An Evaluation of the Pathways of Metabolism of Glucose, Gluconate and 2-Oxogluconate by *Pseudomonas aeruginosa* by Measurement of Molar Growth Yields

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SUMMARY

Pseudomonas aeruginosa, ATCC 9027, was grown in a simple ammonium salts medium with limiting glucose, gluconate or 2-oxogluconate as the sole carbon and energy source. There was little difference in molar growth yield between glucose and gluconate but with 2-oxogluconate the yield was significantly less. Measurement of Y_o values (g. dry weight of cells produced/mole of oxygen consumed) during growth on the three substrates indicated most efficient oxygen utilization with 2-oxogluconate. The results are discussed in relation to the relative metabolic importance of the various pathways of glucose metabolism in the organism.

INTRODUCTION

Several initial pathways of glucose metabolism are possible in *Pseudomonas aeruginosa*. The presence of a direct oxidative pathway via gluconate and 2-oxogluconate has been recognized for many years (for a review, see De Ley, 1960) and evidence has been presented that these oxidations are not linked to nicotinamide or flavin nucleotides (Ramakrishnan & Campbell, 1955; Campbell, Ramakrishnan, Linnes & Eagles, 1956). The presence of enzymes characteristic of phosphorylative pathways, such as hexokinase, gluconokinase and glucose 6-phosphate dehydrogenase, has also been demonstrated in cell-free extracts of glucose-grown *P. aeruginosa* (Claridge & Werkman, 1953, 1954). In the present investigation, molar growth yield determinations (Bauchop & Elsdon, 1960) were carried out with glucose, gluconate and 2-oxogluconate as the sole carbon and energy sources in an attempt to gain some insight into the relative metabolic importance of the various pathways, the assumption being made that glucose metabolism via the direct oxidative pathway would make less energy available to the organism than a pathway via glucose 6-phosphate and 6-phosphogluconate in which the oxidative step is nicotinamide nucleotide-linked. Campbell *et al.* (1956) did report that when *P. aeruginosa*, strain ATCC 9027, was grown on limiting equimolar amounts of glucose, gluconate or 2-oxogluconate identical growth yields were obtained, and they concluded that no energy was gained in the oxidative steps from glucose to 2-oxogluconate. Unpublished observations in our laboratory with a different strain of *P. aeruginosa*, 2F32, indicated that there were some differences in molar growth yield with the three substrates and the present work was undertaken to re-examine the observations of Campbell *et al.* (1956) with strain ATCC 9027.

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METHODS

Organism. *Pseudomonas aeruginosa*, strain ATCC 9027, was maintained on nutrient agar slopes at 30°. It was subcultured at monthly intervals and stock slopes were stored at 4°.

Media. For molar growth yield determinations, the following medium was used: KH_2PO_4 , 9 g.; $(\text{NH}_4)_2\text{SO}_4$, 2 g.; nitrilotriacetic acid, 0.48 g.; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.004 g.; the pH was adjusted to 7.1 with 5 N-NaOH and the volume made up to 1 l. with distilled water. Three volumes of this solution were diluted with two volumes of carbon source solution (see below) and 5 ml. of each of the following two salts solutions added per litre of complete medium. Salts solution 1 contained: concentrated HCl, 13.4 ml.; CaCO_3 , 2 g.; ZnO, 0.406 g.; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 5.4 g.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.99 g.; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.17 g.; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.238 g.; H_3BO_3 , 0.062 g.; distilled water up to 1 l. Salts solution 2 contained: concentrated HCl, 45 ml., MgO, 10.08 g.; distilled water up to 1 l. Details of these salt solutions were originally provided by Mr D. W. Tempest. All carbon sources were sterilized by filtration through a Millipore membrane (Millipore Filter Corporation, Bedford, Massachusetts) and, with the exception of 2-oxogluconate, were added to the medium at 2, 3, 4, and 5 mM final concentration. 2-Oxogluconic acid was prepared by passing a solution of the commercial calcium salt through a column of Dowex 50 cation exchange resin in the hydrogen form. The free acid was eluted from the column with distilled water and its concentration determined by titration against standardized 0.01 N-NaOH in a Conway microburette using phenol red as indicator. CO_2 -free air was bubbled through the titration mixture. The acid was neutralized with NaOH and added to the medium at known concentrations approximating to those used for the other carbon sources.

