Biochem. J. (1973) **132**, 129–140
Printed in Great Britain

Chemostat Studies on the Regulation of Glucose Metabolism in *Pseudomonas aeruginosa* by Citrate

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(*Received* 27 *June* 1972)

The effect of the relative concentrations of citrate and glucose on the regulation of key enzymes of the direct oxidative, phosphorylative, Entner–Doudoroff and pentose-cycle pathways of glucose metabolism in *Pseudomonas aeruginosa* has been investigated in continuous culture under conditions of NH_4^+-limitation. For comparison isocitrate dehydrogenase and aconitase were also assayed. Measurements were made for steady-state and transient conditions and the effect of growth rate was also studied. When cells grew on 75mM-citrate the glucose concentration had to attain 6–8mM before significant induction of enzymes of glucose metabolism occurred; the specific activities increased further as the result of both raising the glucose concentration to 30mM and then subsequently lowering the citrate to 60mM and then to 45mM. The specific activities of the glucose enzymes increased immediately during the transient period between the steady states characteristic of growth on 6mM- and 8mM-glucose, the increase continuing for about two doubling times. The converse experiment of adding increasing citrate concentrations to 45mM-glucose medium revealed an immediate induction of the citrate-transport system, oxidation of citrate following the increase in citrate concentration up to 8mM. Between 8mM- and 16mM-citrate a marked repression of gluconate, glucose 6-phosphate and 6-phosphogluconate dehydrogenases and the Entner–Doudoroff enzymes occurred. Increased growth rate in citrate medium resulted in decreased specific activities of glucose 6-phosphate dehydrogenase and isocitrate dehydrogenase. Increased growth rate in citrate–glucose medium gave decreased specific activities of isocitrate dehydrogenase and aconitase whereas the activities of some of the glucose enzymes decreased initially but then increased at the highest growth rate ($0.5\,h^{-1}$), at which a marked increase in glucose utilization occurred. These observations accord with the regulation of glucose enzymes by induction with glucose or its metabolites and repression by citrate or its metabolic products.

The pathways of glucose metabolism in *Pseudomonas aeruginosa* are complex. The direct oxidation of glucose to gluconate and 2-oxogluconate was demonstrated by Stokes & Campbell (1951) and Claridge & Werkman (1953), and radio-respirometric studies by Wang *et al.* (1959) indicated the operation of the Entner–Doudoroff (1952) pathway and the pentose cycle in the approximate ratio of 70:30 in glucose-grown cells. Terminal oxidation occurs via the tricarboxylic acid cycle (Fig. 1). There is no evidence for the presence of a functional glycolytic system although the organism grows readily on organic acids and gluconeogenesis clearly occurs. The inhibition by succinate and citrate of acid production from glucose by *Pseudomonas aeruginosa* in synthetic media was observed by Liu (1952). Subsequently Hamilton & Dawes (1959) showed that diauxic growth occurred when cells grown on an organic acid as the sole carbon source were inoculated into a medium containing glucose and the organic acid. However, in contrast with the previous reports with *Aerobacter aerogenes* (Ravin, 1952; Dagley & Dawes, 1953; Davis, 1956) the organic acid was the preferentially utilized substrate; this was the first recorded example of a 'reverse' diauxie. Although the tricarboxylic acid cycle is constitutive in *P. aeruginosa*, the entry of various intermediates of the cycle into glucose-grown bacteria is mediated by inducible permeases (Stokes & Campbell, 1951; Clarke & Meadow, 1959).

Hamilton & Dawes (1960, 1961) examined three possible explanations for the reverse diauxie, namely: (1) the enzymes of glucose metabolism are constitutive but entry of glucose is mediated by a permease which is repressed by growth on organic acids; (2) some or all of the glucose-metabolizing enzymes are inducible and their formation and/or activities are repressed or inhibited by the simultaneous presence of an organic acid, but the glucose permease is constitutive; (3) both the glucose enzymes

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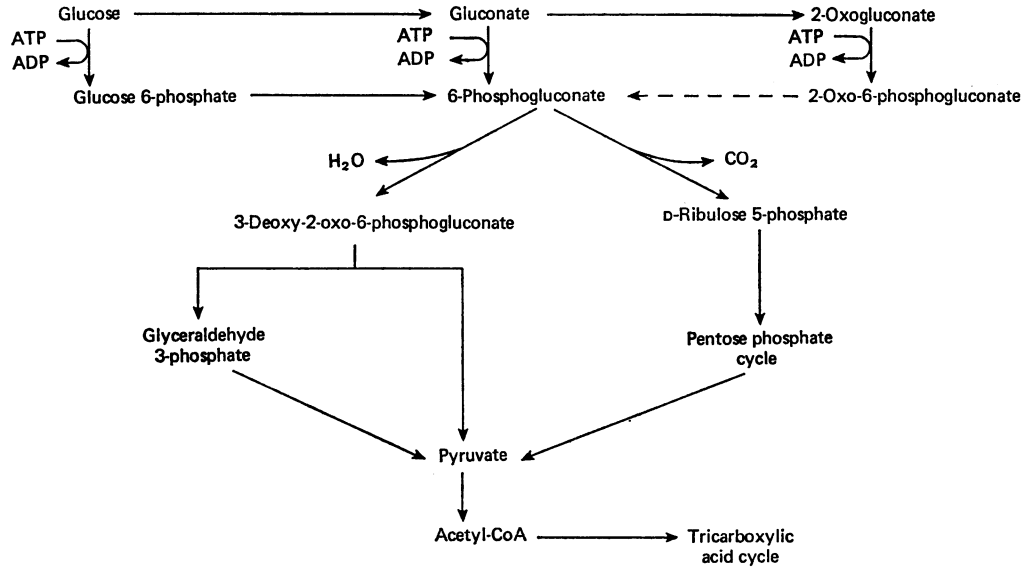

Fig. 1. *Pathways of glucose metabolism in Pseudomonas aeruginosa*

and permease are inducible. Evidence was obtained for the presence of an inducible permease for glucose and cells grown on succinate, citrate or peptone had very low activities of glucose-metabolizing enzymes; incubation of washed suspensions with glucose produced a significant increase in the activities of these enzymes, which did not occur in the presence of chloramphenicol.

