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Oxidative Pathways in a Fluorescent *Pseudomonas*

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During an investigation of the oxidative metabolism of a strain of a fluorescent *Pseudomonas* (KB 1) it was noted that when succinate was oxidized by washed suspensions there was a sharp break in the oxygen-uptake curve corresponding to approximately 1.5 moles oxygen/mole succinate added. Examination of the suspending fluid for possible end products showed the presence of keto acids which were tentatively identified as pyruvate and α -ketoglutarate by partition chromatography. The production of α -ketoglutarate by micro-organisms has been reported by Lockwood & Stodola (1946) using a strain of *Ps. fluorescens*; by Dagley, Fewster & Happold (1952) using *Vibrio*, strain 01; by Hockenhull, Wilkin & Winder (1951) using *Pencillium chrysogenum*; by Walker, Hapton & Hall (1951) using *Aspergillus niger*; and by Hunter (1952) using *Mycobacterium butyricum*. Lockwood & Stodola (1946) showed that when their strain of *Ps. fluorescens* was grown upon a medium containing glucose and with a high carbon/nitrogen ratio, with carefully controlled aeration, up to 18 moles α -ketoglutarate/100 moles glucose consumed accumulated in the growth medium. Dagley *et al.* found that this keto acid was produced when *Vibrio* 01 oxidized either benzoate, phenylacetate, citrate, acetate or succinate, though the amounts formed were not determined.

The formation of α -ketoglutarate from succinate by *Ps. fluorescens* suggested that some, at least, of the reactions of the tricarboxylic acid cycle played a part in the oxidative metabolism of this organism.

A detailed study was therefore made of the oxidation of succinate and other tricarboxylic-acid-cycle intermediates by this organism using whole cells and cell-free extracts. Since the oxidation of some of the substrates appeared to be adaptive, it was also possible to examine the application of the simultaneous adaptation technique (Stanier, 1947) to problems of this type. A preliminary account of this work has already been given (Kogut, 1952a, b).

MATERIALS AND METHODS

Organism. The organism was isolated from soil and had the following properties: Gram-negative motile rods $1.0-2.0 \mu \times 0.4-1.0 \mu$; with a single polar flagellum; a green, fluorescent pigment was produced which diffused into the growth medium; opt. temp. 30° , growth occurred at 37° ; gelatin not liquefied; nitrites not produced from nitrates; catalase positive; indole not produced; litmus milk, no change. These properties are compatible with either *Ps. conveza* or *Ps. ovalis*. These two species are differentiated from one another by Bergey (1948) according to their temperature range. Thus *Ps. ovalis* has an optimum temperature of 25° with poor or no growth at 37° , whereas *Ps. conveza* is said to grow readily at 37° and to have an optimum temperature of 30° . For convenience we propose to use the term *Ps. fluorescens* species group.

Cultures were maintained in the dry state (Stamp, 1947), and when in use, were subcultured monthly on 10% (v/v) yeast autolysate (Barker & Beck, 1942) agar slopes. Unless otherwise stated, the organisms were grown on the following medium: K_2HPO_4 , 0.1%; NH_4Cl , 0.1%; $Mg_2SO_4 \cdot 7H_2O$, 0.05%; all (w/v), in distilled water and supplemented with the following trace elements: 100 $\mu g./l.$ Fe^{2+} as $FeSO_4$, - $(NH_4)_2SO_4$, 6 H_2O ; 200 $\mu g./l.$ B as H_3BO_3 ; 100 $\mu g./l.$ Co^{2+} as

CoSO_4 , $7\text{H}_2\text{O}$; $10\text{ }\mu\text{g./l. Cu}^{2+}$ as CuSO_4 , $5\text{H}_2\text{O}$; $10\text{ }\mu\text{g./l. Mn}^{2+}$ as MnSO_4 , $4\text{H}_2\text{O}$; $100\text{ }\mu\text{g./l. Mo}$ as $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, $4\text{H}_2\text{O}$; $200\text{ }\mu\text{g./l. Zn}^{2+}$ as ZnSO_4 , $7\text{H}_2\text{O}$. Sodium succinate, sodium citrate or other single carbon source, as mentioned in the text, in concentration of 0.4% (w/v). The sterile medium was dispensed in 300 ml. portions in penicillin flasks, and after inoculation was incubated at room temperature ($18\text{--}25^\circ$) with shaking. The inocula were prepared by suspending a loopful of stock culture in 5 ml. of the same medium as that to be used for large-scale culture and incubating at 30° for 5–6 hr. Preliminary investigations showed that the oxidative activity of washed suspensions varied with the growth stage of the organism at the time of harvesting (cf. Wooldridge, Knox & Glass, 1936; Gale & Stephenson, 1938, 1939). The most active suspensions, Q_{O_2} 500–600 ($Q_{O_2} = \mu\text{l. O}_2/\text{mg. dry wt./hr.}$), were obtained when cells were harvested before they had reached half the maximum yield for the medium in question (Fig. 1*a*, *b*). Inocula were therefore so adjusted that the activity after overnight growth (14–18 hr.) was in this range. Cells were harvested by centrifugation, washed twice with and suspended in distilled water to give a concentration of 3 mg. dry wt./ml. Dry weight was determined by measuring the optical density of a suitably diluted sample in the Hilger Spekker absorptiometer with a neutral grey filter Ilford no. H508, and referring to a standard graph relating optical density to dry weight.