Growth experiments. Molar growth yield determinations were carried out at 30° and 37° in two ways:

1. In 12.5 ml. medium contained in 50 ml. Erlenmeyer flasks shaken in reciprocating water baths at 80 strokes/min. with an amplitude of 1.5 in. Growth was followed turbidimetrically at 570 m μ with a Unicam SP 600 spectrophotometer (Unicam Instruments, Cambridge) by using flasks fitted with test-tube side arms. When growth was complete, the cultures were made up quantitatively to 25 ml. in volumetric flasks with 0.067 M-phosphate buffer, pH 7.1, for measurement (see later).

2. In 3 ml. medium in Barcroft flasks (without side arms but with centre wells) on the Warburg apparatus. Oxygen consumption was followed until it ceased to be exponential, at which point the flasks were removed and the cultures made up to 10 ml. with phosphate buffer prior to measurement. Barcroft flask centre wells contained 0.2 ml. of the CO_2 -buffer, diethanolamine (60 %, w/v) containing thiourea (0.1 %, w/v), a solution based on the reagent of Pardee (1949). The use of KOH as centre well fluid resulted in extremely long lag phases (often greater than 24 hr) before growth began. Inocula for growth yield experiments were prepared as follows: the organism was subcultured from a nutrient agar slope into 12.5 ml. of medium in which the carbon source concentration was 0.8 % (w/v). Two further subcultures were carried out under these conditions before transfer to a medium in which the carbon source was present in limiting amount (5 mM). The organism was subcultured at least three times in limiting medium before use in growth yield experiments to ensure adequate training of the cells on the particular energy source and no carry-over of unused

energy source. The organism was not grown on excess 2-oxogluconate but trained through several subcultures on limiting concentrations of 2-oxogluconate, cells grown on gluconate being used for the initial inoculum. Erlenmeyer flasks were inoculated with 0.1 ml. and Barcroft flasks with 0.05 ml. of a limiting culture (5 mM energy source) which had just reached the stationary phase.

Growth was measured in two ways. In the first, the extinction of suspensions was measured at 570 $m\mu$ in the Unicam SP 600 spectrophotometer and the dry weight was read from a standard calibration curve relating extinction to bacterial dry weight over the range 0.05–0.5 mg. cells/ml. In the second, a modification of the method of Johnson (1949) for the colorimetric determination of organic matter was used. Cell suspensions were mixed with an equal volume of 0.8 M-perchloric acid, in glass centrifuge tubes, centrifuged and washed once with a volume of 0.4 M-perchloric acid equal to twice the volume of the original suspension. The precipitates were suspended in 1 ml. of distilled water and 3 ml. of the oxidizing reagent, $K_2Cr_2O_7$ (0.18 %, w/v) in concentrated H_2SO_4 (95 %, v/v), added. The tubes were closed with glass condensers and heated at 100° for 20 min. The solutions were cooled, made up quantitatively to 10 ml. with distilled water and the extinctions measured at 440 $m\mu$ against a reagent blank, reduced chemically by addition of 0.2 ml. $Na_2SO_3 \cdot 7H_2O$ (20 %, w/v). A calibration curve relating extinction at 440 $m\mu$ to bacterial dry weight over the range 0.1–0.8 mg. cells/ml. was prepared. All spectrophotometric measurements of bacterial growth were made with 4 ml. glass spectrophotometer cells with a 1 cm. light path. For preparation of calibration curves, large batches of cells were grown to stationary phase at 30° or 37° under conditions of forced aeration with limiting glucose (5 mM) as carbon source. Dry weight measurements were made by heating concentrated cell suspensions to constant weight in a hot air oven at 104°.

