The Utilization of Aconate and Itaconate by *Micrococcus* sp.

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(Received 4 May 1964)

1. An organism, identified as *Micrococcus* sp., was isolated by elective culture on aconate; it also grew on itaconate. 2. Washed suspensions of the aconate-grown organism readily oxidized intermediates of the tricarboxylic acid cycle, aconate and succinic semialdehyde, but not itaconate. Itaconate-grown cells oxidized tricarboxylic acid-cycle intermediates, succinic semialdehyde and itaconate, but not aconate. Succinate-grown cells oxidized neither itaconate nor aconate. 3. Extracts of aconate-grown cells catalysed the formation of succinic semialdehyde and carbon dioxide, in equimolar amounts, from aconate. In the presence of NAD or NADP, succinic semialdehyde was oxidized to succinate with concomitant reduction of the coenzyme. 4. Extracts of itaconate-grown cells catalysed the formation of pyruvate and acetyl-CoA from itaconyl-CoA. 5. Key enzymes involved in the formation of succinate from aconate, and of pyruvate and acetyl-CoA from itaconate, were distinct and inducible: their formation preceded growth on the appropriate substrate.

Studies on the catabolism of itaconate by *Pseudomonas* sp. (Cooper & Kornberg, 1964) indicated that this compound was utilized as sole carbon source for growth via a pathway that led to acetyl-CoA and pyruvate, and involved itaconyl-CoA and citramalyl-CoA as intermediates. The component enzymes of this route, which were shown to be inducible, were formed also when the organism grew on derivatives of itaconate, such as α -methylsuccinate and citramalate, as carbon and energy sources. Since the pathway thus demonstrated was closely similar to that which effects the oxidation of itaconate by liver mitochondria (Adler, Wang & Lardy, 1957; Wang, Adler & Lardy, 1961), and since liver mitochondria also rapidly oxidize aconate (the lactone derived from hydroxymethylfumaric acid), it was possible that aconate was also utilized by the pathway apparently common to the utilization of itaconate and its derivatives.

This was tested with an organism, identified as a *Micrococcus* sp., capable of growth both on itaconate and aconate. The results obtained show that itaconate is used by this organism by a route essentially similar to that used by pseudomonads, but that aconate utilization proceeds via a pathway which effects the transformation of this substance into succinate. Like the component enzymes of the former route, those of the latter are inducible.

A preliminary report of this work has been

presented to The Biochemical Society (Cooper, Itiaba & Kornberg, 1964).

MATERIALS AND METHODS

Isolation and growth of the organism. The organism was isolated from soil by elective culture on a medium which contained (in 140 ml.): sodium-potassium phosphate buffer, pH 7.1 (12.5 mM), NH_4Cl (12.5 mM), sodium aconate (25 mM), 42 mg. of Difco yeast extract, salts (1.4 mg. of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 2.8 mg. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14 mg. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 0.14 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and 1.4 g. of Oxoid Ionagar no. 2. The organism (kindly identified as a *Micrococcus* sp. by Dr N. S. Mair, Public Health Laboratory, Leicester Isolation Hospital) was maintained by frequent subculture on slopes of the above medium. Cultures were grown at 30° and stored at 2°.

For experimental use, a loopful of cells from a freshly grown slope was used to inoculate 700 ml. of aconate medium (as used for slopes but with the agar omitted) contained in a 2 l. conical flask. The culture was shaken at 30° in a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) and, after 12–14 hr., the bacteria were harvested during the exponential phase of growth (at cell densities about 0.3 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 10 mM-sodium-potassium phosphate buffer, pH 7.0, and were resuspended in this buffer. For the preparation of extracts, the packed cells were suspended in 10 mM-sodium-potassium phosphate buffer, pH 7.0, which also contained EDTA (1 mM) and 2-mercaptoethanol (1 mM).

Preparation of cell extracts. Suspensions of freshly grown cells in the phosphate-EDTA-mercaptoethanol buffer

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were subjected for 5–10 min. at 0° to the output of an MSE ultrasonic disintegrator model 60W, operating at 1.5 A. The suspensions were centrifuged at 24000 *g* for 15 min. at 2°: the supernatant solutions thus obtained were used for experimental purposes.

