

Nitrite and Ammonia Assimilation by Anaerobic Continuous Cultures of *Escherichia coli*

By J. A. COLE, K. J. COLEMAN, B. E. COMPTON,
B. M. KAVANAGH AND C. W. KEEVIL

Department of Biochemistry, University of Birmingham B15 2TT

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SUMMARY

Anaerobic continuous cultures of *Escherichia coli* strains Hfrc and K12-OR75 assimilated nitrite to produce 63.7 g dry mass/g-atom N. Washout occurred when these cultures were aerated, or when the dilution rate was above 0.05 h^{-1} . Nitrite reductase, glutamate dehydrogenase and glutamine synthetase activities and cytochrome c_{552} synthesis were derepressed when growth of strain OR75 was limited by the concentration of either nitrite or ammonia in the reservoir. Glutamate synthase activity was far less than glutamate dehydrogenase, but was highest when nitrite limited growth. These results indicate that enzymes which catalyse glutamate synthesis from inorganic nitrogen compounds are regulated in an interdependent manner, but that glutamine synthetase protein is unlikely to be the aporepressor for glutamate dehydrogenase synthesis in this organism. Possible reasons for apparently opposite regulatory mechanisms in *E. coli* and *Klebsiella aerogenes* are discussed in the context of the selective pressures which are applied to bacteria in continuous culture. We conclude that *E. coli* K12 has alternative pathways for synthesizing glutamate, and because the K_m of glutamate dehydrogenase for NH_3 is 1.6 mM, a growth-limiting concentration of an inorganic nitrogen compound in the reservoir would be assimilated predominantly by the glutamine synthetase-glutamate synthase pathway.

INTRODUCTION

Attempts in various laboratories to demonstrate growth of coliforms with nitrate as sole nitrogen source have yielded conflicting results, possibly because of variations in the media components, culture conditions and the strains used (McNall & Atkinson, 1956; Zarowny & Sanwal, 1963; Fujita & Sato, 1966; Venables & Guest, 1968). There is little doubt, however, that various strains assimilate nitrate in media supplemented with small quantities of organic nitrogen compounds.

Far less is known about the assimilation of nitrite, the toxic product of nitrate reduction. Mutants of *Escherichia coli* K12, deficient in nitrite reductase activity, have recently been isolated amongst bacteria that had survived penicillin treatment when nitrate or nitrite was the only nitrogen compound (J. A. Cole, unpublished). Because penicillin is lethal only to growing bacteria, this enrichment indicated that the wild type should be capable of assimilating nitrite. However, no colonies formed when bacteria were plated on to solid media containing nitrite, even when plates were incubated anaerobically. To develop a selective medium for isolating and characterizing new nitrite reductase mutants, we have investigated whether continuous cultures of *E. coli* can grow with nitrite as sole nitrogen source. We have compared yields of bacteria from anaerobic cultures in which growth was limited by the supply of ammonia or of nitrite in the feed and have measured the activities of enzymes

of nitrogen metabolism in extracts of bacteria grown continuously with limited or excess nitrite or ammonia.

Most micro-organisms assimilate inorganic nitrogen compounds by one or both of two pathways in which glutamate is formed from ammonia and 2-oxoglutarate. Although other pathways have been proposed, for example the amination of fumarate to aspartate by aspartate- NH_3 lyase, they are physiologically unimportant (Berberich, 1972). Some eukaryotes and many prokaryotes synthesize a glutamate dehydrogenase (GDH) that catalyses the reductive amination of 2-oxoglutarate to glutamate. The K_m for GDH is very high (1 to 5 mM), so to maintain an adequate rate of glutamate synthesis when the concentration of NH_3 is less than 1 mM, these organisms must either synthesize more GDH or utilize a more effective NH_3 -scavenging pathway. GDH activity was not detected in some *Bacillus* and *Erwinia* species (Meers, Tempest & Brown, 1970); in these bacteria, glutamate is synthesized by an alternative pathway that involves two enzymes, glutamine synthetase (GS) and glutamate synthase [glutamine (amide): 2-oxoglutarate amino transferase oxidoreductase (NADP^+); GOGAT]. The GS-GOGAT pathway is used preferentially by many prokaryotes and at least some eukaryotes when the nitrogen supply is growth-limiting (Brenchley, Prival & Magasanik, 1973; Brown, Burn & Johnson, 1973). The enzymes for both pathways have been detected in several strains of *E. coli* and the regulation of glutamine synthesis in *E. coli* w has been studied in greater detail than for any other organism, but comparatively little is known about the relative regulation of the two glutamate pathways in this species. If nitrite reduction to NH_3 is a slow, growth-limiting process, synthesis of GS and GOGAT might be derepressed even when the concentration of nitrite in the feed medium is high. This type of regulation has been observed with nitrogen-fixing cultures of *Klebsellia pneumoniae* (Nagatani, Shimizu & Valentine, 1971).