Reagents. Reagents obtained from supply houses were A.R. grade. Acids were neutralized with NaOH before use. α -Ketoglutaric acid was prepared by the modified method of

Clutterbuck (1927), as given by Umbreit, Burris & Stauffer (1949). Great difficulty was experienced in freeing this compound from small traces of succinate, using the method recommended by these authors. Pure samples in very low yield were finally obtained by repeated recrystallization from acetone and benzene. *cis*-Aconitic acid was prepared by the method of Malachowski & Maslowski (1928). DL-isoCitric acid was prepared by hydrolysis of a sample of trichloromethylparaconic acid, and assayed for the natural dextrorotatory isocitrate by the method of Ochoa (1948). All quantities of isocitrate given in this paper refer to the natural dextrorotatory form of the acid as above. A preparation containing approximately 15% triphosphopyridine nucleotide (TPN) and 30% diphosphopyridine nucleotide (DPN) was made from horse liver by the method of Lepage & Mueller (1949) and assayed for TPN by the method of Ochoa (1948) and for DPN by the method of Racker (1950). Adenosine triphosphate as the barium salt was prepared from rabbit muscle by the method of Lohmann (1931) as modified by Needham (1942). Coenzyme A was a preparation obtained from Armour laboratories and contained 10 Lipmann units/mg. Solutions of oxaloacetate were prepared from oxaloacetic acid immediately before use. 2:4-Dinitrophenylhydrazine obtained commercially was recrystallized from *n*-butanol.

Analytical methods. Manometric measurements were carried out in the conventional Warburg apparatus at 30° ; the reactions were stopped by tipping 0.3 ml. 5*N*- H_2SO_4 from the second side arm. Succinic acid was estimated by the method of Krebs (1937). α -Ketoglutaric acid was

Fig. 1. (*a*) Variation of Q_{O_2} succinate ($\mu\text{l. O}_2/\text{mg. dry wt./hr.}$) with growth expressed as mg. dry wt./ml. of cells in the growth medium. Q_{O_2} calculated from the oxygen uptake during the period 5–35 min. after tipping. Manometer cups contained: in the side arm 0.5 ml. of bacterial suspension (1.5 mg. dry wt. of cells), in the main cup 0.5 ml. of 0.066*M* phosphate buffer pH 7.4, 0.6 ml. of 0.2*M* sodium succinate, distilled water to 2.3 ml., in the centre well 0.2 ml. of 20% (w/v) KOH. Temp. 30° . (*b*) Growth curve of cells grown on a mineral medium with 0.4% (w/v) sodium succinate at 25° with shaking. Yield of cells (mg. dry wt./ml.) obtained by measuring the optical density of a suitably diluted sample in the Spekker absorptiometer and referring to a standard graph relating dry weight to optical density.

estimated by the transaminase method of Krebs (1950); the spectrophotometric method of Goodwin & Williams (1952) was used for the estimation of α -ketoglutaric acid and of pyruvic acid.

When the transaminase method was used the acidified reaction mixture was transferred quantitatively to a graduated cylinder and heated in a boiling-water bath for 10 min. to decompose any oxaloacetate, since it reacts in this method; pyruvate does not interfere in this system. After adjusting the pH of the reaction mixture to 5.0, α -ketoglutarate was estimated in a sample. For the method of Goodwin & Williams, 0.5 ml. of the acidified reaction mixture was pipetted into a graduated cylinder, diluted to 5.0 ml. and any oxaloacetate present decomposed as described above. (Recoveries of α -ketoglutaric acid tended to be 5–10% low by this method and those of pyruvic acid correspondingly high.)

Keto acids were identified by paper-partition chromatography of the 2:4-dinitrophenylhydrazones after the method of Cavallini, Frontali & Toschi (1949). The developing solvent was *n*-butanol saturated with water and the papers (Whatman no. 1) were developed by the descending method.

Acidified manometer-cup contents were transferred to centrifuge tubes and the cells centrifuged off. To each of the supernatants were added 2 ml. of 0.1% (w/v) 2:4-dinitrophenylhydrazine in 2*N*-HCl and the mixture was kept at room temperature for 30 min. The solution was extracted three times with 2 ml. of ether and the pooled ethereal extracts were shaken up with 2 ml. of 10% (w/v) Na_2CO_3 . The aqueous layer was removed, acidified and again extracted with ether (2 ml.). The ethereal extract was taken to dryness on the water bath and the residue dissolved in 0.5 ml. ethanol. Samples (50 μl .) of this solution were applied to sheets of Whatman no. 1 filter paper. The 2:4-dinitrophenylhydrazones of pyruvate and α -ketoglutarate were put through the same extraction procedure and the ethanolic solutions so obtained used as controls.

Respiratory quotients (R.Q.) were measured by the method of Dickens & Šimer (1930) as modified by Elsdén & Lewis (1953).

Preparation of cell-free extracts. These were prepared either with the Hughes bacterial press (Hughes, 1951) or with the Mickle disintegrator (Mickle, 1948). When the Hughes press was used the cells were grown on the above medium solidified with 2% (w/v) agar in Roux bottles and incubated at 30° overnight; 15–20 Roux bottles of medium gave 2–5 g. wet weight of cells. After harvesting and washing once in distilled water, the cells were mixed with their own weight of powdered Pyrex glass and transferred to the bacterial press, which had been cooled to -14° , and crushed. After crushing, a smear of the paste was made, fixed and stained with Loeffler's methylene blue and examined microscopically with a 2 mm. objective and a $\times 10$ ocular. The presence of less than ten unbroken cells per field was taken as an indication of a satisfactory crush. The paste of crushed cells and glass was transferred to a tared centrifuge tube, weighed, and 1 ml. of 0.066*M* phosphate buffer (pH 7.4)/g. of paste added. The glass and cell debris was removed by centrifuging for 5 min. at 9000–10000 rev./min. in an M.S.E. high-speed angle centrifuge. The supernatant consisted of a brownish opalescent liquid in which were suspended pink particles; these two phases were mixed and used together. On a few occasions the supernatant was almost clear and pale yellow and contained no pink particles. Such preparations had no oxidizing capacity.

