EXCRETION OF α-KETOGLUTARIC ACID DURING OXIDATION OF ACETATE BY A VIBRIO

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The production of α-ketoglutarate from glucose[1] and from succinate[2] has been demonstrated for *Pseudomonas fluorescens*. DAGLEY, FEWSTER AND HAPPOLD[3] used paper chromatography to detect the presence of α-ketoglutarate during oxidation of benzoate, phenylacetate, citrate, succinate and acetate by a vibrio. Its production from acetate is of special interest since, assuming synthesis by reactions of the tricarboxylic acid cycle, a mechanism must be postulated for continual replenishment of the oxalacetate that condenses with acetylcoenzyme A to initiate the cycle. In the present work we have studied, first the development of the enzyme systems responsible for production of α-ketoglutarate from acetate, and second the conditions under which amounts sufficient for satisfactory characterization can be made to accumulate during oxidation of acetate by particular batches of cells.

EXPERIMENTAL

Methods

The organism used was a vibrio capable of oxidizing various aromatic compounds[1] and members of the tricarboxylic acid cycle[3]. Batches of cells were grown with forced aeration at 30° in media containing: carbon source, 0.01 *M*; KH_2PO_4, 5 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; dist. water 1 l; pH adjusted to 7.0 with 5 *M* NaOH. Keto acids were estimated by the method of FRIEDEMANN AND HAUGEN[5] and were identified by paper chromatography as their 2:4-dinitrophenylhydrazones according to CAVALLINI, FRONTALI AND TOSCHI[6] with the modifications used by DAGLEY *et al.*[3]. Oxygen uptakes were measured in the Warburg apparatus by the techniques described by UMBREIT, BURRIS AND STAUFFER[7].

Development of ability to produce keto acids during oxidation of acetate

Previous work[3] indicated that unless the cells had grown on acetate as sole source of carbon, keto acids did not accumulate when the cells oxidized acetate subsequently. The vibrios were harvested by centrifugation from a medium supplying *p*-hydroxybenzoic acid as sole source of carbon, and after washing twice with phosphate buffer (pH 7.0) were resuspended to give equal turbidities (0.1 mg dry weight of cells/ml) in the following three media: (1) *p*-hydroxybenzoate growth medium ; (2) acetate growth medium; (3) a solution containing sodium acetate, 0.01 *M*; KH_2PO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; distilled water 1 l; pH 7.0; that is, an acetate growth medium from which $(NH_4)_2SO_4$, as source of N for growth, had been omitted. The suspensions were then incubated at 30° and a gentle stream of sterile air bubbled through each. Development of ability to synthesize keto acids from acetate was then measured as follows. A 12 ml

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sample was withdrawn, centrifuged and the cells drained free from supernatant. After resuspension in 5 ml of phosphate buffer (pH 7) containing 0.02 *M* sodium acetate the cells were aerated at 30° for one hour and then removed by centrifugation. The rate of production of keto acids from acetate by this suspension, expressed as μmol per litre per hour, was then obtained by determination of the keto acid concentration in 3 ml of clear supernatant. This procedure was repeated at various time intervals and the results are given in Fig. 1A where it is seen that ability to produce keto acids during acetate oxidation was developed only by the vibrios that were aerated with the complete medium for growth on acetate. When $(NH_4)_2SO_4$ was omitted activity was not developed. Ability to oxidize acetate, after growth on *p*-hydroxybenzoate was also studied (Fig. 1B).