It was important to distinguish between changes in enzyme activities due to changes in the nitrogen supply, and non-specific changes in activities of unrelated pathways due to variations in cell density or catabolite repression. Growth was therefore sulphur- rather than carbon-limited when the nitrogen source was in excess (see Hill, Drozd & Postgate, 1972).

METHODS

Organisms and culture. *Escherichia coli* K12-OR75, a prototrophic Hfr isolated by R. Curtiss III, was a gift from F. B. Ward. *Escherichia coli* HfrC met-NCTC10237 was obtained from the National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen. Both organisms were maintained on nutrient agar slants at 4 °C.

Nutrient broth (2.8 l) was sterilized in a 4 l continuous fermentation vessel (L. H. Engineering Ltd, Stoke Poges, Buckinghamshire) and inoculated with 20 ml of an overnight culture grown aerobically in nutrient broth. The culture was aerated until E_{650} was above 1.0, and then gassed with $\text{N}_2:\text{CO}_2$ (95:5, v/v). The feed medium was added at 80 ml/h ($D = 0.029$). Temperature and pH were maintained at 37 °C and 7.2 ± 0.05 , respectively. The ammonium salts medium contained, per litre distilled water: 5 g KH_2PO_4 , 1 g trisodium citrate, 0.2 ml conc. HCl, 12 g glucose (sterilized separately), and 1 ml of a sulphur-free trace metal solution (prepared by mixing 8.2 g $\text{MgCl}_2 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.4 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.1 g CaCl_2 and 2 ml conc. HCl in 100 ml distilled water). The sulphur source was Na_2SO_4 at 0.1 mM for sulphur-limited cultures, and at 0.4 mM for nitrogen- or carbon-limited cultures. When nitrite was the nitrogen source, HCl was omitted from the feed medium and 6.2 g $\text{K}_2\text{HPO}_4/\text{l}$ replaced 5 g $\text{KH}_2\text{PO}_4/\text{l}$, because nitrite is unstable in acidic solution and gaseous ammonia is liberated from ammonium salts at high pH. Sterile

1 M- NaNO_2 or 1 M- NH_4Cl solutions were added to 8 l batches of sterile feed medium to give a final concentration of 6 mM for nitrogen-limited cultures (unless otherwise noted), or 20 mM for sulphur-limited cultures. No attempt was made to exclude oxygen from the feed medium or from silicone tubing connexions. The purity and E_{650} of the culture were checked with 20 ml samples taken aseptically from a sampling port.

Bacteria for enzymic analyses were taken directly from the fermentation vessel. In a typical set of experiments, the nitrogen source in the feed medium was changed sequentially from 20 mM- NaNO_2 to 20 mM- NH_4Cl , 6 mM- NH_4Cl and finally to 6 mM- NaNO_2 . After 15 l medium had passed through the fermenter, a 2 l sample was harvested. A further 15 l of the same medium was added at 80 ml/h, and a second 2 l sample was harvested. The fermenter was then refilled with the next medium in the sequence until a new steady-state resulted. The 2 l samples were cooled to 4 °C and harvested by centrifugation at 10 000 g for 10 min. The pellets were resuspended in 30 ml 10 mM- MnCl_2 + 50 mM-tris-HCl, pH 7.4, and again centrifuged at 10000 g for 10 min. The washed pellet was drained and stored at -20 °C.

Preparation of bacterial extracts. The bacterial pellet was thawed, mixed to a slurry with an equal volume of TEA buffer pH 8.0 (50 mM-tris HCl, 5 mM-ethylenediamine tetraacetate Na_2^+ , 5 mM-ascorbic acid) and broken in a Hughes (1951) press cooled to -25 °C. High speed supernatant (HSS) and membrane (M) fractions were prepared as described previously (Cole & Rittenberg, 1971) except that TEA buffer was used to prepare HSS extracts. Membranes were resuspended in 50 mM- Na^+K^+ phosphate pH 7.4, sonicated for 3×30 s, sedimented by centrifugation for 20 min at 30000 g, and resuspended in 50 mM-phosphate pH 7.4.

Enzyme assays. Glutamate dehydrogenase activity was measured spectrophotometrically by recording the rate of NADPH oxidation after adding 10 μl HSS (or 10 μl of a suitable dilution in 66 mM-tris-HCl pH 8.0) to 0.85 ml 0.18 mM-NADPH + 50 mM- NH_4Cl + 80 mM-tris-HCl pH 8.0. After 2 min, 150 μl 20 mM-2-oxoglutarate were added, and the rate of NADPH oxidation was again measured. The difference in rates with and without 2-oxoglutarate was proportional to the volume of extract added, but independent of the substrate concentrations. Where no ranges are given for spectrophotometric assays, replicates agreed to within 1 %.