For 'Mickle' extracts the cells were grown on the liquid medium described above, harvested and washed once in distilled water and then suspended in 0.066*M* phosphate buffer, pH 7.4, to a concentration of 15–25 mg. dry wt./ml. The thick suspensions were then shaken in the disintegrator with Ballotini beads, grade 12, until stained smears of the suspension showed no more than ten unbroken cells per microscope field; usually 15–30 min. shaking sufficed. The glass beads were filtered off through a sintered-glass funnel, porosity 2, and the suspension of crushed cells was centrifuged for 5 min. at 9000–10000 rev./min. The supernatants were similar in appearance to those from block-crushed cells.

The total N of the cell-free extracts was determined by the micro-Kjeldahl procedure of Chibnall, Rees & Williams (1943). The ammonia was distilled off in the apparatus of Markham (1942), and the ammonia trapped in 1 ml. of the boric acid reagent of Conway & O'Malley (1942).

RESULTS

The oxidation of succinate by washed suspensions

Fig. 2 shows a typical progress curve for oxidation of succinate by a washed suspension of the organism. It will be noted that there is a sharp break corresponding to approximately 1.5 moles of oxygen/mole of succinate added. Similar ratios were obtained when the suspensions were allowed to respire in the presence of excess of succinate, and the reaction stopped by the addition of acid and the residual

Fig. 2. Oxidation of succinate by washed suspensions grown on succinate. Manometer cups contained: in the side arm 0.5 ml. of bacterial suspension (1.5 mg. dry wt. of cells), in the main cup 0.5 ml. of 0.066*M* phosphate buffer pH 7.4, substrate as indicated, distilled water to 2.3 ml., in the centre well 0.2 ml. of 20% (w/v) KOH. Temp. 30°.

Table 1. *Consumption of succinate and of oxygen by washed suspensions*

(The total cup contents were: 3.0 ml. containing 0.011 M phosphate buffer pH 7.4 and 0.2 ml. of 20% KOH in the centre well, 0.5 ml. of 50% H₂SO₄ in one side bulb and 0.5 ml. suspension in the second side bulb. Substrate as indicated in the main compartment, and water to volume. Temp. 30°.)

Expt. no.	Succinate added (μmoles)	Suspension (mg. dry wt./cup)	Succinate consumed (μmoles)	Oxygen consumed (μmoles)	Ratio: oxygen/succinate
1	20	1.5	9.07	14.8	1.63
2	30	1.0	13.3	17.3	1.30
3	30	0.95	7.15	9.87	1.38
4	30	1.5	14.4	19.1	1.32
	+ 0.001 M sodium azide				

succinate determined (Table 1). Addition of 0.001 M sodium azide or 0.0001 M 2:4-dinitrophenol as assimilation inhibitors (Clifton, 1937) had no effect on this break in the oxygen uptake curve. Campbell & Stokes (1951) have made similar observations with *Ps. aeruginosa* grown on acetate. As the complete oxidation of succinate to carbon dioxide and water requires 3.5 moles oxygen/mole succinate these results suggested that under our conditions the substrate is incompletely oxidized, with the accumulation of one or more intermediates. On the basis of the observed oxygen:succinate ratio of 1.5, two schemes can be postulated: first, the stepwise oxidation of succinate to acetate with the uptake of 1.5 moles oxygen and the production of 2 moles carbon dioxide according to the following equation:

Secondly, the stepwise oxidation of 1 mole of succinate to 'active' acetate, of a second mole to oxaloacetate, followed by the condensation of these products to give citric acid which would then be oxidized to α-ketoglutarate. The overall equation for this reaction scheme is

Measurements of the respiratory quotients of suspensions oxidizing succinate (Table 2) gave values close to 1.2 which does not agree very well with either of these schemes, since the R.Q. for equation (1) is 1.3 and that for equation (2) is 1.0. A small amount of volatile acid, shown by paper chromatography to be acetate, was present in the reaction mixture. Tests for the presence of keto acids were positive. Chromatography of the 2:4-dinitrophenylhydrazones, prepared from cup contents in which succinate had been oxidized, gave three yellow spots, with *R_F*'s 0.162, 0.46 and 0.63, respectively, which were the same as those of the 2:4-dinitrophenylhydrazones of authentic α-ketoglutaric acid and pyruvic acid. Altmann, Crook & Datta (1951), Seligson &

Table 2. *Respiratory quotient of succinate oxidation*

(Double side-bulb cups containing 0.5 ml. of suspension (2.0 mg. dry wt./ml.), 0.5 ml. of 0.066 M phosphate buffer pH 7.0, 0.1 ml. of 0.33 M succinate in dangling tube, 0.3 ml. of 5N-HCl in one side bulb, 0.3 ml. of N-NaOH in second side bulb and water to 2.5 ml. total vol. Temp., 30°.)