Hamlin *et al.* (1967) carried out a detailed investigation of the phenomenon in batch culture but the inherent difficulties of measuring enzyme activities during the first phase of diauxic growth in such cultures, when bacterial densities are very low, led them to study, under nitrogen-limited steady-state conditions in a chemostat, the effect of several relative concentrations of citrate and glucose on various enzymes of glucose metabolism. Evidence was obtained for competitive interaction between glucose as an inducer and a repressor derived from citrate.

We have extended these original observations with nitrogen-limited citrate and glucose-grown cells to include the transient states after changes in substrate concentration and have studied the effect of growth rate to elucidate further the regulatory mechanisms that operate in *P. aeruginosa*. A preliminary report of some of these results was presented to The Biochemical Society (Ng & Dawes, 1967) and results were also provided for a review article by Clarke & Lilly (1969).

Experimental

Culture of organism and extraction

Organism. Pseudomonas aeruginosa 2F32 was originally obtained from Dr. R. W. Kinney of Iowa State College. It was maintained on nutrient–agar slants. For experiments it was fully adapted to the appropriate synthetic medium by daily subculture; for chemostat studies at least ten subcultures were used.

Growth media. For batch cultures the basal medium was prepared from the two following solutions of the compositions shown (g/litre). Solution A: KH_2PO_4, 9; $(NH_4)_2SO_4$, 2; nitrilotriacetic acid, 0.478; trace elements solution 3 (see below), 0.17ml; pH adjusted to 7.1 with 5M-NaOH. Solution B: carbon and energy source at 2.5 times the final concentration required; 12.5ml of each of trace elements solutions 1 and 2 (see below). The solutions were autoclaved separately at 15lb/in^2 (103186N·m^{-2}) for 20min and, after cooling, the sterile trace elements solutions were added and solutions A and B combined (3:2, v/v).

The trace elements solutions were of the following compositions (g/litre). Solution 1: $CaCO_3$, 2; ZnO, 0.406; $FeCl_3,6H_2O$, 5.4; $MnCl_2,4H_2O$, 0.99; $CuCl_2,2H_2O$, 0.170; $CoCl_2,6H_2O$, 0.238; H_3BO_4, 0.062; HCl, 0.155M. Solution 2: MgO, 10; HCl, 0.524M. Solution 3: $Na_2MoO_4,2H_2O$, 24.1 (D. W. Tempest, personal communication). They were sterilized by filtration.

For chemostat work medium was prepared in 20-litre batches in calibrated aspirators from five solutions, which were autoclaved separately and mixed aseptically on cooling. The solutions were of the following composition. Solution A contained (g/17 litres): KH_2PO_4, 108; $(NH_4)_2SO_4$, 52.8; nitrilotriacetic acid, 5.73; trace elements solution 3, 2ml; pH adjusted to 7.1 with 5M-NaOH. Solution B contained, in 1.5 litres: trisodium citrate to required concentration; trace elements solution 1, 100ml. Solution C contained (g/0.5 litre): $(NH_4)_2SO_4$, 52.8. Solution D: trace elements solution 2, 103ml. Solution E: glucose of the appropriate concentration, when required. After the solutions were mixed, the volume was made to 20 litres with sterile water. The required concentrations of citrate and glucose were obtained by appropriate adjustments to solutions B and E. The nitrogen source (NH_4^+) was the growth-limiting factor in all the chemostat media used.

Chemostat. The chemostat was of the 0.5 litre Porton pattern (Herbert *et al.*, 1965) fitted with automatic pH and temperature control and, in certain experiments, with a Mackereth (1964) oxygen electrode. Foaming was prevented by metered additions of antifoam (Polyglycol P-2000; Dow Chemical Co., London, W.1, U.K.). To prevent backgrowth the medium line adjacent to the chemostat was jacketed and water at 60°C circulated continuously. The culture vessel was heated externally by an infrared lamp and the temperature controlled to 37°C.

Sampling. Samples were withdrawn from the chemostat vessel into Universal bottles, which were not cooled since the organism lyses rapidly when chilled to 0°C. The bacteria were centrifuged at 25–37°C at 23000*g* for 5min, washed twice with the appropriate buffer solution and resuspended in buffer.

Manometric experiments. Bacteria were harvested, washed and resuspended in buffer solution as described for chemostat sampling. The complete operation took 45–60min and by maintaining the temperature between 25° and 37°C the viability of the cells was normally not less than 93±5%. Warburg flasks contained 2ml of washed suspension (4–6mg dry wt./ml) in 67mM-KH_2PO_4, pH7.1, supplemented with 0.1% (w/v) $MgSO_4,7H_2O$, and 0.3ml of chloramphenicol solution (1.5mg/ml) in the main compartment; 0.2ml of 20% (w/v) KOH was in the centre well and 0.5ml of 200mM-glucose or citrate in the side arm. Water replaced the substrate in the endogenous controls.