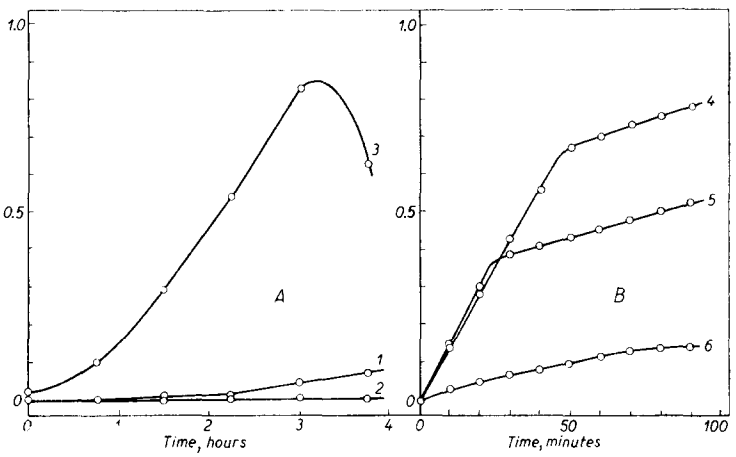

Fig. 1. Data for cells grown on *p*-hydroxybenzoic acid as sole carbon source. A: Development of ability to excrete α-ketoglutarate during oxidation of acetate. Ordinate, rate of keto acid accumulation in m*M* per l per hour. After harvesting, cells were aerated with: 1. *p*-Hydroxybenzoate; 2. Acetate, no ammonium salt present; 3. Acetate, ammonium salt present. Ability of cells to produce α-ketoglutarate from 0.02 *M* acetate was then tested at times indicated. B: Ability to oxidize acetate. Ordinate, O_2 uptake in ml. 4. *p*-Hydroxybenzoate, 5 μ*M*; 5. Acetate, 10 μ*M*; 6. No substrate present.

Although these cells did not give rise to detectable keto acid accumulation when aerated in the presence of acetate, they were clearly capable of oxidizing the latter immediately, without adaptation.

Similar experiments were conducted for cells grown at the expense of citrate, succinate, malate, fumarate, mandelate, benzoate, phenylacetate and phenol. In every case acetate was readily oxidized but in none did a significant amount of keto acid accumulate in the medium. On transfer to an acetate growth medium curves were obtained similar to that of Fig. 1A to show development of a mechanism for keto acid production.

Identity of keto acids produced from acetate

Paper chromatography of 2:4-dinitrophenylhydrazones showed that α-ketoglutarate was the main keto acid produced during acetate oxidation. Experiments were now performed to ascertain the conditions under which the concentration of α-ketoglutarate could be increased above the values hitherto obtained (0.5 to 1.0 m*M*, see Fig. 1A) with the object of obtaining a derivative for characterization. Increase of acetate concen-

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tration up to *ca* 0.06 *M* gave rise to increased α-ketoglutarate formation but higher concentrations were markedly inhibitory. An optimum period for aeration likewise existed. Previous studies of the kinetics of pyruvate formation during oxidation of compounds of the tricarboxylic acid cycle by *A. aerogenes*[8] showed that the keto acid concentration rose to a maximum and then decreased with time. This is a consequence of the fact that pyruvate, like α-ketoglutarate, is an intermediate in the terminal respiratory process and a point is reached when its rate of decomposition first equals, and then exceeds, its rate of formation. Finally, LOCKWOOD AND STODOLA[1] obtained their maximum yields of α-ketoglutarate from glucose by controlling the aeration of the *Ps. fluorescens* culture. We found yields of α-ketoglutarate increased with aeration: thus for a given air flow the yield was considerably greater in a tall cylindrical vessel than for the same volume of culture contained in a flask. The maximum air flow that could be provided by available facilities did not, in fact, exceed the optimum.