To measure GOGAT activity, the rate of oxidation of 0.18 mM-NADPH by 10 to 50 μl HSS extract in 3.3 mM-glutamine and 33 mM-tris HCl pH 8.0 was determined. After 2 min, 2-oxoglutarate was added to 5.0 mM. The increase in the rate of NADPH oxidation was independent of the 2-oxoglutarate concentration, and was proportional to the volume of HSS added. Brenchley (1973) reported that GOGAT from *Klebsiella aerogenes* lost 90 % of its activity in 10 min when incubated with NADPH and glutamine in the absence of 2-oxoglutarate. It was possible, therefore, that we had seriously underestimated the GOGAT activity of our extracts. To check this, GOGAT activity was measured both by adding HSS after 2-oxoglutarate to start the reaction, and by adding 2-oxoglutarate to enzyme which had been incubated for various times with the other reagents. In a typical experiment, GOGAT from *E. coli* lost approximately 20 % of its activity after 10 min without 2-oxoglutarate; it was therefore appreciably more stable than the *K. aerogenes* enzyme. The instability of GOGAT from *E. coli* varied considerably between preparations, and in several experiments the two assay procedures gave identical results. It is possible, however, that some of the GOGAT activities reported in Fig. 3 below are up to 25 % too low. In subsequent experiments rates of NADPH oxidation in the presence or absence of glutamine were determined separately.

Glutamine synthetase activities were measured by the transferase assay in mixed imidazole buffer pH 7.15 (Shapiro & Stadtman, 1970b). The buffer constituents and the pH for this assay

were selected so that, with extracts of *E. coli* W, the rate of γ -glutamyl hydroxamate (γ -GH) formation with 0.3 mM-Mn²⁺ was proportional to the total concentration of active and inactive (adenylylated) GS subunits. With both 0.3 mM-Mn²⁺ and 60 mM-Mg²⁺, adenylylated subunits were inactive and the observed rate was proportional to the concentration of enzymically active unadenylylated subunits (Shapiro & Stadtman, 1970*b*; but see also Prival, Brenchley & Magasanik, 1973).

Reduced NADH-nitrite oxidoreductase activity (EC.1.6.6.4) was assayed with a Cary 14 spectrophotometer (Applied Physics Corp., Monrovia, California, U.S.A.), using Thunberg cuvettes that had been evacuated and filled repeatedly with 'white spot' nitrogen (British Oxygen). Reactions were started by adding 2 to 20 μ l HSS in 100 μ l TEA to 2.9 ml 250 μ M-NADH + 1 mM-NAD⁺ in TEA buffer. The difference in rates of NADH oxidation in the presence or absence of 2 mM-NaNO₂ was proportional to the volume of HSS added (Cornish-Bowden, Ward & Cole, 1973).

Protein concentrations were determined by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951), and nitrite was determined as described by Cole & Ward (1973). Cytochrome concentrations were measured by difference spectroscopy with either a Cary 14 or a Unicam SP1800 (Pye Unicam Ltd, Cambridge) spectrophotometer. Both test and reference samples (3.0 ml; 2 to 10 mg protein/ml) were reduced with a few grains of sodium dithionite, and a base-line was recorded. The reference sample was partially reoxidized by about 1 mg NaNO₂, and the difference spectrum was recorded. A fresh sample of extract was then oxidized by about 0.1 mg K₃Fe(CN)₆ and used as the reference sample for a second difference spectrum. Peak heights at 552, 559 and 628 nm were determined, and cytochrome concentrations were calculated by assuming the molar extinction coefficient $\epsilon = 24.0$ for cytochrome *c*₅₅₂ (Fujita, 1966) and 20.0 for cytochrome *b*₁. Cytochrome *d* (*a*₂) concentrations are expressed as absorbance units/mg protein.

Glutamine (grade III), NADH (grade III), NAD⁺ (grade III), NADPH, L-methionine sulphone and L-methionine-DL-sulphoximine were purchased from Sigma. All other chemicals were commercial samples of the highest available purity.

RESULTS

Yields of bacteria during nitrite assimilation

Escherichia coli strains Hfrc or OR75 maintained steady-state anaerobic growth when the nitrogen source was either nitrite or ammonia. Washout occurred when air was supplied to cultures assimilating nitrite. Bacterial yields from equivalent concentrations of nitrite and ammonia were identical, and proportional to the concentration of nitrogen in the feed (Fig. 1). Similar yields were also obtained when growth was limited by 0.1 mM-sulphate and the nitrogen was either 20 mM-nitrite or 20 mM-ammonia. No nitrite was detected in the culture supernatant under any growth condition, but the concentration of NH₃ in supernatants from sulphate-limited cultures of *E. coli* OR75 was 12.3 mM when nitrite was in excess, and 12.7 mM when ammonia was in excess. Maximum growth rates were not determined for all conditions used, but washout occurred when the dilution rate of nitrite-limited cultures of either strain was increased to 0.05.