Bacterial densities. Bacterial densities were determined turbidimetrically in a Unicam SP.600 spectrophotometer at 570nm; extinctions were converted into bacterial densities from curves relating bacterial dry weight (after drying to constant weight at 105°C) to extinction.

Viability determinations. Bacterial viability was measured by plate counts and by the slide-culture technique of Postgate *et al.* (1961).

Preparation of bacterial extracts. A variety of methods of cell disruption was investigated to secure the most efficient technique. The following standardized method was adopted and always strictly adhered to. Bacteria were harvested by centrifuging as described above, washed twice with 67mM-potassium phosphate buffer, pH7.1, and finally with 120mM-glycylglycine buffer, pH7.1. The whole operation was conducted at temperatures between 25° and 37°C to avoid cold shock lysis of the cells. The cell pellet was resuspended in 60mM-glycylglycine buffer, pH7.1, to give a bacterial concentration of approx. 0.2g wet wt. of packed cells/ml of buffer. This suspension was then cooled in ice before disruption with a MSE Ultrasonic Disintegrator (60W model) operated at 18–20kHz and 1.0–1.5A for 2min. Each portion (10ml) of suspension was treated for three 40s periods separated by 2min intervals; the vessel was surrounded by crushed ice and the temperature not allowed to exceed 15°C. The resulting suspension was centrifuged at 6600*g* for 15min at 0°–2°C and the supernatant, after careful removal to another tube by Pasteur pipette, was centrifuged again under the same conditions. The resulting cell extract, which usually contained approx. 20mg of protein/ml, was used for enzyme assays. Such crude extracts could be stored at −18°C for at least 46h without significant loss of activity but were never held for more than 6h at 4°C.

Enzyme assays

All enzymes were assayed at 37°C under conditions of optimum pH, substrate and cofactor concentration. Assays involving measurements of E_{340} were

Table 1. *Effect of dilution rate on RNA and nitrogen content of P. aeruginosa extracts*

	Concn. in bacterial extract (mg/ml)				
Dilution rate (h^{-1}) ...	0.125	0.170	0.200	0.250	0.500
Total N	2.70	2.77	2.81	2.75	2.84
RNA	1.60	1.96	2.16	2.43	2.76
RNA N	0.26	0.31	0.35	0.38	0.44
RNA N/total N (%)	9.6	11.2	12.5	13.8	15.5

made with a Unicam SP.800 recording spectrophotometer. All specific activities are recorded as μmol of substrate converted/h per mg of N and the values are corrected for the N content of glycylglycine buffer and for RNA N present in the extract (Table 1). The reproducibility of all the assays was within $\pm 8\%$. E_{340} for NADPH was taken as $6.22 \times 10^3 l \cdot mol^{-1} \cdot cm^{-1}$ (Horecker & Kornberg, 1948).

ATP-D-hexose 6-phosphotransferase (*hexokinase*) (*EC* 2.7.1.1). Silica-glass cuvettes contained: 120mM-Tris buffer, pH8.2, 1.0ml; 500mM-D-glucose, 0.4ml; 250mM-$MgCl_2$, 0.12ml; 36mM-ATP, 0.8ml; 10mM-NADP, 0.3ml; glucose 6-phosphate dehydrogenase (15 units/ml), 0.2ml; water, 0.08ml; cell extract, 0.1ml. The increase in E_{340} was measured.

ATP-D-gluconate 6-phosphotransferase (*gluconokinase*) (*EC* 2.7.1.12). Silica-glass cuvettes contained: 120mM-Tris buffer, pH7.6, 1.0ml; 50mM-sodium gluconate, 0.5ml; 250mM-$MgCl_2$, 0.12ml; 36mM-ATP, 0.8ml; 10mM-NADP, 0.3ml; 6-phosphogluconate dehydrogenase (2mg/ml), 0.08ml; water, 0.1ml; cell extract, 0.1ml. The increase in E_{340} was measured.

Glucose 6-phosphate dehydrogenase (*EC* 1.1.1.49). Silica-glass cuvettes contained: 120mM-Tris buffer, pH8.6, 1.0ml; 20mM-glucose 6-phosphate, 0.75ml; 10mM-NADP, 0.5ml; 250mM-$MgCl_2$, 0.18ml; water, 0.47ml; cell extract, 0.1ml. The increase in E_{340} was measured.

6-Phosphogluconate dehydrogenase (*EC* 1.1.1.44). Silica-glass cuvettes contained: 120mM-tris buffer, pH7.6, 1.0ml; 10mM-6-phosphogluconate, 0.4ml; 10mM-NADP, 0.2ml; 250mM-$MgCl_2$, 0.18ml; water, 1.12ml; cell extract, 0.1ml. The increase in E_{340} was measured. There was loss of activity in this assay system unless the cell extract contained at least 6mg of protein/ml.

Isocitrate dehydrogenase (*EC* 1.1.1.42). Silica-glass cuvettes contained: 120mM-glycylglycine buffer, pH8.8, 1.0ml; 10mM-DL-isocitrate, 0.6ml; 10mM-NADP, 0.3ml; 250mM-$MgCl_2$, 0.2ml; water, 0.8ml; cell extract, 0.1ml. The increase in E_{340} was measured.

