NITRATE REDUCTION

II. Utilization of Possible Intermediates as Nitrogen Sources and as Electron Acceptors[1]

EARL G. McNALL and DANIEL E. ATKINSON

Department of Chemistry, University of California, Los Angeles, California

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The reduction of nitrate to nitrite has been studied extensively (Stickland, 1931; de la Haba, 1950; Sacks and Barker, 1952; Virtanen and Rautenan, 1952; Nicholas *et al.*, 1954; Nicholas and Nason, 1954; Verhoeven, 1956; Taniguchi *et al.*, 1956; Nason, 1956) and this step is generally considered to be involved in the reduction of nitrate to ammonia. Hydroxylamine, which appears to be an intermediate in nitrogen fixation (Virtanen and Jarvinen, 1951; Wilson and Burris, 1953) and in the oxidation of ammonia by autotrophic nitrogen bacteria (Hofman and Lees, 1953; Lees, 1952), is also a likely participant in nitrate reduction (Virtanen and Csaky, 1948; Silver and McElroy, 1954). Intermediates between nitrite and hydroxylamine have not been conclusively identified. Investigations of these compounds are hampered by the toxicity of nitrite, nitric oxide, and hydroxylamine. The growth of *Aspergillus niger* and *Nicotiana tabacum* on nitrohydroxylaminate (Steinberg, 1939, 1953, 1956) seems to be the only case in which an inorganic compound intermediate between nitrite and ammonia has been shown to serve as sole nitrogen source for growth of any organism.

Escherichia coli strain Bn was derived from *E. coli* strain B by serial transfer in medium containing both nitrate (at a level which inhibited the growth of the parental strain rather strongly) and an available nitrogen source (McNall and Atkinson, 1956). Although strain Bn was thus selected only for *toleration*, rather than for *utilization*, of nitrate, it was found to differ from the parental strain also in tolerance to nitrite and nitric oxide and in its ability to grow with nitrate or nitrite as sole source of nitrogen (McNall and Atkinson, 1956).

This paper reports the growth of *E. coli* strain Bn with nitrous oxide, hyponitrite, or hydroxylamine as sole source of nitrogen and the reduction of nitrite, hyponitrite, and hydroxylamine at the expense of hydrogen. This strain also grows anaerobically on lactate with nitrate as electron acceptor.

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MATERIALS AND METHODS

E. coli strains B and Bn were grown in glucose-mineral medium (McNall and Atkinson, 1956)[2] in deep standing culture under air except where otherwise noted. Growth was followed turbidimetrically. Multiplication of the reported optical densities by 1.1 gives dry weight of cells in mg per ml. Media containing hydroxylamine, sodium hyponitrite, pyruvic oxime, lactate, or hydrazine were sterilized by filtration. When the atmosphere was other than air, cultures were grown in closed bottles with provision for introduction of the desired gas phase and for measurement of turbidity during growth (McNall and Atkinson, 1956). Hydrogen uptake was measured by standard manometric methods at 28 C. Hydroxylamine was determined essentially by the method of Blom (1928). The nitrous oxide used was anesthetic grade (National Cylinder Gas Company). Sodium hyponitrite was prepared by the method of Jones and Scott (1924). After five recrystallizations from ethanol the product gave a negative Griess test for nitrite. Analysis of the insoluble silver salt gave 98 per cent of the calculated value for silver.

RESULTS

Nitrogen compounds at intermediate oxidation levels as sole sources of N for growth. Since *E. coli* strain Bn utilizes nitrite as the sole source of

[2] Two components, $MgSO_4$ at 200 mg/L and $MnCl_2$ at 10 mg/L, were inadvertently omitted from the description of the medium in the paper cited. In later work, NaCl was usually omitted from the medium.

nitrogen as readily as it uses nitrate (McNall and Atkinson, 1956), other possible inorganic intermediates in nitrate reduction were test d as sources of nitrogen for growth of this strain.

(1) Nitrous oxide:—When strain Bn cells grown with nitrate, nitrite, or ammonia as nitrogen source were transferred to liquid glucose-mineral medium lacking fixed nitrogen under an atmosphere containing nitrous oxide, significant growth was not obtained. However, after 8 serial transfers on agar medium containing glucose, nitrate, and yeast extract under an atmosphere of 35 per cent nitrous oxide and 65 per cent hydrogen or helium, a culture was obtained which could be maintained under the same atmosphere on slants lacking nitrogen. When elementary nitrogen was substituted for nitrous oxide no growth was obtained. The adapted culture grew also in liquid medium with nitrous oxide as sole source of nitrogen (figure 1).

