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Session A 1 : Chemical and biochemical aspects of nitrogen and analytical methods for determination of nitrogen compounds.

Session C 1 : Sources of nitrogen as a water pollutant.

Session C 2 : Public health aspects of nitrate in ground water.

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Session A.1.

MICROBIAL TRANSFORMATIONS OF INORGANIC NITROGEN

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SUMMARY

The main microbiological processes involving inorganic nitrogen are assimilation of ammonia, nitrate and dinitro en for synthesis of cellular material; dissimilation, or respiration, of nitrate and nitrite (denitrification if dinitrogen is formed); and nitrification, oxidation of ammonia to nitrite then to nitrate, releasing energy for cell synthesis.

Relevant aspects of the nutrition, physiology, pathways, kinetics and inhibition of some of these transformations are discussed with special reference to the effect of dissolved oxygen. The extent to which these processes occur, or might be made to occur with advantage, and their importance in the treatment of waste waters and in natural waters are discussed.

Nitrogen is a versatile element and exists in nature in all redox states from +5, the most oxidized, nitrate, to -3, the most reduced, ammonia. The element plays an essential part in all living matter since it is a constituent of cellular protein (enzymes) and nucleic acids (genetic material); the conversion of various forms of inorganic nitrogen to these cellular components takes place in one way or another in all plants and microorganisms. Other transformations of inorganic nitrogen make use of oxygen bound in the compound for respiration and yet others involve oxidation of the compound to provide energy for synthetic reactions.

Most, if not all, of these processes occur in rivers, lakes and waste-water treatment and since they can affect the aqueous environment in many ways they are worthy of examination. Examples of their effects in rivers are oxygen depletion caused by nitrification (as well as direct toxicity to fish by ammonia) and assimilation of nitrate by algae (eutrophication). Further, in waste-water treatment requiring a nitrified effluent, it is desirable to know the optimal conditions for growth of nitrifiers and when most of the nitrogen has to be eliminated a knowledge of conditions for denitrification becomes imperative.

Since the subject was last reviewed (PAINTER, 1970), an extensive review of transformations in natural waters (BREZONIK, 1972) and one on physiological aspects of inorganic nitrogen assimilation (BROWN et al, 1974) have appeared.

THE MAIN PROCESSES

The relationships between the main biological processes involving nitrogen are shown in Fig. 1.

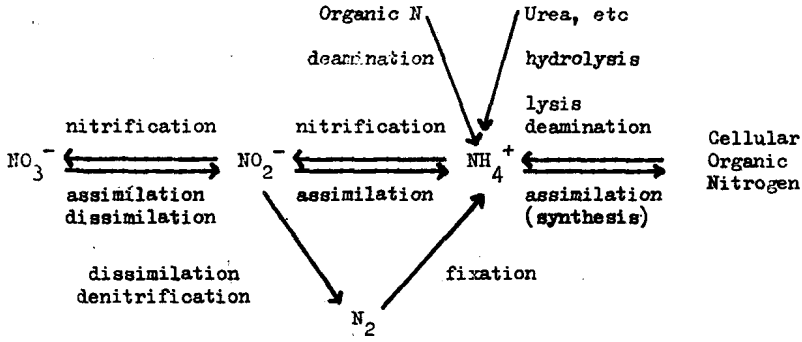

FIG. 1

The main biological processes involving inorganic nitrogen

In this review fixation, nitrification and nitrate metabolism (largely denitrification) are discussed in the sections which follow. The reader is referred to the review by BROWN et al (1974), for details of ammonia assimilation to cellular protein and to the previous review (PAINTER, 1970) for information on deamination and lysis.

FIXATION OF ELEMENTARY NITROGEN

This process, which occurs only during growth, is currently the subject of much intensive research, especially with the object of making more widespread the ability to synthesize protein from dinitrogen* by transferring the

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* 'Dinitrogen' is used for elementary nitrogen in accordance with the IUPAC convention; the enzyme which reduces dinitrogen to ammonia is termed 'nitrogenase'.

appropriate genetic material to a larger variety of bacterial and plant species. Chemically, the search is proceeding to discover metallo-dinitrogen complexes (particularly those containing Mo, found in the enzyme nitrogenase) which will reduce dinitrogen to ammonia catalytically. So far although ammonia has been produced, the complexes were decomposed in the process (CHATT et al, 1975).

There have been many recent reviews on microbial fixation (e.g. POSTGATE, 1971, 1974; HILL et al, 1972; DALTON and MORTENSON, 1972; STEWART, 1973; DALTON, 1974); in this review only those aspects relevant, or thought to be relevant, to the aqueous environment will be discussed.