With these considerations in mind the following procedure was adopted for the isolation of the 2:4 dinitrophenylhydrazone of α-ketoglutaric acid. Twenty litres of a culture grown in acetate medium were harvested, the cells washed with phosphate buffer (pH 7) and then taken up in 2 l of phosphate buffer (pH 7) containing 0.04 *M* sodium acetate. The suspension was divided between two tall glass cylinders (7 cm internal diameter) and each batch of 1 litre was aerated at 30° for 3 hours when the keto acid concentration reached a maximum. The rate of flow of air through each culture was 12 l per minute. The cells were removed by Seitz filtration and after acidification with hydrochloric acid a solution of 2 g 2:4-dinitrophenylhydrazine in 1 l 2*N* HCl was added to the clear filtrate until no further pale yellow precipitate was formed. The solution was incubated for 2 hours at 30°, allowed to stand overnight in the refrigerator, filtered and the precipitate washed several times with acidified distilled water and then suspended in a little water. Subsequent purification procedure followed that developed by KOGUT AND PODOSKI[2] the suspension was extracted with ether, the hydrazone re-extracted with 15% (w/v) Na_2CO_3 and on acidification with conc. HCl a pale yellow crystalline precipitate was formed and then filtered off. Following three crystallizations from ethanol-water mixtures the needle-shaped crystals were dried over $CaCl_2$ *in vacuo* yield, 0.5 g. Analysis of the compound was as follows. Found: C, 38.7; H, 3.7; N, 15.8. Loss of weight on drying *in vacuo* at 50° over P_2O_5 to constant weight, 4.9%. Calc. for $C_{11}H_{10}O_8N_4,H_2O$: C, 38.4; H, 3.5; N, 16.3; H_2O, 5.2%. The hydrazone was dissolved in 15% (w/v) Na_2CO_3 and an equal volume of 1.5 *N* NaOH added to give a concentration of 12.5 μg hydrazone per ml. Its absorption spectrum was then determined using a Unicam quartz spectrophotometer and compared with that of an authentic sample of 2:4-dinitrophenylhydrazone of α-ketoglutarate. The two curves could be entirely superimposed over the wave lengths investigated (400–600 mμ): maximum absorption occurred for both specimens at 420–425 mμ. The authentic specimen, the sample isolated as described and mixtures of the two, all melted with decomposition at 219–220°. These physical constants leave little doubt that the compound isolated was the 2:4-dinitrophenylhydrazone of α-ketoglutarate.

Growth on long-chain aliphatic acids

Hitherto it had been shown that cells were stimulated to excrete α-ketoglutarate during acetate oxidation only when they had grown at the expense of acetate. Exceptions were provided by cells that had utilized acids of longer chain length. Crops were har-

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vested from mineral salt media supplying these compounds individually as carbon sources and ability of the cells to produce α-ketoglutarate was tested as described. In Table I rates of production are compared with those for cells grown at the expense of other substrates.

TABLE I

INFLUENCE OF GROWTH SUBSTRATE ON ABILITY OF CELLS TO PRODUCE α-KETOGLUTARATE FROM ACETATE

Acetate, 0.02 *M*; density of cell suspensions 0.5 mg/ml. The concentrations of α-ketoglutarate were determined after aeration at 30° for 1 hour.

Growth substrate	*Concentration (μmoles per litre)*	*Growth substrate*	*Concentration (μmoles per litre)*
malate	5	butyrate	115
fumarate	5	valerate	135
succinate	0	caproate	100
citrate	0	heptoate	220
benzoate	5	caprylate	380
mandelate	0	pimelate	180
phenylacetate	0	azelate	450
p-hydroxybenzoate	5	sebacate	75

DISCUSSION

Many significant studies on terminal respiration in microorganisms have appeared recently, of which the following are especially relevant to the present work. STONE AND WILSON[9] prepared extracts of *Azotobacter vinelandii* that readily oxidized intermediates of the tricarboxylic acid cycle and so demonstrated that the lag periods obtained when whole cells were used in similar experiments did not argue against the operation of the cycle but might be attributed to cell impermeability. BARRON AND GHIRETTI[10], in particularly extensive researches, found the enzymes for synthesis of citrate and oxidation of isocitrate in all of the variety of microorganisms they studied and concluded that in these cases acetate metabolism could proceed *via* the citric acid cycle. They emphasized, however, that this did not eliminate the possibility that a dicarboxylic acid cycle functions to provide the oxalacetate needed to initiate the tricarboxylic acid cycle. Cell-free extracts were prepared which oxidized acetate in the presence of methylene blue; coenzyme requirements were investigated and it was postulated that dehydrogenation of two molecules of acetylcoenzyme A occurred to give one molecule of succinate. Nevertheless, experiments with radiotracers to support a Thunberg-type condensation of acetate have been criticized by KRAMPITZ and his colleagues[11] who also presented strong positive evidence against the occurrence of such a process under the conditions of their experiments[12].