No.	Oxygen consumed (μmoles)	CO ₂ produced (μmoles)	R.Q.
1	20.7	24.6	1.18
2	19.4	23.9	1.23
3	20.3	24.7	1.21

Shapiro (1952), Walker *et al.* (1951), and Gordon, Wopart, Burnham & Jones (1951) have also observed that the 2:4-dinitrophenylhydrazone of pyruvic acid often gives two spots, due to *syn-anti* isomerism. Since the 2:4-dinitrophenylhydrazone of oxaloacetate has an *R_F* similar to that of the α-ketoglutarate derivative, the above procedure was carried out on duplicate samples of supernatants, one of which was heated for 10 min. in a boiling-water bath to decompose any oxaloacetate present and the hydrazones were separated as described above. The chromatograms from these two supernatants were identical, indicating that the slowly moving spot was in all probability the 2:4-dinitrophenylhydrazone of α-ketoglutarate. No keto acids were found in a control without substrates.

Table 3 shows the results from a number of experiments in which the amounts of α-ketoglutarate formed from succinate by washed suspensions was estimated by the transaminase method and by the method of Goodwin & Williams (1952). The ratio of moles α-ketoglutarate formed/moles oxygen consumed was found to vary between 0.08 and 0.27, whereas if succinate were oxidized quantitatively to α-ketoglutarate according to equation (2), the ratio should be 0.33. Fig. 3 shows the production of α-ketoglutarate and pyruvate, and the consumption of oxygen plotted against time. The experiment was carried out by using a series of cups and stopping the reaction at suitable time intervals.

Table 3. *Production of α -ketoglutarate from succinate*

(All experiments were performed in double side-bulb cups containing 0.0133 M phosphate buffer pH 7.4, 1.5 mg. dry wt. of cells were tipped in from one side bulb. Acid in the second side bulb and substrate as indicated in the main cup. 0.2 ml. 20% (w/v) KOH in the centre well. Temp. 30°.)

Expt. no.	Succinate added (μ moles)	Time of incubation (min.)	Oxygen consumed (μ moles)	α -Ketoglutarate formed (μ moles)	Ratio: α -ketoglutarate formed/oxygen consumed
4	20	20	12.6	1.70	0.135
	20	30	18.2	2.34	0.128
	20	54	30.9	2.65	0.086
	20	69	32.2	3.11	0.097
	20	134	34.6	2.46	0.071
7	60	25	12.6	3.4	0.27
	60	35	18.1	3.65	0.20
	60	79	32.3	4.30	0.134
	60	99	36.4	4.70	0.129
8	100	15	7.0	1.41	0.20
	100	20	10.8	1.49	0.138
	100	30	15.0	2.38	0.158
	100	40	21.2	3.41	0.161
	100	50	26.6	4.55	0.171

Fig. 3. Oxygen consumption and the production of α -ketoglutarate and pyruvate by washed suspensions oxidizing succinate. Experimental conditions are the same as described in Table 3.

Isolation of α -ketoglutaric acid from the growth medium

Initial attempts to detect α -ketoglutaric acid in the ordinary growth medium were unsuccessful. Lockwood & Stodola (1946), in their work on the production of α -ketoglutarate from glucose, used a medium with a carbon:nitrogen ratio of approx. 100. We therefore modified the usual succinate medium so that it contained 3.6% (w/v) sodium succinate and 0.01% (w/v) ammonium chloride (C:N = 400). The α -ketoglutaric acid was isolated as the 2:4-dinitrophenylhydrazone from the culture medium as follows.

Six penicillin flasks, each containing 300 ml. of the above medium, were inoculated and incubated at 25° for 50 hr. with shaking. At the end of this period the organisms were centrifuged off and the α -ketoglutarate content of a sample of the supernatant was estimated; yield 58 μ g./ml. The supernatant was treated with 20 g. of decolorizing charcoal (Hopkin

and Williams) and after filtration was concentrated *in vacuo* to 230 ml. The concentrated solution was adjusted to pH 7.0 with conc. HCl and 51 ml. of conc. HCl were added, followed by 300 mg. of 2:4-dinitrophenylhydrazine in 45 ml. of 2N-HCl. The mixture was left at room temperature for 2 hr. and stored overnight in the refrigerator. The crystalline precipitate was filtered off and washed several times with distilled water. The dry precipitate weighed 740 mg. and apparently contained some succinic acid from the growth medium. It was suspended in a little water, transferred to a separating funnel and 30 ml. of ether were added. After shaking for about 1 min., the two layers were separated, and the aqueous phase was washed with successive portions of 20, 10 and 10 ml. ether. The ethereal extracts were combined and extracted with successive portions of 20, 10, 5 and 5 ml. of 15% (w/v) Na_2CO_3 . The combined carbonate extracts (40 ml.) were acidified with conc. HCl, when a pale-yellow, crystalline precipitate was formed. This was left in the refrigerator for a few hours, and the precipitate filtered off and washed exhaustively with acidified water to remove any succinic acid. The crystals were dried in a desiccator over CaCl_2 : yield, 123.7 mg. Paper chromatography of this preparation showed only one spot which corresponded to the 2:4-dinitrophenylhydrazone of α -ketoglutaric acid. The compound was recrystallized from 10 ml. of hot ethanol plus 15 ml. of hot water and the solution allowed to cool. The pale, lemon-yellow, needle-shaped crystals were filtered off, washed with water, and dried in a desiccator over CaCl_2 : yield, 90 mg. This material had m.p. 219–221° (decomp.) and the mixed melting point with authentic α -ketoglutarate dinitrophenylhydrazone (m.p. 219–220°) (decomp.) was 220–221° (decomp.). (Found: C, 38.7; H, 3.7; N, 15.9; loss of weight on drying *in vacuo* at 50° over P_2O_5 to constant weight, 5.1%. Calc. for $\text{C}_{11}\text{H}_{10}\text{O}_8\text{N}_4$, H_2O : C, 38.4; H, 3.5; N, 16.3; H_2O , 5.2%.) Fig. 4 shows the absorption curves of our sample and that of an authentic specimen of 2:4-dinitrophenylhydrazone of α -ketoglutarate, determined in a Beckman spectrophotometer model DU. The E_{max} for both specimens under these conditions was 1640 at 420–425 μ .