Determination of protein. Soluble protein was measured by the biuret method as modified by Gornall, Bardawill & David (1949). The protein content of dilute solutions was estimated by the Folin-Ciocalteu method as modified by Lowry, Rosebrough, Farr & Randall (1951).

Preparation of substrates. Sodium aconate trihydrate was prepared by the method of Campbell & Hunt (1947). Succinic semialdehyde was prepared as described by Jakoby (1962).

Preparation of CoA derivatives. Succinyl-CoA was prepared by the method of Simon & Shemin (1953), and itaconyl-CoA as described by Cooper & Kornberg (1964).

Electrophoresis. Organic acids were separated by high-voltage paper electrophoresis at 5 kv and 80 ma for 30 min. in 0.5 M-acetic acid–pyridine buffer, pH 4.0 (Gross, 1959).

Other chemicals. Reduced and oxidized nicotinamide nucleotides, crystalline lactate dehydrogenase, CoA, sodium pyruvate and α -oxoglutaric acid were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; L-malic acid was from L. Light and Co. Ltd., Colnbrook, Bucks.; itaconic acid was from Eastman Organic Chemicals, Rochester 3, N.Y., U.S.A.; ATP was from Schwartz BioResearch Inc., Mount Vernon, N.Y., U.S.A. Phenylhydrazine was purchased from British Drug Houses Ltd., Poole, Dorset, and was recrystallized from 25% (v/v) HCl before use. All other reagents were of the highest purity commercially available.

Enzyme assays. (a) Assay of aconate delactonase. The enzymic opening of the lactone ring of aconate was determined as the rate of removal of aconate from solutions. Aconate was assayed with an alkaline hydroxylamine reagent similar to that described by Hestrin (1949), which was prepared, immediately before use, by mixing 10 ml. of 2 M-hydroxylamine hydrochloride with 7 ml. of 3.5 N-NaOH, and diluting to 20 ml. with water. The assay system contained (in 1.0 ml.): 50 μ moles of tris-HCl buffer, pH 8.0,

enzyme (containing about 500 μ g. of soluble protein) and 5 μ moles of sodium aconate. After 10–20 min. incubation at 30°, 1.5 ml. of the alkaline hydroxylamine reagent was added and the mixture was incubated for exactly 1 min. After the addition of 1.5 ml. of the ferric chloride reagent of Lipmann & Tuttle (1945), the solutions were left at room temperature for 10–15 min. and the extinction at 540 m μ was measured. An internal standard was provided by measurement of the extinction given by 5 μ moles of aconate, subjected to the same treatment but in the absence of the enzyme.

(b) Assay of succinic semialdehyde-NAD(P) oxidoreductase. This enzyme was assayed as the rate of increase in extinction at 340 m μ concomitant with the reduction of NADP in the presence of succinic semialdehyde. The assay system contained (in 1.0 ml.): 90 μ moles of tris-HCl buffer, pH 9.0, 2 μ moles of MgCl₂, approx. 0.15 μ mole of NADP, 1 μ mole of succinic semialdehyde and about 30 μ g. of soluble protein. The reaction was started by addition of the succinic semialdehyde.

(c) Assay of pyruvate formation from itaconate. Itaconyl-CoA synthetase, itaconate CoA-transferase, itaconyl-CoA hydratase and citramalyl-CoA lyase were estimated as described by Cooper & Kornberg (1964). In the adaptation experiments, pyruvate formation from itaconyl-CoA was measured in the assay system of Dixon & Kornberg (1959); the increase in extinction at 315 m μ was a measure of pyruvate phenylhydrazone formation, and at this wavelength 1 μ mole of pyruvate phenylhydrazone/ml. had an extinction of 13.1 units.

Spectrophotometric procedures were performed at room temp. (22 $\pm$ 1°) in silica cells (1.5 ml. vol.; 1 cm. light-path) with an Optica model CF4 recording spectrophotometer.