Isotopic experiments with [U - ^{14}C]glucose. 30 ml. of medium were prepared such that the glucose concentration was 4 mM and its specific activity was 0.168 $\mu C/\mu mole$. This medium was sterilized by membrane filtration and 12.5 ml. amounts dispensed into 50 ml. Erlenmeyer flasks fitted with side arms. These were inoculated with 0.05 ml. of a limiting culture as previously described, stoppered with rubber bungs and incubated until growth was complete. The suspensions were made up quantitatively to 25 ml. with distilled water for measurement of turbidity. The radioactivity of (a) the original medium, (b) the cell suspension, and (c) the growth supernatant solution (obtained by centrifuging a sample of the cell suspension at 14,000 g for 15 min.) was determined by spreading portions (0.1 and 0.2 ml.) of suitably diluted solutions on clean nickel planchets having an effective raised area of 1.77 $cm.^2$ and drying under an infra-red lamp. In (b) and (c) the pH was adjusted to at least 7.6 with 0.2 N-NaOH to prevent loss of steam-volatile acids. All estimations were made at infinite thinness using an Ekco Automatic Scalar, Type N530G, and corrections were applied for background. Counts were collected for a period sufficient to ensure that the counting error was less than ± 4 %.

Chemicals. Unless otherwise stated, all chemicals were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex or British Drug Houses Ltd., Poole, Dorset. Wherever possible, A.R. grade reagents were used. Of the growth substrates, glucose was A.R. and sodium gluconate was Laboratory Reagent quality. Calcium 2-oxogluconate was obtained from Nutritional Biochemicals Ltd., Cleveland, Ohio, U.S.A. The purity of the three growth substrates was checked by paper chromatography

using a modification of the method of Grado & Ballou (1961). The solvent system was ethyl acetate-pyridine-water saturated with boric acid (60:25:40 by volume). On shaking this separated into two layers and the top layer was used as solvent. Descending paper chromatograms (Whatman No. 1) were run for 48 hr, and were developed with the benzidine-periodate reagent described by Smith (1961). Each substrate ran as a single spot.

[U- 14 C]Glucose was obtained from the Radiochemical Centre, Amersham, Bucks.

Fig. 1

Fig. 2

Fig. 1. The relation between growth of *P. aeruginosa* at 30° and substrate concentration. Growth substrates: ○, glucose; □, sodium gluconate; △, sodium 2-oxogluconate. Each point is the mean of the number of estimations shown in parentheses; the standard deviations from the mean are represented by the heights of the vertical bars.

Fig. 2. The relation between growth of *P. aeruginosa* at 37° and substrate concentration. Growth substrates: ○, glucose; □, sodium gluconate; △, sodium 2-oxogluconate. Each point is the mean of the number of estimations shown in parentheses; the standard deviations from the mean are represented by the heights of the vertical bars.

RESULTS

Figure 1 shows the relation between total growth of the organism at 30° in open flasks, as measured by extinction, and substrate concentration. Growth was measured over a range of substrate concentrations to ensure that the energy source was the growth-limiting factor. Similar data were obtained for growth at 37° (Fig. 2). In each case the mean *Y* values were calculated from the gradients of the straight lines relating growth to substrate concentration and are summarized in Table 1. The results obtained using the method for the estimation of total organic matter were plotted in a similar manner and are also recorded in Table 1.

A summary of the results obtained from manometric growth experiments is given in Table 2. The importance of making turbidimetric growth measurements as soon as oxygen consumption ceases to be exponential is illustrated in Fig. 3 for glucose-grown cells at 37°. The experiment was performed at a single glucose concentration (4 mM) by removing flasks for growth measurement at the times indicated by the triangles. The oxygen consumption which occurs at the end of exponential growth is presumably due to the oxidation of endogenous materials and therefore not a reliable indication of the 'growth curve' of the culture. Similar relationships between oxygen consump-

Table 1. Summary of molar growth yields obtained with *Pseudomonas aeruginosa* in open flasks

Substrate	Method of measurement		
	Turbidimetric		Total organic matter
	30°	37°	30°
Glucose	77 (48)*	79 (43)	78 (34)
Sodium gluconate	75.5 (46)	74 (47)	77.5 (36)
Sodium 2-oxogluconate	66 (46)	64.5 (24)	—

* Molar growth yields are expressed as g. dry weight of organism per mole of substrate; figures in parentheses represent the total number of determinations made for each substrate.