Activities of enzymes for glutamate synthesis in E. coli OR75

Higher activities of glutamate dehydrogenase were observed when cultures were nitrogen-limited than when they were sulphur-limited (Fig. 2*a*). This apparent derepression of GDH during nitrogen starvation was greatest when nitrite was the nitrogen source. Similar

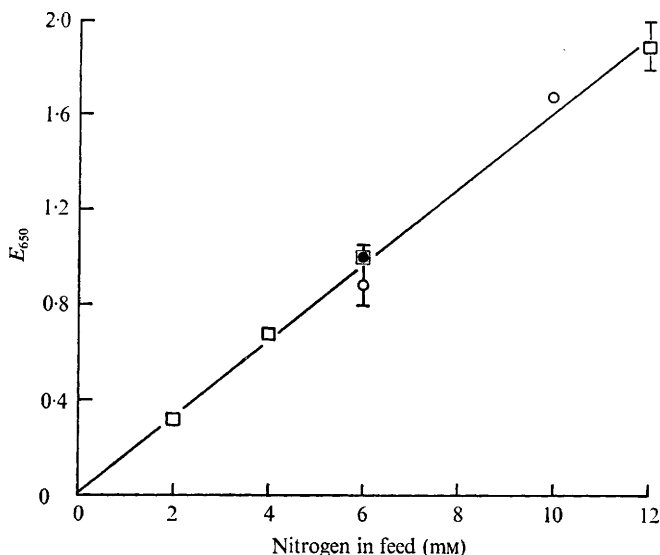


Fig. 1. Effect of the nitrogen concentration in the reservoir on bacterial yield. Growth of continuous cultures of *E. coli* strains HfrC and OR75 was limited by the concentration of NaNO_2 or NH_4Cl in the reservoir (1.2 %, w/v, glucose; $D = 0.029 \text{ h}^{-1}$). An extinction of 1.0 at 650 nm corresponds to 395 mg of bacterial dry wt/l; from the slope of the graph one can calculate that 63.7 g of bacterial dry wt are formed/g-atom N. □, Strain HfrC with limiting NaNO_2 ; ○, strain OR75 with limiting NaNO_2 ; ●, strain OR75 with limiting NH_4Cl . Bars indicate the range observed when any steady state was repeated more than three times.

variations were found in the activities of glutamine synthetase, and the highest activities were observed when extracts of nitrite-limited bacteria were assayed with 60 mM- Mg^{2+} (Fig. 2*b*; Table 1). In contrast, activities of GOGAT varied almost as much in extracts of bacteria grown in any one medium as in those grown in different media, but again highest activities were observed with nitrite-limited bacteria (Fig. 2*c*).

Enzymes for glutamate synthesis differ even in closely related organisms (Brown *et al.* 1973). It was therefore important to establish that assays which had been developed for other strains, species or genera were also valid for *E. coli* K12. The K_m for GDH from a nitrite-limited culture was determined by assaying the enzyme at various NH_4Cl concentrations, but at constant concentrations of enzyme, 2-oxoglutarate and NADPH. The K_m for ammonia was calculated to be 1.6 mM. This high value made it more difficult to eliminate the possibility that the apparent GOGAT activities, which were far lower than the GDH activities, were due to reaction of GDH with traces of NH_3 released from glutamine by glutaminase, or by a more complex reaction sequence. The effects of various concentrations of L-methionine-DL-sulphoximine and L-methionine sulphone on the activities of GDH and GOGAT were therefore measured (Fig. 3). GOGAT was 65 % inhibited by 100 mM-L-methionine-DL-sulphoximine and 76 % inhibited by 10 mM-L-methionine sulphone. GDH activity was unaffected by either compound at any concentration tested.

In many of the previous experiments, GS activity was stimulated rather than inhibited by Mg^{2+} (Table 1; see also Prival *et al.* 1973). This was not surprising, because growth with excess glucose would favour a high intracellular concentration of 2-oxoglutarate which is an inhibitor of the GS adenylylating enzyme and an activator of the deadenylylation enzyme (Shapiro & Stadtman, 1970*a*). To test whether GS activity from *E. coli* OR75 was regulated