RESULTS

Growth on aconate

Oxidation of substrates. Washed suspensions of the aconate-grown *Micrococcus* readily oxidized aconate, succinate, succinic semialdehyde and L-malate (Table 1); the oxidation of pyruvate,

Table 1. *Oxidation of substrates by washed suspensions of Micrococcus sp.*

The main compartments of Warburg manometer flasks contained (in 2.6 ml.): 10 μ moles of sodium–potassium phosphate buffer, pH 7.2, and washed cells (5–6 mg. dry wt.). The centre wells contained 400 μ moles of KOH. Substrates (10 μ moles) 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.)					
	Aconate-grown cells		Succinate-grown cells		Itaconate-grown cells	
	Observed	Corrected for endogenous respiration	Observed	Corrected for endogenous respiration	Observed	Corrected for endogenous respiration
None	10	—	9	—	7	—
Aconate	131	121	16	7	20	13
Succinic semialdehyde	119	109	60	51	167	160
Succinate	157	147	137	128	149	142
L-Malate	140	130	110	101	102	95
Pyruvate	78	68	54	45	104	97
α -Oxoglutarate	41	31	62	53	51	44
Acetate	38	28	46	37	71	64
Itaconate	16	6	14	5	41	34

α -oxoglutarate and acetate occurred at a lower rate; itaconate was oxidized at a negligible rate. For aconate, the quantities of oxygen consumed (corrected for the endogenous respiration) were 52% of those required for complete oxidation; the observed Q_{O_2} of 121 μ l. of oxygen absorbed/mg. dry wt./hr. hence corresponds to the utilization of 2.6 μ moles of aconate/mg. dry wt. of cells/hr.

In contrast, washed suspensions of the organism grown on succinate oxidized succinate and L-malate rapidly, acetate, pyruvate, α -oxoglutarate and succinic semialdehyde less rapidly, and itaconate and aconate at a negligible rate. These observations suggest that aconate-grown cells contain enzymes that catalyse the conversion of aconate into intermediates of the tricarboxylic acid cycle, and that such enzymes are largely absent from cells grown on succinate.

Action of cell-free extracts on aconate. When extracts of aconate-grown *Micrococcus* were incubated with aconate and phenylhydrazine in the assay system of Dixon & Kornberg (1959), a rapid increase in extinction at 315 $m\mu$ indicated that an oxo compound was being enzymically formed; no such increases in extinction were observed when aconate or the cell extract was omitted, or when a boiled extract was used. However, unlike the oxo compound formed when itaconate-grown *Pseudomonas* extracts were incubated with cofactors and itaconate in this assay system (Cooper & Kornberg, 1964), the material derived from aconate did not require cofactors for its formation, and was not pyruvate but was succinic semialdehyde. The evidence for this view was derived from a comparison of the properties of solutions of pyruvate, succinic semialdehyde and the unknown material; the solution of the unknown material was obtained by incubating 200 μ moles of aconate overnight at pH 6.8 with an ultrasonic extract of aconate-grown *Micrococcus*, removing protein with 4% (v/v) perchloric acid and adjusting to pH 7.0 with potassium hydroxide.

It was observed that:

(i) Whereas pyruvate was rapidly reduced when incubated with lactate dehydrogenase and $NADH_2$, neither authentic succinic semialdehyde nor the unknown material promoted a decrease in extinction at 340 $m\mu$.

(ii) The 2,4-dinitrophenylhydrazone of the unknown compound, twice recrystallized from water, melted at 198–200° (uncorr.), as did the derivative of succinic semialdehyde and a mixture of both 2,4-dinitrophenylhydrazones.

(iii) The 2,4-dinitrophenylhydrazone of the unknown material, unlike that of pyruvate, gave only one spot in the solvent system of El Hawary & Thompson (1953), and ran identically to the derivative of succinic semialdehyde.

(iv) Whereas the alkaline 2,4-dinitrophenylhydrazone of pyruvate exhibited extinction peaks at 445 and 520 $m\mu$ (Friedemann & Haugen, 1943), those of authentic succinic semialdehyde and of the unknown compound exhibited peaks at 432 and 510 $m\mu$; the ratio of extinction at 432 $m\mu$ to that at 510 $m\mu$ was, in both cases, 2.1. Further, whereas the red colour of the alkaline pyruvate 2,4-dinitrophenylhydrazone was stable for at least 16 min., that of the alkaline 2,4-dinitrophenylhydrazones of succinic semialdehyde and of the unknown compound decreased by about 40% in this time.

(v) The phenylhydrazones of the unknown material and of authentic succinic semialdehyde exhibited identical spectra, with an extinction peak at 272 $m\mu$ and a shoulder at 300 $m\mu$; in contrast, the derivative formed from pyruvate gave a major peak at 315 $m\mu$ and a minor peak at 290 $m\mu$.