Table 2. Summary of results from manometric growth experiments with *Pseudomonas aeruginosa*

Substrate	Growth temperature	Molar growth yield (g./mole)	Percentage of substrate oxidized*	Mean generation time (min.)	Oxygen consumption (mole/mole of substrate)†	Y_{O_2} (g. dry weight/mole of oxygen consumed)†
Glucose	30°	78.1 ± 1.5 (16)	40.6 ± 1.4 (16)	77.6 ± 6 (15)	2.44	32.0
	37°	76.2 ± 2.0 (12)	42.6 ± 1.8 (12)	68.9 ± 8 (12)	2.55	29.9
Sodium gluconate	30°	75.5 ± 1.2 (15)	40.2 ± 1.4 (15)	84.0 ± 4.4 (15)	2.21	34.2
	37°	73.1 ± 1.8 (14)	41.3 ± 1.7 (14)	77.0 ± 5 (14)	2.28	32.1
Sodium 2-oxogluconate	30°	64.6 ± 1.2 (11)	36.0 ± 1.3 (11)	90.8 ± 4 (11)	1.80	35.9
	37°	62.0 ± 2.1 (12)	36.7 ± 2.0 (12)	75.4 ± 6 (12)	1.85	33.8

* Based on the theoretical oxygen consumption for complete oxidation of substrate to CO₂ and water.

† Based on the average values for the percentage of substrate oxidized.

The figures in parentheses represent the number of estimations carried out.

tion and molar growth yield were obtained with gluconate and 2-oxogluconate, also at 4 mM at 30° and 37°. Oxygen consumption subsequent to cessation of exponential growth and the concomitant drop in molar growth yield were always more marked at 37°. The results in Table 2 are expressed in terms of molar growth yields and also as the grams dry weight of cells produced per mole of oxygen consumed (Y_{O_2}). The Y_{O_2} concept was introduced by Whitaker and Elsdon (1963) to compare yields of a number of organisms as a function of oxygen consumption under conditions where the energy source concentration was the growth-limiting factor. Of relevance to the problem here were their observations on Y_{O_2} as a measure of the efficiency of oxygen utilization during growth, e.g. they showed that when *Pseudomonas fluorescens* was

grown on benzoate or nicotinate, the Y_{O_2} values were less than those obtained when the organism was grown on equimolar mixtures of the products of ring fission, in agreement with the view that the oxygen used for ring fission does not participate in oxidative phosphorylation. The use of Y_{O_2} as a measure of the efficiency of oxygen utilization during glucose metabolism by *P. aeruginosa* will be discussed later.

To measure the percentage assimilation of glucose by the cells during growth and

Fig. 3. The relation between oxygen consumption, period of growth and molar growth yield for cultures of *P. aeruginosa* grown on 4 mM glucose at 37°. ●, oxygen consumption; ▲, molar growth yield.

Table 3. Growth of *Pseudomonas aeruginosa* on [$U^{14}C$]glucose at 30° and 37°

Radioactive fraction	30°		37°	
	A	B	A	B
Total counts (a)	1,623,000	100	1,668,000	100
Cells + supernatant (b)	919,700	56.7	931,300	55.8
Supernatant (c)	92,300	5.7	111,800	6.7
Cells (b-c)	827,400	51.0	819,500	49.1
Carbon dioxide (a-b)	703,300	43.3	736,700	44.2

Column A: counts/12.5 ml. medium/min.

Column B: percentage of total counts present.

For growth at 30°, the specific activity of glucose was 31,100 counts/min./μmole and for growth at 37°, 32,800 counts/min./μmole.

The molar growth yield (g./mole measured turbidimetrically) was 77 at 30° and 75.5 at 37°.

to determine whether there was appreciable excretion of materials into the growth medium, the organism was grown at 30° and 37° in the presence of [U-¹⁴C] glucose (Table 3). The results agree well with the data from the manometric experiments and show only small amounts of radioactive material in the culture supernatant. In view of its low amount, the nature of this material was not further investigated.