Although the precise nature of the mechanism remains a matter for discussion it is evident from the present work and from other considerations that conversion of C_2 to C_4 units must play an important part in acetate metabolism in addition to the tricarboxylic acid cycle. The latter is a generally accepted link between carbohydrate and protein metabolism and in fact KREBS, GURIN AND EGGLESTON[13] have suggested that the reactions of the cycle in microorganisms serve primarily the needs of synthesis. If this is so it may be anticipated that growth at the expense of a given substrate may result in adjustment of reaction rates, or development of additional reactions, so that

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concentrations of α-ketoglutarate and other key compounds in synthesis may be built up in excess of the catalytic amounts required for oxidation *via* the tricarboxylic acid cycle. Accordingly we have shown that ability to excrete α-ketoglutarate during oxidation of acetate resulted from growth at the expense of acetate and was not a consequence merely of ability to oxidize the substrate. It is evident that continual removal of α-ketoglutarate for synthetic purposes leads to complete depletion of oxalacetate in a culture oxidizing acetate unless reactions operate for its replenishment from acetate. For growth on the latter as sole source of carbon such reactions may serve not merely as a "priming" mechanism but may have a metabolic function as indispensable as those that constitute the tricarboxylic acid cycle itself. Similar conclusions can be drawn from experiments in which it is possible to demonstrate a build-up of any component of the tricarboxylic acid cycle substantially beyond the catalytic amounts needed for acetate oxidation. In addition to the present isolation of α-ketoglutarate from acetate at least two other cases are on record. BARRON, ARDAO AND HEARON[14] demonstrated accumulation of succinate when *Corynebacterium creatinovorans* oxidized acetate in the presence of malonate; this was confirmed in later work[10]. JAYASURIYA[15] showed that citric acid accumulated from glycollic acid during oxidation by an oxalate-decomposing organism with fluoracetate present and pointed out that citrate synthesis by the tricarboxylic acid cycle implied, in addition, synthesis of a C_4 unit from a C_2 unit. Recent extensions of this work indicate that the α-carbon of glycollate may give rise to a C_1 unit in the synthesis of citrate[16].

Growth on various fatty acids conferred ability to excrete α-ketoglutarate during acetate oxidation. STADTMAN AND STADTMAN[17] have provided alternative explanations for previous results[18,19,20] suggesting that β-oxidation of these substrates by bacteria did not occur, and in consequence it seems probable that a process similar to the "fatty acid cycle"[21] may operate. If this is so, development by cells so grown of mechanisms of synthesis solely from C_2 units is to be expected. The mere presence of $-CH_2 \cdot COOH$ in the substrate molecule, as in phenylacetic acid, does not confer activity: the microbial metabolism of this substrate does not involve β-oxidation but occurs by way of homogentisic acid[22].

SUMMARY

Excretion of α-ketoglutaric acid by a vibrio during oxidation of acetate has been established by isolation and characterization of the 2:4-dinitrophenylhydrazone. Cells grown on a wide variety of substrates were able to oxidize acetate but they did not excrete α-ketoglutarate in the process. A mechanism for excretion of this compound developed during growth on acetate and other fatty acids. The necessity for postulating other mechanisms in addition to the tricarboxylic acid cycle is discussed in the light of these observations.

RÉSUMÉ

L'excrétion de l'acide α-cétoglutarique par un vibrion pendant l'oxydation de l'acétate a été établie en isolant et en caractérisant la 2:4-dinitrophenylhydrazone de l'acide. Pendant la croissance, dans une grande variété de milieux, les cellules sont capables d'oxyder l'acétate, mais, pendant ce processus, elles n'excrètent pas d'acide α-cétoglutarique. Pour l'excrétion de ce composé, un mécanisme se développe pendant la croissance en présence d'acétate et d'autres acides gras. Par suite de ces observations les auteurs suggèrent la nécessité de postuler certains autres mécanismes, à côté du cycle tricarboxylique.

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ZUSAMMENFASSUNG

α-Ketoglutarsäure wurde als ein Ausscheidungsprodukt eines Vibrios während der Acetatoxydation als 2:4-Dinitrophenylhydrazon isoliert und charakterisiert. Zellen, die auf den verschiedensten Substraten gezüchtet wurden, konnten Acetat oxydieren, sie schieden jedoch kein α-Ketoglutarat im Laufe des Prozesses aus. Ein Mechanismus für die Ausscheidung dieser Verbindung hat sich im Vibrio während seines Wachstums auf Acetat und anderen Fettsäuren entwickelt. Die Notwendigkeit neben dem Tricarbonsäurecyclus noch andere Reaktionsmechanismen anzunehmen wird im Lichte dieser Beobachtungen erörtert.

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