Glucose dehydrogenase (*EC* 1.1.1.47). Silica-glass cuvettes contained: 100mM-veronal-acetate buffer, pH6.6, 1.0ml; 75mM-D-glucose, 0.55ml; 15mM-KCN, 0.1ml; dichlorophenolindophenol (0.5mg/ml), 0.2ml; water, 1.05ml; cell extract, 0.1ml. The decrease in E_{600} was measured. E_{600} for dichlorophenolindophenol at pH6.6 was determined as $19.5 \times 10^3 l \cdot mol^{-1} \cdot cm^{-1}$.

Gluconate dehydrogenase (*EC* 1.1.99.3). Silica-glass cuvettes contained: 100mM-sodium acetate buffer, pH5.5, 0.6ml; 75mM-sodium gluconate, 0.25ml; 15mM-KCN, 0.1ml; dichlorophenolindophenol (0.5mg/ml), 0.5ml; water, 1.45ml; cell extract, 0.1ml. The decrease in E_{576} was measured. E_{576} for dichlorophenolindophenol at pH5.5 was determined as $9.1 \times 10^3 l \cdot mol^{-1} \cdot cm^{-1}$.

Table 2. *Effect of relative citrate:glucose concentrations in inflowing medium on steady-state enzymic activities of citrate-grown cells*

The dilution rate was $0.25 h^{-1}$. The values recorded are for the steady states established at each substrate concentration ratio.

Citrate:glucose concentration ratio in medium (mM)	Specific activity (μmol/h per mg of N)								
	Glucose 6-phosphate dehydrogenase	Hexokinase	6-Phosphogluconate dehydrogenase	Gluconokinase	Entner–Doudoroff enzymes	Glucose dehydrogenase	Gluconate dehydrogenase	Isocitrate dehydrogenase	Aconitase
75:0	17.5	8.5	0.6	—	2.0	1.2	9.7	302.3	26.0
75:0.5	16.5	13.1	0.4	—	3.8	1.7	16.0	269.4	30.0
75:1	14.7	9.4	0.6	—	4.3	0.9	18.7	295.5	33.2
75:2	19.7	16.0	0.6	—	5.2	1.9	23.6	274.6	31.5
75:4	22.4	15.4	2.7	0.1	5.4	1.6	30.7	290.6	27.2
75:6	30.3	19.5	3.9	0.3	7.6	2.2	41.1	358.2	40.9
75:8	73.6	29.8	18.6	1.0	16.8	2.7	38.9	332.9	35.8
75:10	83.1	35.0	20.1	4.2	17.4	2.4	64.5	325.8	36.0
75:15	94.2	37.7	20.6	3.8	21.8	2.5	74.9	352.6	40.4
75:20	98.7	37.4	24.5	4.1	28.8	2.9	78.1	357.9	43.3
75:30	101.4	39.2	24.7	6.7	30.3	3.1	86.9	338.2	44.0
60:30	116.5	44.3	28.1	4.9	32.7	3.5	78.8	276.2	43.3
45:30	147.6	45.5	30.1	5.2	34.1	11.1	83.1	277.4	43.8

6-Phosphogluconate dehydratase (*EC* 4.2.1.12) *and 3-deoxy-2-oxo-6-phosphogluconate aldolase* (*EC* 4.1.2.14) (*Entner–Doudoroff enzymes*). These enzymes were assayed together because 3-deoxy-2-oxo-6-phosphogluconate was not available as a substrate. Test tubes (150mm×22mm) contained: 120mM-glycylglycine buffer, pH7.8, 2.0ml; 1M-hydrazine, pH7.4, 0.5ml; 200mM-sodium arsenite, 0.2ml; 50mM-sodium phosphogluconate, 0.6ml; water, 1.2ml; cell extract, 0.5ml. The reactants, minus cell extract, were incubated at 37°C for 10min before the reaction was initiated with cell extract. Samples (1ml) were withdrawn at 0, 3, 6 and 10min into 5ml centrifuge tubes and the reaction was halted with 0.3ml of 40% trichloroacetic acid. The precipitated protein was removed by centrifuging at 4000*g* for 5min and the supernatants were assayed for pyruvate.

Aconitate hydratase (*aconitase*) (*EC* 4.2.1.3). Silica-glass cuvettes contained: 60mM-trisodium citrate–50mM-KH_2PO_4 buffer, pH7.6, 2.9ml; cell extract, 0.1ml. The increase in E_{240} was measured. E_{240} for *cis*-aconitate was taken as $3.43\times10^3 l\cdot mol^{-1}\cdot cm^{-1}$.

NADPH oxidase (*EC* 1.6.99.1). Silica-glass cuvettes contained: 120mM-tris buffer, at each pH value used for all the assays which employ NADP, 1.0ml; 1mM-NADPH, 0.4ml; 250mM-$MgCl_2$, 0.18ml; water, 1.32ml; cell extract, 0.1ml. The decrease in E_{340} was measured and any activity noted was used to correct the appropriate assays that employ NADP reduction.

Chemical analyses

Glucose was determined by the method of Nelson (1944), pyruvate by the toluene-extraction technique of Friedemann & Haugen (1943), citrate by the method of McArdle (1955) and P_i by the method of Fiske & SubbaRow (1925). Ammonia was determined by nesslerization and protein by the Stickland (1951) method. Total N was by the micro-Kjeldahl technique. RNA was determined by the method of Fleck & Munro (1962).