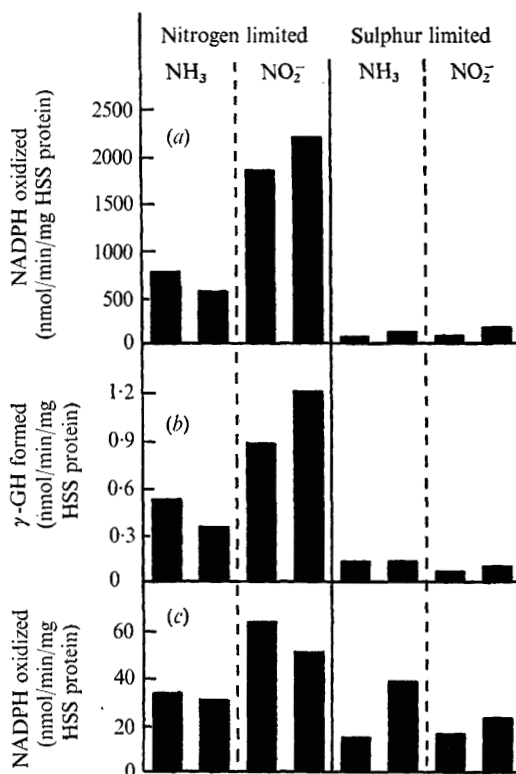


Fig. 2. (a) Glutamate dehydrogenase, (b) glutamine synthetase, and (c) glutamate synthase activities in *E. coli* OR75. Growth in anaerobic continuous cultures was limited by the concentration of NaNO₂ or NH₄Cl in the feed, or by the concentration of Na₂SO₄ when NaNO₂ or NH₄Cl were in excess. Each bar in the histogram shows the mean activity of duplicate assays for independent HSS preparations. Nitrogen-limited cultures were supplied with 6 mM-NH₄Cl or 6 mM-NaNO₂, and 0.4 mM-Na₂SO₄ as sulphur source. Concentrations of NH₄Cl and NaNO₂ were increased to 20 mM for sulphur-limited cultures, and the Na₂SO₄ concentration was reduced to 0.1 mM. $D = 0.029 \text{ h}^{-1}$.

Table 1. The effect of 60 mM-Mg²⁺ on glutamine synthetase activities in *E. coli* OR75

Each line of data is the mean activity of 4 to 9 determinations on one bacterial extract.

Nitrogen source	Limiting nutrient	Glutamine synthetase activity (nmol γ-GH formed/min/mg protein)	
		+ Mg ²⁺	- Mg ²⁺
6 mM-NH ₄ Cl	N	575	522
		450	380
6 mM-NaNO ₂	N	1960	1130
		1450	2800*
20 mM-NH ₄ Cl	S	130	130
		120	120
20 mM-NaNO ₂	S	63	63
		136	93

* This measurement was repeated 3 times (18 assay tubes) because only this enzyme preparation from six independent samples was inhibited by Mg²⁺; the datum is included because the same extract contained 'typical' activities of all other enzymes and cytochromes whose values are presented in other Figures or Tables.

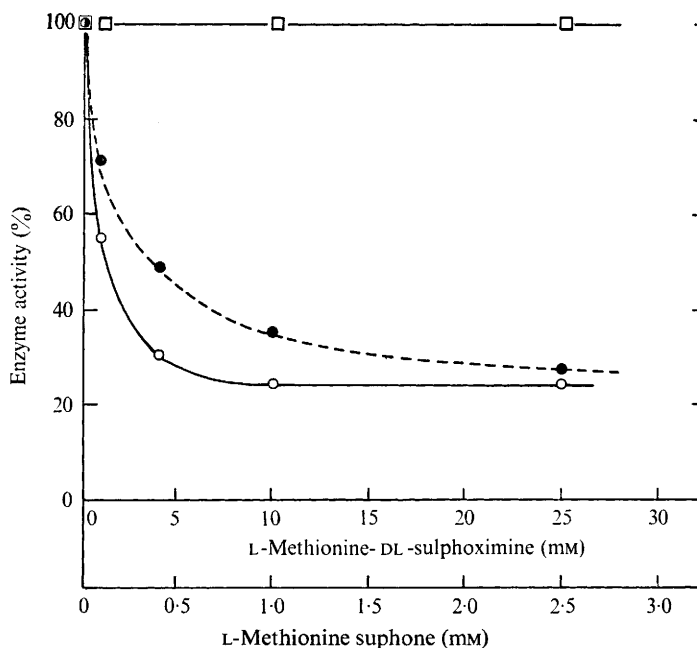


Fig. 3. Effect of L-methionine-DL-sulphoximine and L-methionine sulphone on the activities of glutamate synthase and glutamate dehydrogenase. Enzyme activities are expressed as the percentage of the activity in the absence of inhibitor. Each point is the mean of duplicate assays. □, Glutamate dehydrogenase with either inhibitor; ●, GOGAT with L-methionine-DL-sulphoximine; ○, GOGAT with L-methionine sulphone.

Table 2. The effects of glutamate and carbon-limitation on the activities of enzymes for glutamate synthesis in *E. coli* OR75

Nitrogen source	Limiting nutrient	Enzyme activity*			
		Glutamate dehydrogenase	Glutamate synthase (GOGAT)	Glutamine synthetase	
				+ Mg ²⁺	- Mg ²⁺
10 mM-NaNO ₂	N	1630	62.0	1100	800
5 mM-NaNO ₂ + 5 mM-Na ⁺ glutamate	N	2900	72.0	890	1940
60 mM-NH ₄ Cl	C	990	56.5	120	250

* Units are nmol NADPH oxidized or γ -glutamyl hydroxamate formed/min/mg protein.

by an adenylation-deadenylation cycle, anaerobic growth was limited sequentially by 10 mM-NO₂⁻, 5 mM-NO₂⁻ and 5 mM-glutamate, and by 0.6% (w/v) glucose when 60 mM-NH₄Cl was the nitrogen source (Table 2). The Mn²⁺-dependent GS activity increased when part of the nitrogen was supplied as glutamate, but much of this enzyme was inhibited by 60 mM-Mg²⁺. Extracts from glucose-limited cultures contained far less GS activity, and 53% of this was inhibited by Mg²⁺ (Table 2). GDH activity was significantly greater when half of the limited nitrogen supply was sodium glutamate (Table 2).