(2) Hyponitrite:—When strain Bn was inoculated into medium containing sodium hyponitrite as sole source of nitrogen, growth occurred after a short lag when the concentration of hyponitrite was 0.005 M or less. Maximal growth on this nitrogen source without appreciable lag was obtained consistently on the second and later transfers. When nitrous oxide was added during the log phase of growth on a limiting concentration of hyponitrite, enhanced growth was obtained (figure 2), suggesting that adaptation to nitrous oxide is facilitated in cells using hyponitrite, possibly because of exposure to N_2O arising by

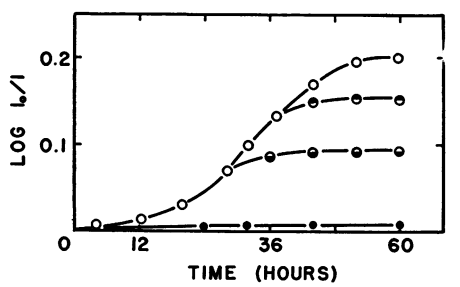

Figure 1. Growth of *Escherichia coli* strain Bn with nitrous oxide as sole source of nitrogen. Inoculum previously grown on 0.002 M $NaNO_3$ plus 0.2 per cent yeast extract in the presence of 0.35 atm of N_2O for 8 transfers. Gas phase: ● = N_2; ○ = 0.1 atm N_2O and 0.9 atm N_2; ◒ = same, but evacuated and filled with N_2 at 28 hr; ◓ = same, evacuated and filled with N_2 at 36 hr.

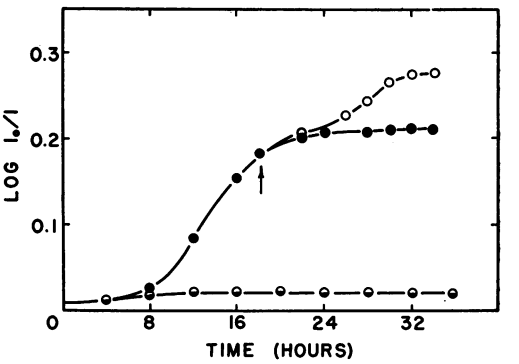

Figure 2. Growth of *Escherichia coli* strain Bn with hyponitrite as sole source of nitrogen. Inoculum grown on 0.005 M hyponitrite. Initial pH, 7.8. Nitrogen source: ◒ = none; ● = 0.005 M sodium hyponitrite; ○ = 0.005 M sodium hyponitrite with 0.1 atm of N_2O added at 18 hr.

decomposition of the hyponitrite. Strain B failed to grow on hyponitrite.

(3) Nitramide:—As recently reported by Kluyver and Verhoeven (1954) and Mozen and Burris (1955), nitramide supplied as the solid material in the sidearm of a Warburg flask is decomposed nearly quantitatively within 5 min after mixing with buffer. This behavior, which we also had noted using recrystallized nitramide prepared by the method of Marlies *et al.* (1939), precludes the use of this compound either as nitrogen source for growth or as electron acceptor in manometric experiments.

(4) Hydroxylamine:—Heavy inocula of strains B and Bn were transferred to medium containing 10^{-3} M hydroxylamine as sole nitrogen source. Both strains metabolized this compound (figure 3), presumably reducing it to ammonia at the expense of glucose. Growth of strain Bn, which began when the hydroxylamine concentration had been reduced to about 10^{-5} M, was equal to that on an equimolar level of ammonia. Strain B failed to grow even with an inoculum of about 10^7 cells per ml, although hydroxylamine was removed nearly quantitatively. The production of ammonia was confirmed by a similar experiment in which an aliquot of a strain B culture taken at 28 hr was found on aeration and Nessler analysis to be 9.3×10^{-4} M in ammonia (93 per cent of the initial hydroxylamine molarity). In a duplicate uninoculated flask at 30 hr, 88 per cent of the initial hydroxylamine was found as such, while only 4 per cent was recovered as

ammonia. The other products of decomposition were presumably nitrogen and nitrous oxide (Remy, 1956).