Materials

Chemicals. Glucose 6-phosphate (potassium salt), 6-phosphogluconate (sodium salt), ATP, NADP (sodium salt), 2,6-dichlorophenolindophenol and crystalline bovine albumin were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Isocitric acid (DL-, trisodium salt) was obtained from Sigma (London) Chemical Co. (London, S.W.6, U.K.) and *cis*-aconitic acid (A grade) from California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). Chloramphenicol was a gift from Parke, Davis and Co. (Hounslow, Middx., U.K.).

All other chemicals were of analytical grade.

Enzymes. Glucose 6-phosphate dehydrogenase was obtained from BDH Chemicals Ltd. and 6-phosphogluconate dehydrogenase from Boehringer und Soehne (Mannheim, Germany).

Results

Enzymes representative of the direct oxidative, phosphorylative, Entner–Doudoroff and pentose phosphate pathways were selected for assay. For comparative purposes two enzymes of the tricarboxylic acid cycle, isocitrate dehydrogenase and aconitase, were also assayed.

Table 3. *Steady-state utilization of glucose and citrate in the chemostat by citrate-grown cells*

Citrate:glucose concentration ratio in inflowing medium (mM)	Glucose		Citrate	
	Residual (mM)	Utilized (mmol/h per g dry wt.)	Residual (mM)	Utilized (mmol/h per g dry wt.)
75:0	0	0	26.7	7.547
75:0.5	0.4	0.015	25.8	7.703
75:1	0.8	0.031	27.4	7.437
75:2	1.7	0.047	27.6	7.406
75:4	3.7	0.047	27.0	7.500
75:6	4.1	0.297	28.5	7.265
75:8	4.3	0.578	28.7	7.234
75:10	5.3	0.734	29.4	7.125
75:15	9.5	0.859	30.0	7.344
75:20	13.2	1.062	30.6	6.938
75:30	22.7	1.140	25.3	7.765
60:30	19.0	1.718	22.1	4.359
45:30	8.0	3.437	11.4	2.906

Table 4. *Changes in enzymic specific activities during the transient period in the chemostat after increase of glucose concentration from 6 mM to 8 mM in the presence of 75 mM-citrate*

The dilution rate was 0.25 h^{-1}. The specific activities were measured at intervals after the glucose concentration of the inflowing medium was changed from 6 mM to 8 mM.

Time after change (h)	Specific activity (μmol/h per mg of N)								
	Glucose 6-phosphate dehydrogenase	Hexokinase	6-Phosphogluconate dehydrogenase	Gluconokinase	Entner–Doudoroff enzymes	Glucose dehydrogenase	Gluconate dehydrogenase	Isocitrate dehydrogenase	Aconitase
0	34.4	18.6	4.8	1.0	8.1	2.7	32.5	361.4	34.7
2	27.1	15.1	6.6	—	10.0	2.5	31.6	223.1	29.0
4	61.2	24.2	13.6	2.0	12.8	3.8	40.7	292.5	37.9
7	74.3	—	17.2	—	16.0	3.5	43.7	309.4	39.2
9	70.0	30.0	20.8	4.0	—	3.0	41.3	—	—
13	62.1	26.3	20.6	2.4	16.8	2.4	36.1	294.1	30.4
24	68.2	29.8	19.0	1.4	14.8	2.2	45.6	335.4	30.0

Table 5. *Effect of relative glucose:citrate concentrations in inflowing medium on the steady-state enzymic specific activities of glucose-grown cells*

The dilution rate was 0.25 h^{-1}. The values recorded are for the steady states established at each concentration ratio.

Glucose:citrate concentration ratio in medium (mM)	Specific activity (μmol/h per mg of N)								
	Glucose 6-phosphate dehydrogenase	Hexokinase	6-Phosphogluconate dehydrogenase	Gluconokinase	Entner–Doudoroff enzymes	Glucose dehydrogenase	Gluconate dehydrogenase	Isocitrate dehydrogenase	Aconitase
45:0	110.1	22.4	19.8	4.7	27.9	17.7	292.6	143.7	26.9
45:4	107.0	21.8	19.5	3.4	22.9	11.2	312.3	147.0	26.3
45:8	119.6	21.7	21.9	4.1	28.1	14.0	301.9	176.7	31.4
45:16	72.9	19.9	13.0	4.4	13.9	14.0	211.2	181.0	26.1
45:32	76.9	16.6	11.2	3.6	8.7	11.2	198.7	176.6	24.5
45:64	40.1	12.5	2.2	3.8	3.0	4.4	46.3	180.7	26.5
0:64	5.8	3.2	1.0	1.9	0.6	4.5	13.9	174.0	26.8

Effect of increasing glucose concentration on the steady-state specific activities of citrate-grown cells

The effect on the specific activities of selected enzymes of glucose metabolism of increasing the concentration of glucose in a medium providing 75mM-citrate, with NH_4^+ as the growth-limiting nutrient, was investigated. The results recorded (Table 2) refer to the steady-state values attained at each concentration ratio.

The glucose enzymes displayed relatively low specific activities in cells grown on citrate alone and there was little significant increase until the inflowing glucose concentration was raised to 6–8mM. Clearly a marked inductive effect of glucose was manifest between these concentrations and, although the glucose enzyme specific activities increased further as the glucose concentration was raised, the subsequent increases were less pronounced. When the glucose concentration had been raised to 30mM the citrate concentration was dropped from 75 to 60mM and then to 45mM (Table 2), which resulted in further increases in specific activities of the glucose enzymes. The utilization of glucose by the culture reflected the changes in specific activity observed (Table 3).