(vi) $NADPH_2$ was rapidly formed from NADP when solutions of succinic semialdehyde and of the unknown material were incubated with the co-enzyme and ultrasonic extracts of γ -aminobutyrate-grown *Escherichia coli*, which were rich in succinic semialdehyde-NAD(P) oxidoreductase (Jakoby, 1962); pyruvate did not react.

These observations are interpreted as indicating that the oxo compound enzymically formed from aconate was succinic semialdehyde.

Succinic semialdehyde and carbon dioxide production from aconate. The direct formation of succinic semialdehyde from aconate would be expected also to yield an equivalent amount of carbon dioxide. This was tested by incubating an extract of aconate-grown cells with aconate in a Warburg manometer at pH 6.5, and measuring the quantities of gas evolved. Perchloric acid (final concn. 4%) was added after gas evolution ceased to release retained carbon dioxide quantitatively and to precipitate the protein. The cup contents were removed and centrifuged, and the supernatant solution was adjusted to pH 7.0 with potassium hydroxide and recentrifuged. As mentioned under (vi) above, samples of the supernatant solutions so obtained were assayed for succinic semialdehyde by measuring the $NADPH_2$ formed in the presence of NADP and succinic semialdehyde-NAD(P) oxidoreductase. The results (Table 2) show that aconate gave rise to carbon dioxide and succinic semialdehyde in equimolar amounts.

Formation of succinate from aconate. It was likely that the succinic semialdehyde formed from aconate entered the tricarboxylic acid cycle after oxidation to succinate. A succinic semialdehyde-NAD(P) oxidoreductase, which catalysed this oxidation of succinic semialdehyde with concomitant reduction of NADP, was found to be abundant in extracts of aconate-grown *Micrococcus*: the formation of $NADPH_2$ occurred both

Table 2. *Equivalence of succinic semialdehyde and carbon dioxide formation from aconate*

The evolution of CO₂ was measured manometrically, and succinic semialdehyde was measured spectrophotometrically by the formation of NADPH₂ in the presence of succinic semialdehyde-NAD(P) oxidoreductase. For conditions of assay see the text.

Aconate added (μmoles)	Succinic semi- aldehyde formed (μmoles) (A)	CO ₂ evolved (μmoles) (B)	B/A ratio
4.5	3.8	4.5	1.2
9.0	7.5	8.6	1.1
13.5	12.2	13.4	1.1

with authentic succinic semialdehyde and with aconate as substrate. NAD also served as coenzyme for this reaction though, since ultrasonic extracts of *Micrococcus* were rich in NADH₂-oxidase activity, quantitative assays with NAD were difficult to carry out. However, by taking advantage of the reoxidation of NADH₂ through the oxidase, it was feasible to isolate the organic acid formed from succinic semialdehyde and to identify it as succinate. The reaction catalysed by an ultrasonic extract of aconate-grown *Micrococcus* (0.5 mg. of protein) incubated with 60 μmoles of aconate and 12 μmoles of NAD was allowed to go to completion by keeping the reaction mixture at room temperature overnight. The acidified mixture was extracted continuously with ether for 14 hr. and samples of the ether extract were subjected to paper chromatography in ether-formic acid-water (16:1:1, by vol.) solvent, and to high-voltage paper electrophoresis (see the Materials and Methods section). In both systems, the reaction product behaved identically with authentic succinic acid, and differently from aconic acid and succinic semialdehyde.

Enzymes involved in aconate utilization. The results obtained suggested that aconate was catabolized to succinate via succinic semialdehyde. This was supported by the finding that the amounts of component enzymes in extracts of aconate-grown *Micrococcus* were more than sufficient to account for the utilization of aconate by whole cells. It was observed that the delactonization of aconate, as measured by the removal of aconate from solutions, occurred readily at 30° and pH 7.6; under the conditions of the aconate-delactonase assay procedure, 0.3 μmole of aconate was removed/mg. of soluble protein/min. This reaction was linear with time for the removal of at least 65% of the aconate present.

Similar rates of aconate removal were obtained by measurement of the rates of carbon dioxide formation concomitant with the decarboxylation of (presumably delactonized) aconate. Again, ultra-

Table 3. *Influence of growth substrates on amounts of enzymes in extracts of Micrococcus sp.*

The rates of reactions are expressed as specific activities (μmole of substrate converted/min./mg. of protein).