Despite the nearly quantitative reduction of hydroxylamine to ammonia by strain B, no growth occurred and none followed the introduction at 28 hr of additional ammonium ion equivalent to the original hydroxylamine concentration (thus bringing the ammonium molarity to 1.93×10^{-3}). Evidently growth of strain B is irreversibly inhibited by hydroxylamine, while the effect on strain Bn is reversed when the concentration is sufficiently reduced.

Strain Bn inocula which had been subcultured in the presence of hydroxylamine initiated growth more promptly in media containing hydroxylamine (figure 4) than did the unexposed strain Bn cells described above. The adapted inocula initiated growth virtually as rapidly on hydroxylamine at the concentration used in the earlier experiments as on ammonia. When the concentration of hydroxylamine was followed during growth of such adapted subcultures, it was found that their earlier growth resulted in part from an increased rate of hydroxylamine

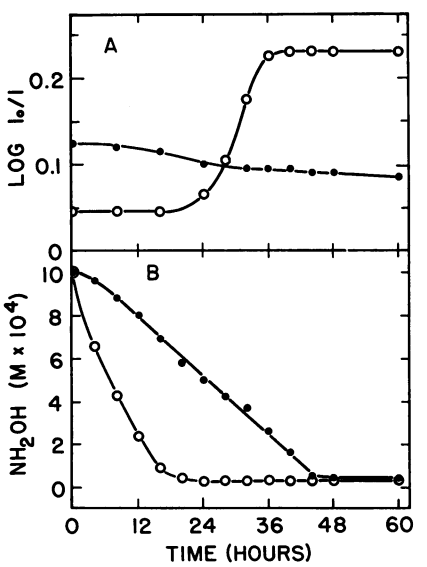

Figure 3. Growth (*A*) and metabolism of hydroxylamine (*B*) by two strains of *Escherichia coli* with hydroxylamine as sole source of nitrogen. Initial NH_2OH concentration, 10^{-3} M; pH 7.8. ○ = strain Bn; inoculum growth on 10^{-3} M $NaNO_3$. ● = strain B; inoculum grown on 5×10^{-4} M $(NH_4)_2SO_4$.

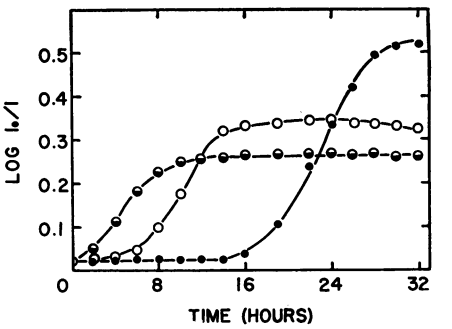

Figure 4. Effect of hydroxylamine concentration on growth of *Escherichia coli* strain Bn. Inoculum previously grown on 0.005 M NH_2OH for 5 transfers. NH_2OH concentration: ◒ = 0.001 M; ○ = 0.005 M; ● = 0.01 M.

reduction, but mainly from an increase in the threshold level for growth from about 10^{-5} M in the unadapted culture to about 3×10^{-3} M. No inhibition, except for the initial lag, resulted from initial hydroxylamine concentrations as high as 0.02 M.

After 3 serial transfers in medium lacking hydroxylamine, the previously adapted culture responded to hydroxylamine in the same way as a nonadapted culture of strain Bn. The increased tolerance thus does not seem to involve a permanent hereditary change in the organism.

Growth with hydroxylamine as sole nitrogen source was slow at pH values below 7 and the terminal level of growth was low. Above pH 7 growth was rapid and was characterized by marked clumping. Tween 80 (polyoxyethylene sorbitan monooleate), added as a possible anti-clumping agent, inhibited growth in the alkaline range but had little effect on the normally diffuse growth in more acid media. With 0.005 M hydroxylamine as nitrogen source, the optical densities at 20 hr in control flasks at pH 6.0 and 7.5 were 0.17 and 0.33, respectively. When 0.005 per cent Tween 80 was added, the corresponding values were 0.15 and 0.01. The same concentration of Tween 80 did not affect growth on either ammonium or nitrate at pH 6.0 or 7.5.