Changes in specific activities of glucose enzymes during transient periods

The specific activities of the enzymes were measured for the transient periods after these step-changes in glucose concentration (mM): 4–6, 6–8, 8–10, 10–15, 15–20 and 20–30. As the most marked differences occurred during the change from 6mM- to 8mM-glucose only the results for that transition period are recorded (Table 4). Key increases in the specific activities of glucose 6-phosphate, gluconate and 6-phosphogluconate dehydrogenases and the Entner–Doudoroff enzymes were observed; the responses were immediate and the new values were attained within 7–9h.

Effect of increasing citrate concentration on the steady-state specific activities of glucose-grown cells

The converse experiment of adding increasing concentrations of citrate to cells growing in the chemostat on 45mM-glucose is recorded in Table 5. Between 8mM- and 16mM-citrate a marked repression of glucose 6-phosphate, 6-phosphogluconate and gluconate dehydrogenases and the Entner–Doudoroff enzymes occurred and this effect increased as the citrate concentration was raised to 64mM. Finally glucose was withdrawn from the medium and the specific activities fell further. These changes in specific activity were again reflected by the glucose utilized by the culture (Table 6). As glucose-grown cells carry out terminal oxidation via the tricarboxylic acid cycle, the increase in citrate utilization which occurred as the citrate concentration in the medium was increased is a consequence of the induction of the citrate permease. Rates of oxidation of glucose and citrate were measured with bacteria taken from each steady state (Fig. 2) and these also illustrate the induction of the citrate permease.

Effect of growth rate on enzymic specific activities

Citrate-grown cells. The specific activities of citrate-grown cells were measured as a function of the growth rate in the chemostat (Table 7). Since the citrate utilized varied with growth rate, to ensure that the residual citrate concentration in the chemostat vessel remained reasonably constant at 27–30mM under all conditions, the citrate concentration in the inflowing medium was adjusted for each dilution rate

Table 6. *Steady-state utilization of glucose and citrate in the chemostat by glucose-grown cells*

The values refer to the experiment of Table 5. The dilution rate was $0.25\,h^{-1}$.

Glucose:citrate concentration ratio in inflowing medium (mM)	Glucose		Citrate	
	Residual (mM)	Utilized (mmol/h per g dry wt.)	Residual (mM)	Utilized (mmol/h per g dry wt.)
45:0	12.3	5.109	0	0
45:2	10.1	5.453	0.8	0.188
45:4	10.7	5.359	1.5	0.391
45:8	11.9	5.172	2.8	0.812
45:16	17.6	4.281	4.0	1.875
45:32	24.7	3.172	9.6	3.500
45:64	32.3	1.984	34.0	4.688
0:64	0	0	15.7	7.547

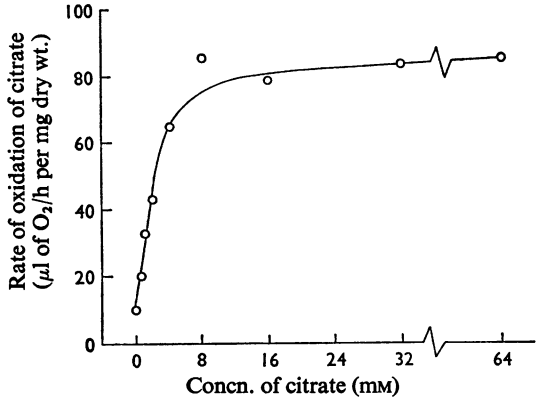

Fig. 2. *Effect of increasing the citrate concentration in glucose medium on the induction of citrate permease*

The glucose concentration in the inflowing medium was 45mM and the rate of glucose oxidation was 212–260μl/h per mg dry wt. throughout. Chloramphenicol was present in the Warburg vessels.

(see Table 8). The specific activity of glucose 6-phosphate dehydrogenase decreased as the growth rate decreased and the same was true of isocitrate dehydrogenase and aconitase.

Citrate–glucose-grown cells. The effect of growth rate was also studied with cells growing in the presence of both substrates. The concentration of inflowing glucose was 4mM at all dilution rates but the inflowing citrate concentration was adjusted (Table 8) to ensure a residual citrate concentration of 27–30mM in the chemostat vessel under all conditions. Table 9 reveals that the specific activities of isocitrate dehydrogenase and aconitase fell steadily as the growth rate increased whereas those of some of the glucose enzymes fell initially but then rose at the highest dilution rate ($0.5\,h^{-1}$), which is reflected by the glucose utilization (Table 8). The analytical data for this experiment are summarized in Table 8 and the very marked increase in the rate of glucose utilization when the dilution rate was increased from 0.25 to $0.50\,h^{-1}$ is noteworthy.

A comparison is made in Table 10 of the effect of dilution rate on the rates of oxidation of citrate and glucose by cells grown on citrate or on citrate–glucose from the experiments of Tables 7 and 9. With the exception of glucose oxidation by cells grown on the mixed substrates, the rates of oxidation of citrate and glucose decreased with increasing dilution rate. The rates of glucose oxidation for both types of cell were considerably lower than for glucose-grown cells to which an increasing concentration of citrate was added (Fig. 2).

Table 7. *Effect of dilution rate on enzymic specific activities of citrate-grown cells*

The values recorded are for the steady states established at each dilution rate. For comparison the specific activities recorded in batch culture (Hamlin *et al.*, 1967) with citrate-, glucose- and peptone-grown cells are appended.