In Pure Cultures

Species, detection and pathways

Since the introduction of the detection technique using the reduction of acetylene to ethylene (STEWART et al, 1967), the numbers of organisms which have been readily shown to fix dinitrogen have increased, though some species have been eliminated from the list. The distribution of fixers is widespread among free-living microbes and most families now have representatives. The main aerobic bacterial species are Azotobacter, Azomonas, Azotococcus, and Mycobacterium, while the anaerobic* fixers include species of Clostridium, Klebsiella, Bacillus, Desulphovibrio and Chlorobium. Species of heterocystous and unicellular blue-green algae as well as filamentous algae can fix dinitrogen. Studies have been extended to the intestinal contents of animals and man, but it is concluded that little, if any, fixation occurs under normal conditions (BERGSEN and HIPSLEY, 1970; GRANHALL and OTSZUK, 1971).

The ability of nitrogenase to reduce acetylene to ethylene, which can be readily detected and determined by GLC, provides a rapid, reliable and sensitive test (down to the n mol level) for the enzyme and hence for deciding whether a given organism or biological sample can fix dinitrogen. Ethylene is a metabolite of some organisms, but since fixation is suppressed by growth on ammonia the source of ethylene can be readily checked. Other points, relative to technique, concerning this and other methods, which must be observed to avoid misleading results are (a) shaking and thus aerating samples may cause aerobic fixers to 'switch-off' their nitrogenase activity to give a falsely low or nil value for ethylene production; (b) trichloracetic acid, used to stop the

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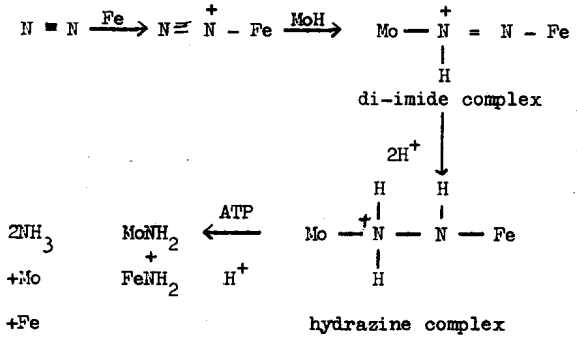
* 'Anaerobic' here is taken to mean 'in the absence of dissolved molecular oxygen'.

biological reaction, has been shown to produce ethylene from serum cap liners; (c) cultures contaminated with species which fix would lead to erroneous conclusions; (d) presence in the medium of fixed nitrogen, especially ammonia from the atmosphere; (e) confirmation, after ethylene production, should be made by using $^{15}N_2$; (f) in the case of anaerobic fixers alleged to be aerobic, the presence of an aerobic non-fixing contaminant could deplete the medium of dissolved oxygen.

So far, ammonia is the only inorganic nitrogen compound identified in the conversion of dinitrogen to cells. Many pathways have been suggested for fixation involving reduction, oxidation and hydration, but none has yet been established. One suggestion (HARDY and BURNS, 1968) involves metal complexes of dinitrogen, di-imide and hydrazine:-



Requirements

Heterotrophic fixers require an oxidizable substrate, e.g. sucrose, as a source of energy while autotrophs require carbon dioxide. Special requirements for fixation, compared with growth on ammonia are iron, molybdenum, cobalt (or vitamin B_{12}) and possibly manganese, zinc, copper and biotin (NICHOLAS, 1963). In cells of Klebsiella pneumoniae previously grown on ammonia, the enzyme had to be induced and the time of induction was greatly decreased by the presence of certain amino acids (YOCH and PENGRA, 1966). Anaerobes yielded a soluble enzyme while in aerobes the enzyme was attached to membranes. Two protein components of nitrogenase have been identified; protein 1 ('molybdoferredoxin') contains molybdenum, iron and sulphur and is fairly sensitive to oxygen; and protein 2 ('azoferredoxin') which contains iron and is extremely sensitive to oxygen.

Because of the inherent oxygen-sensitivity of nitrogenase, free-living micro-organisms which fix dinitrogen do so either anaerobically or have developed special means for excluding oxygen from the enzyme, e.g. confining the enzyme to the heterocyst or other separate part of the cell. Behaviour towards oxygen can be summarized as (DALTON, 1974):-

strict aerobes ..... grow and fix N_2 anaerobically e.g. Azotobacter, heterocystous blue-green algae,

aerobes ..... grow aerobically but fix N_2 micro-aerophilically, e.g. Mycobacterium flavus, non-heterocystous blue-green algae,

facultative aerobes ..... grow aerobically but fix N_2 only anaerobically, e.g. Klebsiella, Bacillus,

anaerobes ..... grow and fix N_2 anaerobically, e.g. Clostridium, Desulphovibrio.