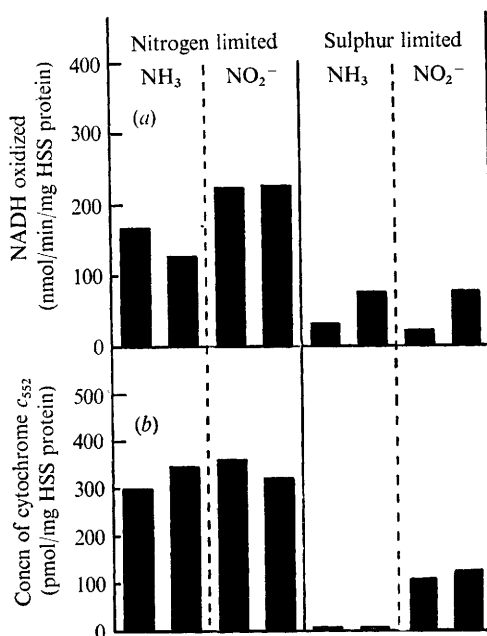


Fig. 4. (a) Nitrite reductase activity, and (b) concentration of cytochrome c_{552} in *E. coli* OR75 from nitrogen- or sulphur-limited continuous cultures. Experimental details were as in Fig. 3.

Table 3. Concentrations of cytochromes *b* and *d* (a_2) in membranes, and of cytochrome c_{552} in extracts

Each line of data refers to an independent sample of bacteria. Cytochrome concentrations are the mean of the two determinations.

Nitrogen source	Limiting nutrient	Cytochrome c_{552} (NO_2^-)* (pmol/mg protein)	Cytochrome c_{552} (Fe^{3+})† (pmol/mg protein)	Cytochrome <i>b</i> (Fe^{3+})† (pmol/mg protein)	Cytochrome <i>d</i> (NO_2^-)* (E_{628} /mg protein)
6 mM- NH_4Cl	N	251	300	492	0.0019
		312	345	252	0.0018
6 mM- NaNO_2	N	292	358	210	0.0045
		270	310	200	0.0045
20 mM- NH_4Cl	S	0	0	157	0.0011
		0	0	282	0.0010
20 mM- NaNO_2	S	103	125	352	0.0033
		114	108	355	0.0026

* Reference sample reduced by dithionite, and then partially reoxidized by NaNO_2 (in cuvettes open to the air).

† Reference sample oxidized by $\text{K}_3\text{Fe}(\text{CN})_6$.

Nitrite reductase activities and cytochrome concentrations

NADH-nitrite oxidoreductase activity was highest in HSS extracts of bacteria from nitrogen-limited cultures when either nitrite or ammonia was the nitrogen source (Fig. 4a). Far lower activities were detected when excess nitrogen was supplied, but again there was no significant difference in this activity in bacteria grown with excess nitrite or excess ammonia.

Cytochrome c_{552} has been implicated as a component of the major pathway for nitrite