Whether or not previously adapted to hydroxylamine, strain Bn inocula initiated growth more promptly with pyruvic oxime than with hydroxylamine as nitrogen source. A typical response of an adapted inoculum is shown in figure 5.

(5) Hydrazine:—No growth of either strain

was obtained in liquid or on agar medium when hydrazine was supplied as sole nitrogen source at concentrations from 10^{-4} to 2×10^{-2} M. The addition of hydrazine at 5×10^{-5} M to media containing 0.2 per cent yeast extract caused a 45 per cent reduction in the initial growth rate of strain Bn (measured at 6 hr). No increase in rate of growth resulted from 12 serial transfers in this medium and the resulting culture did not grow with hydrazine as sole source of nitrogen.

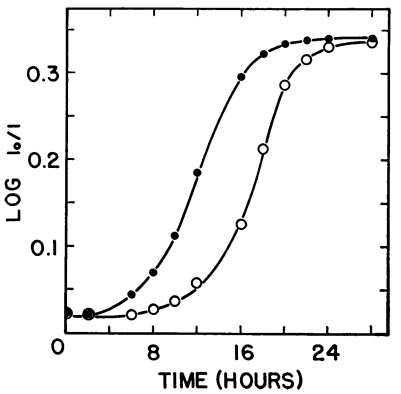

Figure 5. Growth of *Escherichia coli* strain Bn with pyruvic oxime or hydroxylamine as sole source of nitrogen. Inoculum previously grown on 0.005 M NH_2OH for 5 transfers. Nitrogen source: ● = 0.005 M pyruvic oxime; ○ = 0.005 M NH_2OH.

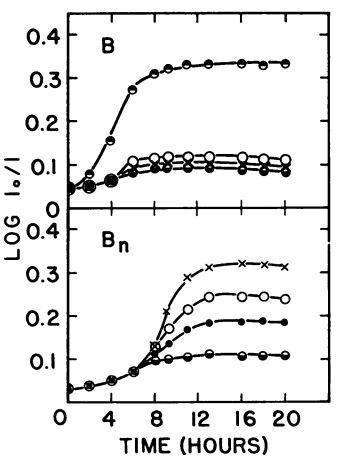

Figure 6. Anaerobic growth of two strains of *Escherichia coli* with lactate as carbon source and nitrate as electron acceptor. Basal medium plus 0.01 M $(NH_4)_2SO_4$ and 0.06 M DL-lactate under N_2 except as noted. Nitrate concentrations: ◒ = 0; ● = 0.001 M; ○ = 0.005 M; × = 0.01 M; ◓ = 1 per cent glucose under air, no nitrate or lactate.

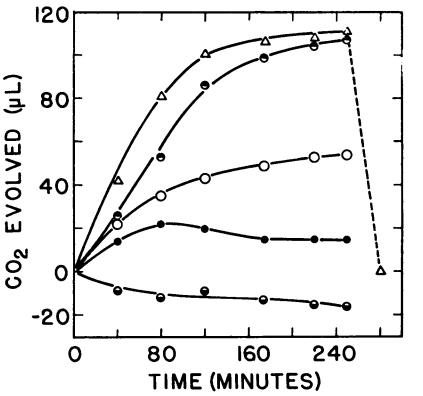

Figure 7. Oxidation of lactate with nitrate as electron acceptor. Each flask contained 0.7 mg (dry wt) of *Escherichia coli* strain Bn cells grown anaerobically on lactate and nitrate. ● = 7.5 µmoles of KNO_3; ◒ = 5 µmoles L-lactate; ○ = 5 µmoles of L-lactate and 2.5 moles of KNO_3; △ = 5 µmoles of L-lactate and 7.5 µmoles of KNO_3, 0.2 ml of 10 per cent KOH added through the vent plug at 250 min; ◓ = 5 µmoles of D-lactate and 7.5 µmoles of KNO_3.

The effect of hydrazine was similar in glucose-mineral medium with ammonium ion as nitrogen source.