Fig. 4. Absorption spectra of two specimens of the 2:4-dinitrophenylhydrazone of α -ketoglutarate. $\times$ — $\times$, authentic specimen of α -ketoglutaric acid derivative. $\circ$ — $\circ$, specimen of α -ketoglutaric acid derivative isolated from the growth medium. The hydrazones were dissolved in 15% (w/v) Na_2CO_3 and an equal volume of 1.5N- NaOH was added. Each solution contained 12.5 μg . of the hydrazone/ml.

Oxidation of tricarboxylic acid-cycle intermediates by washed suspensions

Since it seemed possible that α -ketoglutarate was formed from succinate by reactions involving the tricarboxylic acid cycle, the oxidation of various cycle intermediates by washed suspensions grown on succinate was next tested. Several of the intermediates were oxidized only after a lag period, suggesting that cells grown upon succinate had to adapt to these substrates. It was found that the lags were very much longer (1–2 hr.) when cells were grown on a liquid medium than when they were grown on the same medium solidified with agar, and consequently all further experiments of this type were carried out with cells which had been grown on solid media dispensed in 100 ml. portions in Roux bottles and incubated at 30° overnight. Fig. 5 (a) shows the oxygen uptake plotted against time for the various cycle intermediates by suspensions of cells grown on a succinate medium. From this figure it appears that the cells grown on succinate can oxidize succinate, fumarate, malate and oxaloacetate immediately and linearly, but attack citrate, *cis*-aconitate, *isocitrate* and α -ketoglutarate only after a lag period. Table 4 summarizes a number of experiments in which the oxidation of

various cycle intermediates was tested by suspensions of cells grown on different tricarboxylic acid cycle compounds. In these experiments all initial inflexions in the oxygen uptake curves, however slight, have been interpreted as lag periods, on the basis of our experience that the duration and extent of these can fluctuate considerably from experiment to experiment.

The question arises whether the lag periods observed in the oxidation of some of these substrates by washed suspensions represent enzymic adaptation, i.e. the synthesis of enzymes, and it was thought that an answer might be obtained by use of a specific inhibitor of adaptive-enzyme formation, should one such exist.

Inhibition of adaptation

Irradiation with ultraviolet light seemed to offer possibilities as a specific inhibitor of enzymic adaptation. Swenson & Giese (1950) showed that ultraviolet irradiation inhibited the adaptive formation of galactozymase in yeast. Stanier & Lederberg (1951) have shown that this irradiation inhibits adaptation without affecting oxidative assimilation. Jacob, Torriani & Monod (1951) found that doses which inhibit adaptation in

Figs. 5 (a) and (b). Oxidation of tricarboxylic acid-cycle intermediates by washed suspensions of cells grown on succinate. Oxygen uptake followed manometrically. Manometer cups contained: in the side arm 0.5 ml. bacterial suspension (3.0 mg. dry wt. of cells); in the main cup 0.5 ml. of 0.066M phosphate buffer pH 7.4, 4 μ moles substrate as indicated (4–15 μ moles of *isocitrate*), distilled water to 2.0 ml.; in the centre well 0.2 ml. of 20% (w/v) KOH. Temp. 30°. Fig. 5 (a) = unirradiated suspension. Fig. 5 (b) = the same suspension irradiated with u.v. light for 12 min.

Table 4. Oxidation of tricarboxylic acid cycle intermediates by washed suspensions

(Cup contents: 2.7 ml. containing 0.5 ml. of 0.066M phosphate buffer pH 7.4; 3.0 mg. dry wt. of cells in side bulb; and 5 μ M substrate, 0.2 ml. 20% KOH in centre well. Temp. 30°.)

Growth substrate	Substrate for oxidation									
	Succinate	Fumarate	Malate	Oxaloacetate	Pyruvate	Acetate	Citrate	<i>cis</i> -Aconitate	<i>iso</i> -Citrate	α -Ketoglutarate
Succinate	+	+	+	+	0	0	0	0	0	0
Fumarate	+	+	+	+	.	.	0	.	.	0
Malate	+	+	+	+	.	.	0	.	.	0
Acetate	0	0	.	.	+	+	0	.	.	0
Citrate	0	0	+	.	0	0	+	0	0	0
<i>iso</i> Citrate	0	0	+	.	.	.	+	+	+	0
α -Ketoglutarate	0	0	.	.	0	0	0	.	.	+

+ = Substrate oxidized linearly from the moment of tipping.
0 = Substrate not linearly oxidized initially.

bacteria do not affect the ability of the cells to reproduce bacteriophage. Brandt, Freeman & Swenson (1951) found that ultraviolet irradiation inhibited the formation of galactozymase in yeast, but had no effect on the activity of preformed galactozymase, or on glucozymase. Swenson (1952) determined the action spectrum of inhibition of galactozymase formation by ultraviolet irradiation, and found that it had a maximum at 260 m μ .