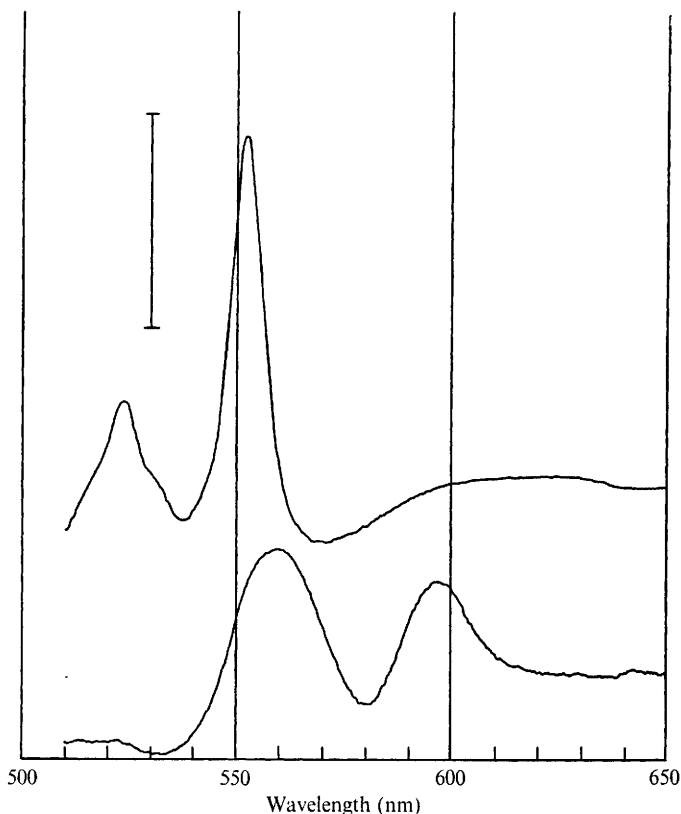


Fig. 5. Difference spectra of soluble cytochromes from *E. coli* strain OR75 grown in continuous culture with limited or excess NH_4Cl as nitrogen source. Test and reference samples (2.0 ml) were reduced with a few grains of sodium dithionite, and the reference sample was partially reoxidized with about 1 mg NaNO_2 . Upper curve: HSS (14.8 mg protein/ml) from a culture limited by 6 mM- NH_4Cl . Lower curve: HSS (27.9 mg protein/ml) from a culture limited by 0.1 mM- Na_2SO_4 (20 mM- NH_4Cl). The vertical bar corresponds to an absorbance increment of 0.05. Spectra were recorded at 18 to 20 °C with a Unicam SP1800 spectrophotometer.

reduction in *E. coli* (Cole & Ward, 1973). Highest concentrations of this cytochrome were found in HSS extracts of bacteria grown with limiting nitrite or limiting ammonia, but none was found in bacteria grown with excess ammonia (Fig. 4*b*). The ratio of the cytochrome c_{552} concentration to the nitrite reductase activity varied with different growth conditions, but high cytochrome concentrations were never found in extracts with low nitrite reductase activity. The concentration of cytochrome *b* in M extracts varied less than cytochrome c_{552} in HSS extracts, and the observed changes did not correlate with the type of nitrogen source or its abundance (Table 3). Cytochrome *d* was most easily detected when the reference sample for M spectra was oxidized by nitrite (Table 3). Peak heights at 628 nm were smaller and irreproducible when ferricyanide was the oxidant.

Although HSS extracts of bacteria grown with excess NH_4Cl contained little or no cytochrome c_{552} , broad peaks in their difference spectra were observed at 559 to 560 nm and 595 to 597 nm (Fig. 5). When nitrite was growth-limiting, M cytochromes which had been reduced by dithionite were partially reoxidized by NaNO_2 (Fig. 6). Peaks at 552 and 628 nm were observed, but the 552 nm peak (presumed to be due to membrane-bound

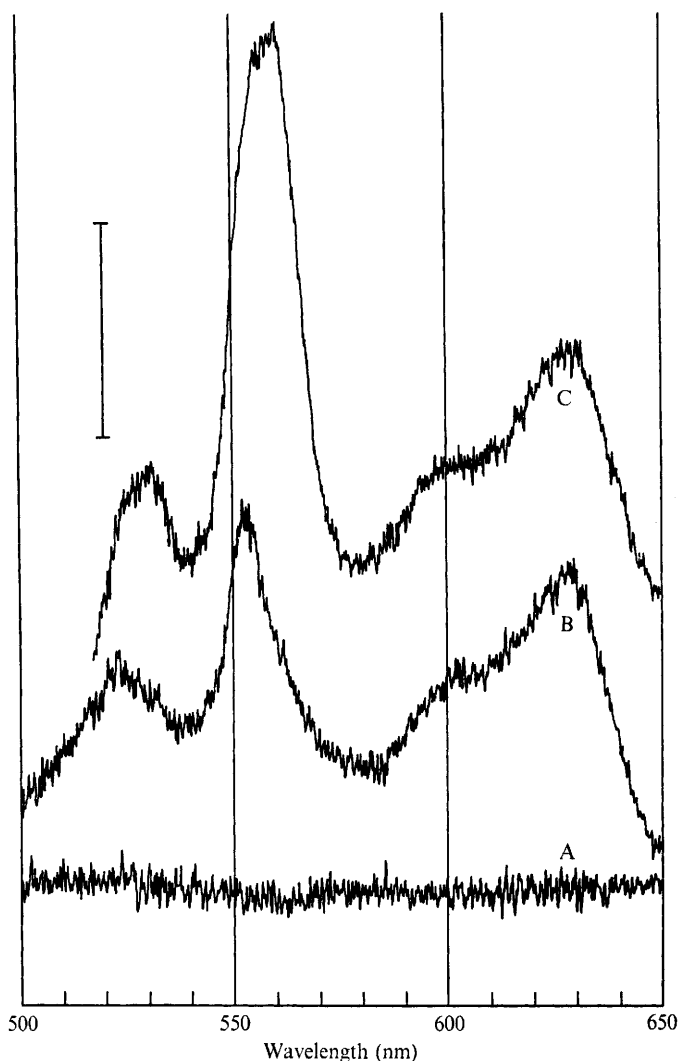


Fig. 6. Difference spectra of membrane-bound cytochromes from *E. coli* Hfrc grown with a limiting concentration of NaNO_2 (6 mM). Curve A, base line with test and reference sample reduced by a few grains of sodium dithionite. Curve B, reference samples partially reoxidized by about 1 mg NaNO_2 . Curve C, fresh reference sample oxidized by $\text{K}_3\text{Fe}(\text{CN})_6$. Protein concentrations were 6.8 mg/ml. The vertical bar corresponds to an absorbance increment of 0.05.

cytochrome c_{552}) was far more pronounced with M extracts from *E. coli* strain Hfrc than from strain OR75. When the reference sample of the same preparation had been oxidized by $\text{K}_3\text{Fe}(\text{CN})_6$, peaks at 559 and 628 nm were observed, together with a poorly defined cytochrome a_1 peak at 595 nm (Fig. 6).

