylation of *cis*-aconitic acid (Bentley & Thiessen, 1957*a, b, c*), the direct formation of an intermediate of the cycle from itaconate, possibly by reversal of this reaction, has not been demonstrated. Two indirect routes of itaconate utilization have been suggested. The first, postulated to occur in a pseudomonad, involves a molecular rearrangement of itaconate, or some compound derived from itaconate, to yield a straight-chain intermediate which would enter the tricarboxylic acid cycle as succinate (Brightman & Martin, 1961). A second route, whose operation in liver mitochondria is established, leads to the formation of pyruvate and acetyl-CoA from itaconate via itaconyl-CoA and citramalyl-CoA (Adler *et al.* 1957; Wang *et al.* 1961).

The evidence presented above indicates that the second of these pathways (Scheme 1) also represents the major route of itaconate utilization in *Pseudomonas B₂aba*, although there appear to be some minor differences between the bacterial and mitochondrial systems. Thus, unlike *Pseudomonas B₂aba*, liver mitochondria do not oxidize citramalate and have not been reported to possess the itaconate CoA-transferase (enzyme C); similarly, neither the itaconyl-CoA hydratase (enzyme A) nor the citramalyl-CoA lyase (enzyme B) of mitochondria require Mg^{2+} ions for activity, whereas this metal ion is needed for the activity of at least one of the

bacterial enzymes. However, in both the animal and microbial systems, the formation of itaconyl-CoA from itaconate, ATP and CoA is catalysed by an enzyme (D) apparently identical with succinyl-CoA synthetase.

The quantitative importance of this pathway, which has also been indicated by the work of Nagai (1963), is emphasized both by the demonstration that the key enzymes A, B and C are present in high activity in extracts of itaconate-grown *Pseudomonas B₂aba* and by the finding that they are synthesized to only a negligible extent during growth on lactate, pyruvate or intermediates of the tricarboxylic acid cycle. After the transfer of cells grown on succinate to a medium containing itaconate as sole carbon source, the formation of these enzymes preceded the onset of growth and their cellular concentrations reached those normally obtaining in itaconate-grown cells before the maximum growth rate was achieved.

SUMMARY

1. Washed suspensions of *Pseudomonas B₂aba*, grown on itaconate, oxidized itaconate, DL-citramalate and mesaconate, but these substances were not oxidized by succinate-grown cells.

2. Extracts of the itaconate-grown organism catalysed the formation of pyruvate and acetyl-coenzyme A when incubated with itaconate, adenosine triphosphate, coenzyme A and Mg^{2+} ions. The quantities of pyruvate formed were proportional to those of coenzyme A added. Synthetic itaconyl-coenzyme A replaced the requirements for itaconate, adenosine triphosphate and coenzyme A.

3. The formation of pyruvate and acetyl-coenzyme A from DL-citramalate, in the presence of extract and Mg^{2+} ions, necessitated the presence either of succinate, coenzyme A and adenosine triphosphate, or of succinyl-coenzyme A.

4. Both synthetic itaconyl-coenzyme A and enzymically produced citramalyl-coenzyme A

yielded pyruvate and acetyl-coenzyme A in equimolar amounts.

5. The enzymes involved in these reactions were isolated and identified. Their inducibility supports their importance in the utilization of itaconate.

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New Procedures for Isolating Polypeptides and Proteins from Tissues

METABOLIC INCORPORATION OF L-[¹⁴C]VALINE INTO FRACTIONS OF INTERMEDIATE MOLECULAR WEIGHT IN BROAD-BEAN (*VICIA FABA* L.) LEAVES

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'Deproteinized' tissue extracts are often demonstrably free from protein. The converse proposition, that the residues from the preparation of such extracts are free from 'non-protein' materials, is not often discussed, still less tested experimentally. Reasons have been given for doubting its applicability to peptides, other than those of very low molecular weight in which hydrophilic side chains predominate (Synge, 1953, 1955, 1959). When we

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undertook the present work, we wished to investigate the occurrence and metabolic behaviour of peptide-like compounds in leaves under conditions in which ¹⁴C-labelled amino acid was being incorporated into protein. Instead of using only conventional deproteinizing procedures, we preferred to dissolve both protein and non-protein materials as completely as possible, and then to isolate from solution those components of the mixture having rather low molecular weight. These were to be isolated under conditions in which their tendency to associate with larger molecules, especially by hydrogen bonds, could be minimized