Figure 5 (b) shows the behaviour of the same washed suspensions as used in Fig. 5 (a) towards tricarboxylic acid-cycle intermediates after ultraviolet irradiation. Washed cell suspensions were irradiated with a Sterilamp (General Electric Co.). The lamp was housed in a wooden cabinet

fitted with a glass window and a pair of holes for manipulation. The cell suspension (5–6 ml.), containing 6 mg. dry wt. cell/ml., was pipetted into a Petri dish (diameter 11 cm.) and shaken by hand for 12 min. at a distance of 33 cm. from the lamp. This treatment was found by experiment to inhibit the adaptive oxidation of benzoate by cells grown upon phenylacetate without affecting in any way the oxidation of phenylacetate. Figs. 6 and 7 show the course of the oxidation of citrate, succinate and α -ketoglutarate by irradiated and non-irradiated suspensions of cells grown on citrate.

In no case did the irradiated suspensions show any augmentation in the rate of oxygen uptake over the initial rate, such as was exhibited by non-

irradiated suspensions. This must mean that ultraviolet irradiation inhibited the process or processes responsible for the increase in oxidation rate in response to substrate, presumably the formation of new enzyme. On the other hand, it can be seen that irradiation affects neither the initial rate of oxygen uptake nor the breaks in the oxygen uptake curves, and Table 5 shows that irradiated cells form α -ketoglutarate from succinate in the same way as do non-irradiated cells.

Fig. 6. Oxidation of citrate and succinate by a washed suspension of cells grown on citrate. Experimental conditions as in Fig. 5. $\times$ — $\times$, suspension irradiated with u.v. light; $\bullet$ — $\bullet$, non-irradiated control.

Fig. 7. Oxidation of citrate and α -ketoglutarate by a washed suspension of cells grown on citrate. Experimental conditions as in Figs. 5 and 6. $\times$ — $\times$, suspension irradiated with u.v. light; $\bullet$ — $\bullet$, non-irradiated control.

Table 5. Production of α -ketoglutarate from succinate by irradiated cells

(Double side-bulb cups containing 0.5 ml. of 0.066M phosphate buffer pH 7.4, 1.5 mg. dry wt. of cells in one side bulb and acid in the second side bulb. Substrate (60 μ moles) in the main cup, 0.2 ml. 20% (w/v) KOH in centre well. Temp. 30°.)

Time of incubation (min.)	Oxygen consumed (μ moles)	α -Ketoglutarate formed (μ moles)	Ratio: α -ketoglutarate/oxygen consumed
20	9.0	0.96	0.107
30	15.1	2.58	0.171
35	19.6	3.18	0.163
45	24.2	4.25	0.175
50	30.2	3.78	0.125
0	0	0.34	—

Oxidation of tricarboxylic acid cycle intermediates by cell-free preparations

Cell-free preparations made by the two methods were tested for their ability to oxidize the various tricarboxylic acid cycle intermediates. Table 6 shows the results of a number of such experiments with cell-free preparations made from cells grown on either a succinate medium or on a citrate medium. The activities of the extracts, when expressed as $q_{O_2}N$ (μ l. O_2 /mg. N/hr.) vary considerably from preparation to preparation, and a precise quantitative comparison between the different extracts is therefore impossible. However, it can be seen that the rates of oxidation of succinate by extracts from succinate-grown cells and by extracts from citrate-grown cells are of the same order. The table also gives the activities of the extracts, relative to that with succinate, towards other tricarboxylic acid-cycle intermediates. It will be seen that the growth conditions had no significant effect on the rate of oxidation of these substrates by the extracts. Two 'Mickle' extracts failed to oxidize citrate, and the rate of citrate oxidation by other such extracts was much lower than when extracts prepared with the Hughes press were used. This may be due to inactivation of aconitase during preparation, since tests for the presence of isocitric dehydrogenase by the spectrophotometric method of Ochoa (1948) were positive when DL-isocitrate was the substrate, but negative when citrate was the substrate. All attempts to prepare extracts which would oxidize acetate failed, whether the Hughes press or the Mickle disintegrator was used, and whether the cells were grown on succinate, citrate or acetate.

The foregoing observations mean that cells, whether grown on succinate or citrate, contain most of the enzymes of the tricarboxylic acid cycle, despite the fact that washed suspensions of the same cells show adaptive phenomena towards some of the tricarboxylic acid-cycle intermediates. Similar observations have recently been published by Barrett, Larson & Kallio (1953).

Table 6. *Oxidations by cell-free extracts*

(Rates, calculated from the O_2 uptake during the first 30 min. after equilibration, are expressed as percentage of the rate on succinate, after subtracting values for blanks. Cup contents were: 2.5 ml. containing 0.5 ml. of 0.066M phosphate buffer pH 7.4, substrate as indicated, 1 μ M $MnSO_4$, 2 μ M $MgCl_2$, approximately 100 μ g. TPN and 200 μ g. DPN. 0.5-0.7 μ M ATP, 0.2 ml. 20% (w/v) KOH in centre well, and 0.5 ml. extract in the side bulb. Temp. 30°.)