DISCUSSION

Our results indicate that when *Pseudomonas aeruginosa* is grown on limiting glucose or gluconate almost identical molar growth yields are obtained. In contrast with the observations of Campbell *et al.* (1956), the molar growth yield with limiting 2-oxo-gluconate is significantly less. A direct comparison with the work of Campbell *et al.* is not possible as their growth data were recorded (a) as a percentage of light transmission, and (b) as mg. protein per 100 ml. of medium. However, in a later paper, Warren, Ells & Campbell (1960) recorded a protein content of 57% for the organism when grown on 0.2% glucose, the concentration used in the earlier growth yield work. At 0.2% glucose, Campbell *et al.* reported a growth yield of 24 mg. protein per 100 ml. medium from which it is possible to calculate a molar growth yield of 37.8, a value approximately one half the figure reported here. Y_{glucose} values have been measured for a number of bacteria grown aerobically in simple medium and have been found to fall in the range 70–90 (e.g. Morris, 1960; Whitaker, 1962; Hadji-petrou, Gerrits, Teulings & Stouthamer, 1964). The Y_{glucose} value reported here for *P. aeruginosa* is thus within this range whereas the figure calculated from the data of Campbell *et al.* is much lower.

In considering the relative metabolic importance of the various pathways of glucose degradation, the fact that glucose and gluconate give similar growth yields indicates that they are probably metabolized by pathways which yield identical amounts of ATP and metabolic intermediates. This would happen if glucose was metabolized to 6-phosphogluconate via gluconate and if the oxidative step from glucose to gluconate was energetically wasteful to the organism. Supporting this concept is the observation that the Y_{O_2} value for gluconate is higher than that for glucose, indicating that a portion of glucose oxidation by the organism is wasteful as regards energy production. The lower molar growth yield with 2-oxogluconate could be interpreted as follows: 2-oxogluconate is phosphorylated to 2-oxo-6-phosphogluconate and then, by analogy with the system operative in *Pseudomonas fluorescens* (Frampton & Wood, 1961), reduced in a nicotinamide nucleotide-coupled reaction to 6-phosphogluconate. Biologically available energy occurs in two forms, ATP and reduced nicotinamide nucleotides, and, since 2-oxogluconate has an energy requirement to initiate its metabolism quite separate from the ATP which all three substrates require for their kinase-mediated phosphorylation, this may explain why $Y_{\text{2-oxogluconate}}$ is less than Y_{glucose} and $Y_{\text{gluconate}}$. The other possibility, that glucose and gluconate are mainly oxidized via 2-oxogluconate, could only be accepted if there was significant ATP production during the oxidation of gluconate to 2-oxogluconate. This seems unlikely since Ramakrishnan & Campbell (1955) could not detect conventional coenzyme requirements for partially purified gluconic acid dehydrogenase from *P. aeruginosa*.

This interpretation of the results may not, however, be quite as simple, since it does not take into account the presence of hexokinase and gluconic acid dehydrogenase in

extracts of glucose-grown cells. The Y_{O_2} value for 2-oxoglucuronate is higher than the corresponding gluconate value, indicating the possibility of some uncoupled oxidation with gluconate as well as with glucose.

Molar growth yield determinations were carried out with all three substrates at 30° and 37° to investigate the possibility of a greater maintenance energy requirement by the cells at the higher temperature. Since the Y values for each substrate were indistinguishable at the two temperatures we conclude that this is not the case. Senez (1962) reached a similar conclusion for *Aerobacter aerogenes* grown aerobically on glucose at various temperatures between 23° and 37°.

An interesting feature of the methodology of carrying out molar growth yield determinations in the Warburg apparatus when simultaneous measurement of oxygen consumption is required, is the long lag periods which we encountered with KOH in the centre well. Belief that this could be caused by a CO_2 deficiency led us to substitute a solution based on the CO_2 -buffer of Pardee (1949), which ensures the maintenance of a partial pressure of CO_2 in the atmosphere; the lag periods were then eliminated. This procedure would seem to be of general applicability for measurements of this type.

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