Growth substrate	Aconate delactonase	Succinic semialdehyde-NAD(P) oxidoreductase
Aconate	0.30	0.35
Succinate	0	0.04
Itaconate	0	0.02
γ-Aminobutyrate	0	0.55
Lactate	0	0.04
Acetate	0	0.02

sonic extracts of aconate-grown cells catalysed the evolution of 0.3 μmole of carbon dioxide/mg. of soluble protein/min. when incubated with aconate.

The quantities of succinic semialdehyde-NAD(P) oxidoreductase present in ultrasonic extracts of aconate-grown cells were also more than adequate to account for aconate utilization by whole cells, and catalysed the formation of 0.35–0.4 μmole of NADPH₂/mg. of soluble protein/min. when incubated with NADP and succinic semialdehyde. Aconate could also be used as substrate but, as expected, the rate observed was somewhat less than with succinic semialdehyde.

To confirm the importance of these enzymes for growth on aconate, extracts of *Micrococcus* grown on aconate, succinate, itaconate, lactate, acetate and γ-aminobutyrate were assayed for the delactonase and the oxidoreductase. The delactonase was present only in aconate-grown cells, though oxidoreductase at high specific activity was found in γ-aminobutyrate-grown cells as well as in aconate-grown cells (Table 3). This shows that both enzymes are inducible and suggests that they are not coordinately linked.

Further support for the necessary role of these enzymes in aconate metabolism is provided by measurements of their differential rates of synthesis during growth on aconate and succinate. The high concentrations of both enzymes remained constant when aconate-grown cells were used as an inoculum for continued growth on aconate: the straight lines relating the total amounts of the enzymes in the culture to the increase in cell mass could be extrapolated to pass through the origins and each had a slope with *P* 13. In contrast, no increase in the amount of delactonase and only a slight increase (*P* 0.5) in that of oxidoreductase were observed when the aconate-grown inoculum continued to grow for almost three generations on succinate.

Comparison of growth on aconate and itaconate

The route of aconate utilization in *Micrococcus* sp., though clearly different from the route of

itaconate metabolism in liver mitochondria and in *Pseudomonas B₂aba*, bears some resemblance to a pathway for itaconate dissimilation proposed by Brightman & Martin (1961). Since *Micrococcus* sp. grew both on aconate and itaconate, it was possible to study the relationship between the two pathways in one organism.

Oxidation of substrates. The oxidation of various substances by washed cells of itaconate- or aconate-grown *Micrococcus* sp. (Table 1) shows differences between aconate- and itaconate-grown cells. Aconate-grown cells oxidized aconate very rapidly, but oxidized itaconate hardly at all. Itaconate-grown cells, surprisingly, did not oxidize itaconate very rapidly, though they readily oxidized succinate and succinic semialdehyde. Initially, such cells oxidized aconate slowly, but after a lag of 60–80 min. began to oxidize aconate rapidly. This increase in oxidation rate was abolished by 0.12 mM-chloramphenicol and was shown to be associated with the induction of aconate delactonase.

Pathway of itaconate utilization. A pathway for itaconate utilization, which involves the formation of itaconyl-CoA, its hydration to citramalyl-CoA and cleavage of this material to acetyl-CoA and pyruvate, has been established for itaconate-grown *Pseudomonas B₂aba* (Cooper & Kornberg, 1964). The enzymes catalysing the reactions of this sequence were also present in extracts of the itaconate-grown *Micrococcus*. The specific activities observed (Table 4) are of the same order as those reported for itaconate-grown *Pseudomonas B₂aba*, though there are two differences: in *Micrococcus* extracts, itaconate was apparently not directly converted into itaconyl-CoA in the presence of ATP and CoA but was formed by CoA transfer from succinyl-CoA, and succinyl-CoA did not serve as a CoA donor to citramalate. However, these findings do not affect the validity of the conclusion that the pathway for itaconate utilization in *Micrococcus* is closely similar to that used by *Pseudomonas B₂aba*, and is entirely different from the route of aconate utilization. Confirmation of this was obtained by measuring the formation of the various enzymes

when cells grown on itaconate were used as inoculum for further growth on itaconate or aconate.