Expt. no.	Substrate (μ moles)	μ l. O_2 /mg. N/hr.		Rate of oxidation as % of rate with succinate						
		Blank	Succinate	Extracts from cells grown on succinate						
				Fumarate	DL-Malate	Oxalo-acetate	Citrate	cis-Aconitate	isoCitrate	α -Keto-glutarate
‘Mickle’ crush										
1	50	.	.	.	23.3	.	0	.	.	17.1
2	50	.	.	.	.	.	38.0	.	.	.
3	5	.	.	.	.	.	.	.	29.7	.
4	40	.	.	.	.	.	25.8	.	.	.
5	30	5	138	67.0	63.0	.	.	.	27.0	19.6
6	30	.	.	29.6	28.0	.	.	.	17.8	17.5
7	5	7	113	60.6	65.5	17.2	0	27.6	71.5	60.5
8	5	12.9	157	69.5	61.0	23.6	17.0	46.5	45.3	54.5
Block crush										
9	50	13.4	113	.	.	.	60.0	.	.	41.7
10	5	15.9	51	.	.	.	65.0	.	70.0	85.5
‘Mickle’ crush										
11	50	.	.	.	92.0	.	6.5	.	.	40.5
12	50	.	.	.	.	.	15.6	.	.	.
13	30	5.7	191	19.2	82.4	.	.	.	94.0	37.6
14	5	9.4	142	40.0	81.0	15.0	6.4	23.4	60.0	40.8

Extracts from cells grown on citrate

DISCUSSION

We have shown that succinate is oxidized by washed suspensions of a fluorescent *Pseudomonas*, strain KB1, with an oxygen uptake of approximately 1.5 moles/mole of succinate, compared with 3.5 moles required for the complete oxidation of succinate. This observation led us to look for α -ketoglutarate as one of the end products of this reaction. It is of interest that Campbell & Stokes (1951), working with washed suspensions of *Ps. aeruginosa*, have published graphs which show that succinate was incompletely oxidized and from which it can be calculated that the oxygen uptake was about 1.5 moles oxygen/mole succinate. However, these workers expressed the opinion that the deficit in oxygen consumption was due to assimilation of part of the substrate, despite the fact that none of the inhibitors of oxidative assimilation tested decreased this deficit.

The production of α -ketoglutarate from succinate can be explained in two ways: either it is formed by the reductive carboxylation of succinate, that is to say, a reversal of the α -ketoglutarate oxidase, or it can be produced by a mechanism involving the tricarboxylic acid cycle. These two pathways can be summarized in the following way:

With washed suspensions of this organism, the above two equations were never completely satisfied, for not only was the amount of α -ketoglutarate found always less than the theoretical, but there was also some pyruvate produced, and the R.Q. was of the order of 1.2 as opposed to the theoretical value of unity.

Dagley *et al.* (1952) detected α -ketoglutarate when *Vibrio strain* 01 oxidized succinate and suggested that it could be produced either by reductive carboxylation of succinate or via the tricarboxylic acid cycle. Our experiments do not enable us to decide between these two mechanisms, but we have assumed, as a working hypothesis, that the second mechanism is the one which operates in these organisms, and that α -ketoglutarate accumulates as a result of its being formed faster than it is removed. This hypothesis implies that the terminal oxidation of 2-carbon fragments in these organisms proceeds through the tricarboxylic cycle.

Tests of cell-free extracts prepared in different ways showed the following enzyme systems to be present: succinic dehydrogenase, fumarase, malic dehydrogenase, aconitase (in some preparations) isocitric dehydrogenase and α -ketoglutaric oxidase. We have not tested for the condensing enzyme, but Ochoa, Stern & Schneider (1951) have reported the extraction of the condensing enzyme from *Ps. fluorescens*. Unlike the cell-free preparations from *Azotobacter vinelandii*, examined by Stone & Wilson (1952), our preparations would not oxidize acetate, even when reinforced with ATP, coenzyme A and catalytic amounts of either fumarate or succinate. Thus, whilst the demonstration that most of the enzymes of the tricarboxylic acid cycle occur in this organism is consistent with the hypothesis that α -ketoglutarate is formed from succinate according to the reactions summarized in scheme (2), it still remains to be shown that the tricarboxylic acid cycle is the only, or the major, mechanism for the complete oxidation of succinate. Quantitative evidence, provided by the ratios of α -ketoglutarate formed/oxygen consumed, show that the formation of this intermediate could account for up to 70 % of the oxygen uptake on succinate. Whether the remainder represents the oxygen used for removal of α -ketoglutarate or whether it represents another mech-

anism, acting simultaneously, for the oxidation of succinate, or some of its oxidation products, remains to be investigated. Attempts to study this question by the use of various inhibitors, such as malonate or arsenite, met with no success. Neither of these had any effect on the oxidation of succinate by washed suspensions possibly because the enzymes concerned were inaccessible; on the other hand, since cell-free extracts did not produce α -ketoglutarate from succinate, the effect of these inhibitors on such preparations was not tested.

When the oxidative capacity of whole cells was examined, we found that cells grown on a succinate medium oxidize citrate, *cis*-aconitate, isocitrate and α -ketoglutarate only after a lag period; but when the cells were grown on a citrate medium there was a pronounced lag in the oxidation of α -ketoglutarate. These activities, however, do not reflect the enzymic constitution of the cell, since extracts of cells, no matter how cultured, contained the appropriate

oxidizing enzymes. When suspensions were irradiated with ultraviolet light the augmentation in the rate of oxygen uptake on any of the substrates tested was completely inhibited whilst it did not affect the initial rate of oxygen uptake, nor the location of the breaks in the oxygen uptake curve, nor the production of α -ketoglutarate from succinate. If one assumes that irradiation with ultraviolet light inhibits specifically the synthesis of new enzyme, then the above results can only mean that the lag periods are manifestations of adaptive enzyme formation.