Nitrate as terminal electron acceptor. Strain Bn grew anaerobically with lactate as carbon source and nitrate as electron acceptor, while strain B showed only negligible growth under these conditions (figure 6). Since all media contained an adequate level of ammonia, the growth response to nitrate reflected the need for an electron acceptor rather than for a nitrogen source. In the presence of 0.01 M nitrate, growth of strain Bn on lactate compared favorably with that of either strain on glucose. Cultures in lactate-nitrate media in fermentation tubes produced carbon dioxide in amounts which appeared to depend on the level of nitrate. The oxidation of lactate to carbon dioxide at the expense of nitrate reduction was also demonstrated manometrically (figure 7). The evolved gas was identified as carbon dioxide by its quantitative absorption in potassium hydroxide. The slight early evolution in the flask lacking added substrate presumably resulted from oxidation of endogenous substrate.

Reduction of nitrogen compounds by hydrogen. Under an atmosphere of hydrogen, nitrate is reduced to ammonia by strain Bn, but only to nitrite by strain B (McNall and Atkinson, 1956).

TABLE 1

Consumption of hydrogen in the reduction of inorganic nitrogen compounds by two strains of Escherichia coli

Substrate	Hydrogen Uptake (Moles/Mole of Substrate)		
	Required for reduction to NH_3	Observed	
		Strain B	Strain Bn
$NaNO_3$	4	0.95	3.9
$NaNO_2$	3	0	2.9
$(NaON)_2$	4	0	4.0
NH_2OH	1	0	0.98

Strain B cells grown with ammonium ion as nitrogen source; strain Bn grown on nitrate, except on hyponitrite for test of hyponitrite reduction. Substrate levels per Warburg flask: $NaNO_3$ and $NaNO_2$, 2.5 μmoles; $(NaON)_2$, 1.25 μmoles; NH_2OH, 5.0 μmoles.

Strain Bn also reduces nitrite, hyponitrite, and hydroxylamine to ammonia with the uptake of stoichiometric amounts of hydrogen (table 1). Hyponitrite is reduced at an appreciable rate only by cells grown in the presence of hyponitrite; however cell free preparations of strain Bn readily reduce hyponitrite regardless of the nitrogen source for growth (unpublished results). Similar preparations of strain B fail to reduce hyponitrite.

DISCUSSION

Growth of *E. coli* with nitrate as terminal electron acceptor was reported by Quastel *et al.* (1925), using a mineral medium plus a single carbon source (e.g., lactate), and by Takahashi *et al.* (1956), using a peptone-yeast extract medium. Verhoeven (1956, p. 199) was unable to repeat this result and questioned the possibility of "true dissimilatory nitrate reduction" (Verhoeven and Goos, 1954) in *E. coli*, presumably because of the toxicity of the nitrite which would be accumulated. Quastel's strain was shown in fact to accumulate large amounts of nitrite, to which it was apparently relatively tolerant; differences in sensitivity to nitrite may explain the failure of other strains to grow under these conditions. *E. coli* strain B behaved like the strains used by Verhoeven, but strain Bn, which reduces nitrite to ammonia and thus does not accumulate toxic reduction products, grew well with nitrate as electron acceptor. Thus this strain, like Quastel's but for a different reason, is capable of "true dissimilatory nitrate reduction" or nitrate respiration in the more descriptive terminology of Taniguchi *et al.* (1956).

Hyponitrite has been reported in growing cultures of nitrifying organisms (Corbet, 1934), but the specificity of the assay has been questioned (Rao *et al.*, 1938). This compound has also been considered as a possible intermediate in nitrate reduction. The ready adaptation of the nitrate-utilizing strain Bn to growth on hyponitrite suggests that this compound or one to which it is readily converted is an intermediate in nitrate reduction. Other compounds at the oxidation level of hyponitrite which have been proposed as intermediates either in nitrate reduction to ammonia or in denitrification are nitramide (Allen and Van Niel, 1952; Allen and Najjar, 1953), nitrous oxide (Kluyver and Verhoeven, 1954), and nitroxyl (Kluyver and Verhoeven, 1954; Verhoeven, 1956). Nitramide and nitroxyl are not sufficiently stable for test by the methods used here. Free nitrous oxide does not seem to be an intermediate in nitrate reduction by *E. coli* strain Bn, since nitrate-grown inocula failed to grow with the oxide as sole nitrogen source. Adaptation to nitrous oxide presumably results from acquisition of ability to convert this compound into one which is a normal intermediate.