The high concentrations of the enzymes necessary for the overall formation of pyruvate (and acetyl-CoA) from itaconyl-CoA remained constant when itaconate-grown cells were used as inoculum for continued growth on itaconate: the straight line relating the total amounts of these enzymes in the culture to the increase in cell mass could be extrapolated to pass through the origin and had a slope of $P\ 21.6$ (Fig. 1). However, only a slight increase in enzyme content was noted when the itaconate-grown inoculum continued to grow for almost three generations on aconate as sole carbon source: under these conditions the straight line had a slope of $P\ 0.25$ (Fig. 1), which indicated that the differential rate of synthesis of the enzymes (or of a key enzyme in the sequence) during growth on aconate was less than 1.5% of that during growth on itaconate.

Transfer of the itaconate-grown inoculum to aconate did not cause a pronounced growth lag, although it took 4 hr. for the maximum growth rate (mean generation time 2.5 hr.) to be achieved. During this initial period the specific activity of a key enzyme of the aconate pathway (aconate delactonase) rose rapidly and reached the high value noted during growth on aconate (Fig. 2). This enzyme was not detected when the itaconate-grown inoculum continued to grow on itaconate.

Table 4. Activities of the enzymes catalysing the conversion of itaconate into acetyl-CoA and pyruvate

Extracts prepared from itaconate-grown *Micrococcus* sp. were assayed as described by Cooper & Kornberg (1964). Specific activities are expressed as μ mole of substrate transformed/min./mg. of soluble protein.

Enzyme	Specific activity
Itaconyl-CoA synthetase	0.00
Itaconate CoA-transferase	0.26
Itaconyl-CoA hydratase	0.48
Citramalyl-CoA lyase	0.41

Fig. 1. Formation of enzymes that catalyze pyruvate formation from itaconyl-CoA by itaconate-grown *Micrococcus* sp. continuing to grow on itaconate (O) or aconate (●) as carbon source. Samples (containing approx. 6 mg. dry wt. of bacteria total) were taken during the course of growth and the relevant enzymes contained in ultrasonic extracts of these cells were assayed as described in the text.

These results indicate that itaconate and aconate specifically evoked the formation of sets of different enzymes, appropriate to the utilization of the particular growth substrate.

DISCUSSION

In order that micro-organisms may grow on analogues of intermediates of the tricarboxylic acid cycle, when such compounds are supplied as the sole sources of carbon, reactions must occur that, by appropriate enzymic transformations, enable these growth substrates to enter the main energy-supplying and biosynthetic routes of the cell. These essential preliminary transformations are often catalysed by inducible enzymes: it is frequently observed that, when organisms are transferred from growth on one substrate to growth on another, the synthesis of the component enzymes of the pathway for utilization of the former substrate virtually ceases and that of a different set of enzymes is elicited (cf. Kornberg, 1961).

Such is the case when *Micrococcus* sp. grows on itaconate or aconate as sole carbon source. The utilization of itaconate proceeds via an enzymic sequence (Scheme 1a) involving the activation of itaconate to itaconyl-CoA, and the entry of that material (after hydration to citramalyl-CoA) into the tricarboxylic acid cycle as pyruvate and acetyl-CoA. However, although itaconic acid (methylene-succinic acid) exhibits some structural similarities to aconic acid (the lactone of hydroxymethylfumaric acid), aconate is utilized via an entirely different enzymic sequence (Scheme 1b), which does not involve CoA derivatives and which enables this substrate to enter the tricarboxylic acid cycle as succinate. The component enzymes of these pathways are inducible and their formation precedes growth on the appropriate substrate.

Fig. 2. Formation of aconate delactonase by itaconate-grown *Micrococcus* sp. continuing to grow on itaconate (●) or aconate (○) as carbon source. Samples (containing approx. 6 mg. dry wt. of bacteria total) were taken during the course of growth and the aconate delactonase contained in ultrasonic extracts of these cells was assayed as described in the text.

Scheme 1. Postulated pathways for the utilization of itaconate (a) and aconate (b) by *Micrococcus* sp. Itaconate enters the tricarboxylic acid cycle as pyruvate and acetyl-CoA, whereas aconate is transformed into succinate.

We thank Miss Joy Whitelam for technical assistance. This work was supported by the U.S. Air Force (Grant no. AFEOAR63-17), European Office of Aerospace Research, and by the Medical Research Council under a Grant for Scientific Assistance.

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