	Specific activity (μmol/h per mg of N)								
Dilution rate (h^{-1})	Glucose 6-phosphate dehydrogenase	Hexokinase	6-Phosphogluconate dehydrogenase	Gluconokinase	Entner–Doudoroff enzymes	Glucose dehydrogenase	Gluconate dehydrogenase	Isocitrate dehydrogenase	Aconitase
0.125	27.3	13.8	0.7	4.3	0.7	0.6	4.7	400.7	41.4
0.170	22.5	12.0	0.8	2.8	1.6	0.3	3.5	392.6	41.2
0.200	19.8	12.1	0.9	2.7	1.8	0.7	3.8	363.7	43.5
0.250	20.1	9.9	0.7	—	1.5	0.9	7.4	346.3	29.8
0.500	12.5	11.2	0.7	3.0	1.2	1.1	4.0	381.5	27.8
Batch culture									
Citrate	4.4	27.5	1.3	—	4.4	1.3	3.2	240.0	46.3
Glucose	126.3	38.1	31.3	—	30.0	4.4	8.2	228.8	23.8
Peptone	15.0	10.0	3.8	—	9.4	4.4	6.3	128.1	11.9

Table 8. *Effect of dilution rate on substrate utilization of cells grown in citrate–glucose media*

Nitrogen was the growth-limiting factor at all dilution rates.

Dilution rate (h^{-1})	0.125	0.170	0.200	0.250	0.500
Citrate:glucose molar concn. ratio, inflowing	91:4	86:4	82:4	75:4	69:4
NH_4^+ inflowing (mM)	20	20	20	20	20
NH_4^+ utilized (mmol/h per g dry wt.)	1.190	1.659	2.100	3.124	3.875
Citrate, residual (mM)	27–30	27–30	27–30	27–30	27–30
Citrate utilized (mmol/h per g dry wt.)	3.631–3.810	4.644–4.892	5.474–5.790	6.563–7.500	12.58–13.52
Glucose, residual (mM)	3.6	3.4	3.1	3.1	0.2
Glucose utilized (mmol/h per g dry wt.)	0.024	0.050	0.095	0.141	1.226
Bacterial dry wt. (mg/ml)	2.0–2.2	1.8–2.3	1.6–2.2	1.5–1.7	1.5–1.6
Bacterial protein (mg/ml)	1.10–1.17	1.12–1.20	1.08–0.96	1.06–0.95	1.02–0.98
Protein (fraction of dry wt.)	0.54	0.55	0.55	0.63	0.65

Discussion

The limitations of batch culture are particularly apparent when it is necessary to compare the effect of the relative concentrations of two carbon and energy sources on the enzymic activity and composition of a bacterial cell; under such growth conditions the continually changing environment introduces variables which cannot be adequately controlled. We have therefore applied the continuous cultivation technique to investigate the effect of the relative concentrations of citrate and glucose on the enzymes of glucose metabolism in *P. aeruginosa* under both steady-state and transient conditions. Nitrogen-limitation was chosen to examine the competitive effects of the two carbon sources since catabolite repression is accentuated under these conditions.

When the bacteria grew with citrate (75mM) as the carbon source and glucose was introduced, a threshold concentration (6–8mM) of glucose had to be attained before substantial increases in the specific activities of glucose enzymes occurred (Table 2). Investigation of the transient period between the steady states on 6mM- and 8mM-glucose revealed that the increases were immediate and continued for approximately two doubling times (Table 4).

The converse experiment of increasing the citrate concentration in glucose medium (45mM) showed a rapid induction of the citrate-transport system and the maximum response was evoked by 8mM-citrate (Fig. 2). Above this concentration of citrate repression of the glucose enzymes occurred (Table 5), the magnitude of repression increasing with increasing citrate concentration; the specific activities fell further when glucose was completely withdrawn from the medium.

These experiments with varying ratios of citrate and glucose (Tables 2 and 5) clearly demonstrate that the specific activities of glucose enzymes can be increased either by increasing the glucose concentration or decreasing the citrate concentration in the medium. Such observations accord with regulation of the glucose enzymes by induction with glucose or its metabolites and repression by citrate or its metabolic products. Similar competition between inducer and repressor has been demonstrated for several inducible enzymes such as the amidase of *P. aeruginosa* (Clarke & Brammar, 1964) and the mandelate pathway of *Pseudomonas fluorescens* (Stanier *et al.*, 1965; Mandelstam & Jakoby, 1965; Stevenson & Mandelstam, 1965).

The observation of a threshold inducing concentration for glucose suggested the induction of the glucose-transport system, evidence for the existence of which was earlier adduced by Hamilton & Dawes (1960). Since permeation can be a major regulatory process, the nature of carbohydrate transport by *P. aeruginosa* 2F32 has been investigated in detail and two distinct components identified, one of which transported glucose and methyl α-glucoside whereas the second was complex, involving the extracellular activity of glucose dehydrogenase (Midgley, 1972; Midgley & Dawes, 1973). The methyl α-glucoside–glucose-transport system was repressed when the organism was grown in the absence of glucose and the induction of the system (and its activity, once induced) were inhibited by products of citrate metabolism when citrate-grown cells were exposed to glucose.

A comparison of the specific activities of the glucose enzymes reveals that glucose 6-phosphate and gluconate dehydrogenases display the highest activities under the conditions investigated and that in

Table 9. *Effect of dilution rate on enzymic activities of citrate–glucose-grown cells*

The inflowing glucose concentration was 4 mM at all dilution rates but the citrate concentration in the inflowing medium was adjusted to secure a residual concentration in the chemostat vessel of 27–30 mM (see Table 8) under all conditions. The values recorded are for the steady states established at each dilution rate.