Assuming this to be true, what is the nature of the enzymes synthesized? There are two possible explanations. First, that growth on a given substrate *A*, which is oxidized via intermediates, *B*, *C*, *D*, etc., will lead to the formation of a certain quantity of enzymes, *E_b*, *E_c*, *E_d*, etc., to deal with these intermediates. Incubation of washed suspensions with relatively high concentrations of one of these intermediates might then require the formation of further enzyme for its oxidation at maximum rate. Against this hypothesis are the following considerations. (1) The difference in behaviour of cells grown on succinate towards fumarate, malate and oxaloacetate on the one hand, and citrate, *isocitrate* and α -ketoglutarate on the other hand. That is to say, growth on succinate appears to lead to the synthesis of sufficient fumarase, malic dehydrogenase and oxaloacetate decarboxylase to deal with high concentrations of these substrates supplied in the medium, but not to the synthesis of enough aconitase, *isocitric* dehydrogenase or α -ketoglutarate oxidase to deal with equivalent amounts of citrate, *isocitrate* or α -ketoglutarate. (2) Cell-free extracts from succinate-grown cells oxidize *isocitrate* or α -ketoglutarate at rates comparable with those of citrate-grown extract, although the washed suspensions of succinate-grown cells show a complete lag of 30 min. on citrate or *isocitrate*.

The second possibility is that the adaptation involved in these experiments is not concerned with the actual oxidative steps, but with making the substrates accessible to the enzyme, either across the cell membrane or to some special site within the cell. On this hypothesis the adaptive process would consist of the formation of the particular enzyme necessary for the movement of substrate.

Campbell & Stokes (1951) observed that washed suspensions of *Ps. aeruginosa*, prepared from cultures grown on acetate, only oxidized certain of the tricarboxylic acid-cycle intermediates after a lag period, whereas dried preparations made from cells grown on the same medium oxidized these same intermediates. They commented as follows: 'Apparently the cell membrane of *Ps. aeruginosa* was initially impermeable to the compounds mentioned, and the period of adaptation

encountered with resting cells was the time necessary for the elaboration of the system for transferring the substrates across the membrane'. Stone & Wilson (1952) observed a similar phenomenon in *Az. vinelandii* and remarked: 'Results from simultaneous adaptation experiments must be interpreted with considerable reservation, unless the factor of permeability can be eliminated. Unfortunately, this is seldom possible, since "permeability" here implies not only ability to penetrate the cell wall, but also to get to the enzyme surface at which the reaction occurs.' Neither of these groups of workers, however, investigated whether the lag periods they observed involved the formation of new enzyme.

Gary & Bard (1952) observed lag periods in the oxidation of gluconate and pentose by resting cells of *B. subtilis* grown on a simple medium containing glucose, and interpreted these as 'corresponding to the time required for the breakdown of a permeability barrier, or its equivalent, the synthesis or activation of the respective kinases'. This statement was not supported by any direct evidence on the nature of the lag. Barrett *et al.* (1953) studied the effect of various inhibitors of enzymic adaptation on the lag period which occurs when washed suspensions of *Ps. fluorescens*, grown on fumarate, are given citrate to oxidize. They found that the inhibitors tested prolonged the lag period indefinitely. They also compared the rates of citrate oxidation by extracts of cells grown on fumarate and by extracts of cells adapted to citrate, and found no significant difference in the two extracts. They commented thus: 'It may be that some type of transport or carrier system is being synthesized by fumarate-grown cells and its completion would enable citrate to be carried across the osmotic barrier and perhaps oriented on the enzyme surface.' A 'carrier' concept has also been discussed by Doudoroff (1951) and by Osterhout (1952), but not in connexion with adaptive enzyme formation.

In view of the above observations by ourselves, and by the other workers cited, the interpretation of simultaneous adaptation experiments must be modified to include the possibility of a 'permeability' adaptation. Stanier (1950) has pointed out that the validity of the simultaneous adaptation technique rests on the assumption that the cell is permeable to all substrates tested, but it has so far been insufficiently realized that the adaptation itself may be concerned with making the substrate accessible to the enzyme system.

SUMMARY

1. A strain of the *Pseudomonas fluorescens* species group (KB 1), isolated from soil, was found to oxidize succinate incompletely, with the uptake

of approximately 1.5 moles oxygen/mole succinate and with the accumulation of α -ketoglutarate and pyruvate. The yield of α -ketoglutarate formed from succinate was variable, but could account for up to 70 % of the oxygen consumed.

2. α -Ketoglutaric acid as the 2:4-dinitrophenylhydrazones was isolated from a growth medium containing succinate as sole source of carbon.

3. Washed suspensions of cells grown with succinate as the sole source of carbon oxidized citrate, isocitrate, *cis*-aconitate and α -ketoglutarate after a lag period. These adaptations were inhibited by irradiating the suspensions with ultraviolet light.

4. Washed suspensions of cells grown with citrate as the sole source of carbon-oxidized succinate and α -ketoglutarate only after a lag period and irradiation of the suspension with ultraviolet light inhibited these adaptations.

5. Cell-free extracts of cells grown on a medium containing either succinate or citrate were found to

contain the following enzymes: succinic dehydrogenase, fumarase, malic dehydrogenase, oxaloacetic decarboxylase, aconitase, isocitric dehydrogenase and α -ketoglutaric oxidase.

6. The role of the tricarboxylic acid cycle in the oxidation of succinate by this organism is discussed.

7. The use of the technique of simultaneous adaptation for the study of the tricarboxylic acid cycle in micro-organisms is discussed.

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