DISCUSSION

Identical yields of *E. coli* strains Hfrc and OR75 were obtained from equimolar concentrations of nitrite or ammonia (63.7 g/g-atom N at $D = 0.029$) (Fig. 1). It is unlikely, therefore, that nitrogen atoms were lost as gaseous denitrification products when growth

was limited by nitrite. The high activities of enzymes for nitrite reduction and glutamate and glutamine synthesis in nitrogen-limited cultures, and their repression when nitrogen was in excess, provide further evidence that various pathways of nitrogen metabolism are regulated in an interdependent manner (Meers *et al.* 1970; Nagatani *et al.* 1971; Brenchley *et al.* 1973; Prival *et al.* 1973). Furthermore, nitrite reductase activity and cytochrome *c*₅₅₂ synthesis appear to be regulated like other enzymes for glutamate synthesis: they are not derepressed in proportion to the concentration of nitrite in the feed as one might expect if their primary function was to detoxify nitrite. The physiological significance of membrane-bound cytochromes *c* and *d* in bacteria assimilating a limited supply of nitrite will be discussed in a subsequent report.

The correlation between high activities of GDH and GS in nitrogen-limited cultures was unexpected because it is inconsistent with one suggested mechanism for GDH regulation. Brenchley *et al.* (1973) described mutants of *K. aerogenes* that lacked GOGAT activity and were unable to grow in minimal media with less than 1 mM-NH₃. A subsequent mutation resulted in constitutive synthesis of GS and complete repression of GDH. It was suggested that GS protein was the key component for regulating the synthesis of enzymes supplying glutamate. They suggested that GS was formed when the intracellular concentration of glutamine was low, and that this enzyme repressed GDH synthesis. Such an explanation clearly cannot be extended to continuous cultures of *E. coli* K12, even if one suggests that it is the adenylylated, Mg²⁺-sensitive form of GS that represses GDH synthesis. Although both nitrogen- and sulphur-limited bacteria yielded GS that was insensitive to Mg²⁺ and was therefore presumably unadenylylated, this form of the enzyme would also have been favoured by the growth conditions used to demonstrate the reciprocal regulation of GDH and GS in other bacteria. Similar arguments make it unlikely that the repressor complex is either glutamine or glutamate bound to GS protein. It has been emphasized that, although regulatory functions have been conserved during evolution, regulatory mechanisms differ even in closely related organisms (Shapiro & Stadtman, 1970*a*). Also, mutant strains with altered regulatory mechanisms might be selected during prolonged experimentation with continuous cultures, even during the delay while a new steady state is being achieved. For these reasons, we are reluctant to extrapolate our observations to other organisms, but offer two explanations of them. One is that GS is derepressed, when the intracellular concentration of NH₃ is low, to synthesize glutamate by the more effective GS-GOGAT pathway; however, the low intracellular concentration of glutamate and the high concentration of 2-oxoglutarate also favour derepression of GDH. An alternative explanation of our data is that the high activities of GDH and GS exert a mass-action effect to maintain sufficient rates of glutamate and glutamine synthesis when the supply of nitrogen is limiting. GOGAT would then be a redundant enzyme, and one should question the significance and validity of its reported activity. Three lines of evidence suggest that the GOGAT assay measured an enzyme other than GDH. First, although the GOGAT activity was low, it was not a constant proportion of the GDH activity. It is therefore unlikely that the slow oxidation of NADPH was due to GDH reacting with traces of NH₃ added as an impurity of the glutamine, or released from glutamine by glutaminase. Second, the apparent GOGAT activity did not vary significantly with different growth conditions: this was as expected (Miller & Stadtman, 1972). Finally, GOGAT activity was substantially inhibited by concentrations of L-methionine-DL-sulphoximine and L-methionine sulphone that had no effect on GDH (Fig. 3). The low activity of GOGAT relative to GDH prevented us from establishing whether the two activities were 'non-additive', as has been reported for *E. coli* strain B/r (Savage, Kotre & Sakamoto, 1972).

We conclude, therefore, that anaerobic cultures of *E. coli* K12 have alternative pathways for synthesizing glutamate from NH_3 , and that the GS-GOGAT pathway would be used preferentially when the nitrogen supply is growth-limiting. The molecules which regulate the synthesis of enzymes of inorganic nitrogen metabolism have yet to be identified.

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