Dilution rate (h^{-1})	Specific activity (μmol/h per mg of N)									
	Glucose 6-phosphate dehydrogenase	Hexokinase	6-Phosphogluconate dehydrogenase	Gluconokinase	Entner–Doudoroff enzymes	Glucose dehydrogenase	Gluconate dehydrogenase	Isocitrate dehydrogenase	Aconitase
0.125	35.6	14.2	9.3	3.7	3.6	0.7	21.0	449.2	79.0
0.170	33.5	15.2	9.5	3.0	4.3	2.0	30.6	451.7	65.5
0.200	27.4	19.3	2.6	1.5	5.8	4.0	32.0	378.8	51.9
0.250	24.9	17.1	3.0	0.2	6.0	1.8	34.1	322.7	30.0
0.500	62.0	25.0	9.1	2.9	5.4	4.3	37.4	321.0	21.6

glucose-grown cells the gluconate dehydrogenase specific activity is almost threefold that of glucose 6-phosphate dehydrogenase. Lessie & Neidhardt (1967) purified 50-fold glucose 6-phosphate dehydrogenase from *P. aeruginosa* ATCC 7700 and showed it was allosterically controlled by ATP, which binds to at least two sites. They suggested that the regulation of glucose 6-phosphate dehydrogenase activity is necessary to attain the most efficient pattern of hexose phosphate consumption in this organism. Lessie & Neidhardt (1967) also briefly commented that both glucose- and succinate-grown cells possessed low activities for the direct oxidation of glucose, which, they suggested, could account for only about 10% of the total glucose utilized. In contrast, we have found that chemostat-grown glucose cells have significantly higher glucose dehydrogenase activities than citrate-grown cells. Further, at high glucose concentrations, since glucose dehydrogenase acts extracellularly, about ten times more glucose is oxidized to gluconate than is transported into the cell (Midgley & Dawes, 1973). The fact that there is little significant difference between the molar growth yields of *P. aeruginosa* on glucose and gluconate (MacKechnie & Dawes, 1969) lends some support to this belief, since the oxidation of glucose to gluconate does not yield energy to the organism whereas the oxidation of glucose 6-phosphate to 6-phosphogluconate does so. The lower activities reported by Lessie & Neidhardt (1967) in comparison with our own may well be a reflection of the higher centrifugal field they used in preparing cell extracts, since glucose dehydrogenase is a membrane-bound enzyme.

The activities of glucose enzymes in chemostat-grown citrate cells were in general lower than Hamlin *et al.* (1967) recorded for batch culture; the highest activity was observed with glucose 6-phosphate dehydrogenase and this decreased as the growth rate was increased, suggesting an increased concentration of repressor in faster-growing cells (Table 7). However, when both citrate and glucose were present in the medium (Table 9) the specific activities of glucose 6-phosphate dehydrogenase, hexokinase and gluconate dehydrogenase all increased significantly at faster growth rates, indicating that induction or derepression occurred in the presence of glucose, although for glucose 6-phosphate dehydrogenase this was true only at the highest growth rate ($0.5\,h^{-1}$).

The behaviour of isocitrate dehydrogenase and aconitase, which are required for both the utilization of citrate and the terminal oxidation of glucose, is of interest. The specific activities of both enzymes increased when the glucose concentration was raised against a constant citrate concentration, aconitase showing the greatest effect. With citrate as the sole carbon source (Table 7) aconitase was repressed as the growth rate increased; with citrate plus glucose

Table 10. *Effect of dilution rate on metabolism of citrate and glucose by cells grown on citrate or on citrate–glucose medium*

In all experiments with citrate–glucose media the residual citrate in the chemostat was held at 27–30mM; glucose was added at 4mM (see Table 8). Chloramphenicol was present during manometric determinations of rates of oxygen consumption.

Dilution rate (h^{-1})	Rate of oxygen uptake (μl/h per mg dry wt.)			
	Citrate-grown		Citrate–glucose-grown	
	Citrate	Glucose	Citrate	Glucose
0.170	188	70	153	103
0.200	153	84	147	135
0.250	151	45	139	152
0.500	117	48	119	146

(Table 9) aconitase displayed a higher specific activity at the lower growth rates but was repressed to about the same extent as on citrate at the higher rates. Isocitrate dehydrogenase was also repressed to a greater extent when the growth rate on citrate–glucose was increased. Both enzymes are clearly under regulatory control but co-ordinate repression is not apparent. The tricarboxylic acid cycle fulfils the dual role of energy generation and production of biosynthetic intermediates and when growth occurs in peptone medium the latter role is subsidiary; under these conditions isocitrate dehydrogenase and aconitase are markedly repressed (Table 7), suggesting that nitrogenous compounds may be of greater importance than glucose in the regulation of these enzymes. The aerobe *P. aeruginosa* thus resembles the facultative *Escherichia coli*, in which enzymes of the tricarboxylic acid cycle are repressed by both glucose and organic nitrogenous compounds (Gray *et al.*, 1966).

We are greatly indebted to the Medical Research Council for a grant-in-aid. F. M.-W. N. was the holder of a University of Hull Research Scholarship during part of this work. The skilled workmanship of Mr. N. Dixon in the construction of the chemostat is gratefully acknowledged.

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