Hydroxylamine is generally accepted as a precursor of ammonia both in nitrogen fixation (Virtanen and Jarvinen, 1951; Wilson and Burris, 1953) and in nitrate reduction (Virtanen and Csaky, 1948), although its participation is not yet conclusively demonstrated. Some hydroxylamine may also be metabolized to protein nitrogen via oximes (Silver and McElroy, 1954). Reduction of hydroxylamine to ammonia at the expense of reduced diphosphopyridine nucleotide has been reported with cell free preparations of *Bacillus subtilis* (Klausmeier and Bard, 1954) and *Neurospora crassa* (Zucker and Nason, 1955). The activity in the latter case was found only in mold grown in the presence of nitrate or nitrite, which suggests that hydroxylamine arose in the reduction of these compounds. Hydroxylamine was reduced to ammonia at the expense of hydrogen by intact cells of *Clostridium perfringens* and a strain of *E. coli* (Woods, 1938) and, on the addition of a viologen dye, by a cell free *E. coli* preparation (Back *et al.*, 1946). None of these

organisms was shown to use hydroxylamine as nitrogen source for growth, presumably because of its toxicity. Similar behavior is shown by our culture of *E. coli* strain B, which reduces hydroxylamine at the expense of glucose but fails to grow although ammonium ion accumulates in the medium. Strain Bn, however, reduces hydroxylamine at the expense of glucose and uses the ammonia thus produced for growth which, after a lag, is equal in rate and extent to that obtained when an equimolar amount of ammonia is supplied initially. This strain appears to be the first organism observed to utilize hydroxylamine as the sole source of nitrogen for growth.

Hydroxylamine dismutates at a moderate rate to ammonia, nitrogen, and nitrous oxide (Remy, 1956), and medium initially containing hydroxylamine has been shown after 2 or 3 weeks to support growth of a fresh inoculum of *Azotobacter vinelandii*, presumably at the expense of ammonia formed by dismutation (Segal and Wilson, 1949). Since only ⅓ to ½ of the hydroxylamine is converted to ammonia in this process, dismutation cannot account for the mole-for-mole equivalence of hydroxylamine and ammonia as nitrogen sources for *E. coli* strain Bn. The conclusion that hydroxylamine was reduced enzymically to ammonia with dismutation playing a very minor role was confirmed by the analyses on a nongrowing strain B culture and on an inoculated control flask.

Strain Bn was derived from strain B by selection only for nitrate tolerance (McNall and Atkinson, 1956). In the course of this selection strain Bn simultaneously acquired: a) increased tolerance to nitrite, nitric oxide, and hydroxylamine; b) the ability to reduce nitrate, nitrite, hyponitrite, and hydroxylamine to ammonia at the expense of hydrogen; and c) the ability to use these same compounds as sole sources of nitrogen for growth. This simultaneously acquired battery of characteristics is inherited together even when strain Bn is grown on ammonium ion as nitrogen source. These observations suggest that the increased tolerance of nitrate is caused not so much by a decrease in actual sensitivity to toxic intermediates as by the capacity to dispose of these compounds by further reduction to ammonia. This capacity, selected for only as a protective mechanism against nitrate poisoning, incidentally allows strain Bn to utilize nitrate and its reduction products as nitrogen sources for growth or as electron acceptors for the oxidation of organic substrates. The results presented in this and the preceding paper (McNall and Atkinson, 1956) thus provide independent support for the involvement of nitrite, hyponitrite or a closely related compound, and hydroxylamine as intermediates in nitrate reduction.

SUMMARY

Escherichia coli strain Bn, previously shown to grow with nitrate or nitrite as sole source of nitrogen, can similarly utilize hyponitrite, hydroxylamine, or nitrous oxide. The rates of adaptation suggest that nitrous oxide is not an intermediate in nitrate reduction. Although the parental strain B in the presence of glucose reduces hydroxylamine nearly quantitatively to ammonia, it fails to grow in the presence of hydroxylamine, even if an ammonium salt is added. Under an atmosphere of hydrogen, strain Bn, but not strain B, reduces nitrite, hyponitrite, and hydroxylamine to ammonia. Strain Bn differs from the parental strain also in its luxuriant anaerobic growth with lactate as sole carbon source and nitrate as electron acceptor.

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