Even obligate aerobes like Azotobacter are readily inhibited by excessive aeration when fixing dinitrogen, though not when utilizing ammonia; Mycobacterium flavus also grew better at low partial pressures of oxygen when fixing dinitrogen (POSTGATE, 1971). The inhibition of oxygen to A. chroococcum depended on whether the culture was limited by carbon, phosphorus or dinitrogen and also on the growth rate. The ambient dissolved-oxygen concentration in dinitrogen-limited cultures giving maximum growth was about 20 μM (0.6 mg/l) and inhibition occurred in excess of 30 μM (~1 mg/l). By contrast, the highest dissolved-oxygen concentration which could be tolerated by carbon-limited cultures was 10 μM (0.3 mg/l). Two mechanisms of protection appear to have been developed by such organisms:-

(a) respiratory protection in which the respiration rate is adjusted to keep up with the external availability of dissolved oxygen to the functioning nitrogenase; this of course cannot be done in carbon-limited cultures and hence the greater toxicity of oxygen under these conditions,

(b) conformational protection in which some steric arrangement prevents oxygen from getting to oxygen-sensitive sites or in which the sites are stabilized by conformational features of the enzyme complex.

Effect of N nutrients

Since it is a key intermediate, it is to be expected that the presence of adequate concentrations of NH_4^+ would inhibit fixation of dinitrogen and this is found to be so with blue-green algae (FOGG, 1962). The degree to which other inorganic nitrogen compounds inhibit fixation depends on the ease with which the particular organism can convert them to NH_4^+. For example, inhibition by urea is usually complete (ALLEN, 1956) but that by nitrate is sometimes incomplete. Also, Anabaena cylindrica has first to be adapted to nitrate before it can be readily assimilated. In bacteria, too, NH_4^+ usually inhibits the formation of the enzyme, for example DROZD et al (1972) reports 50% repression of nitrogenase in A. chroococcum at 14 mg NH_4-N/l and complete repression at 28 mg/l; but in contrast to the algae, Azotobacter can fix dinitrogen at concentrations of NH_4-N up to 100 mg/l (NEWTON et al, 1953). However, in Clostridium pasteurianum, pre-formed nitrogenase is not inhibited by the presence in the medium of excess NH_4^+ (DAESCH and MORTENSON, 1972). Recently, it has been found that mutants of Azotobacter vinelandii (GORDON and BRILL, 1972) and Klebsiella pneumoniae, formed by gene transference (STREICHER et al, 1974) were able to produce nitrogenase and fix dinitrogen in the presence of NH_4^+.

Inhibition

Apart from NH_4^+, many substances inhibit fixation but not all are specific. Hydrogen, carbon monoxide and nitric oxide are all competitive inhibitors. Nitrous oxide and azide (at 0.14 mg N/l) reversibly inhibit fixation, although at lower concentrations azide is stimulatory. Azotobacter was inhibited by cyanate at 0.14 mg N/l and by nitrite above about 1.4 mg N/l, though again at lower concentrations nitrite was stimulatory (AZIM and ROBERTS, 1956).

Potassium orthophosphite at 0.01 M was completely inhibitory to Azotobacter and the inhibition could be reversed by adding ammonium acetate (BULLEN and FREAR, 1957). Similarly, the inhibition of Clostridium pasteurianum by trichloromethylsulphenyl benzoate (5 mg/l) was reversed by the addition of sodium molybdate and that by compounds such as liporic acid (0.1 g/l) or 1:2-diacetylethylene (10 mg/l) could be nullified by adding 10 µg/l biotin (CARNAHAN et al, 1960).

Kinetics

The highest specific growth rates reported are those of ALEXANDER and WILSON (1954) who found μ_{max} for *Azotobacter* to be 0.3–0.4 h^{-1}, doubling time 1.8–2.3 h. By raising the amount of oxygen supplied by 28-fold the amount of dinitrogen fixed rose from about 14 mg N/l h to 175 mg N/l h, but the amount fixed for each unit of substrate utilized remained at about 12–13 mg N/g sucrose, compared with about 80 mg NH_4–N/g glucose by a laboratory-grown coliform organism. The low specific amounts of fixed dinitrogen are reflected in the lower yield coefficients of cells from the carbon substrate as compared with the yields when grown on NH_4^+. For example, *Azotobacter chroococcum* on mannitol gave a molar yield of 60 with NH_4^+ and 38.2 on dinitrogen, and *Klebsiella pneumoniae* on glucose gave a yield of 30 on NH_4^+ and 12.1 on dinitrogen (HILL *et al*, 1972).

Fixation in the Aqueous Environment

Much fixation occurs in the soil by both symbiotic systems and free-living organisms and a recent estimate has put the amount as high as 10^8–10^9 tons/y (BURNS and HARDY, 1973) but although it is well-established that fixation occurs in lakes, largely by blue-green algae, estimates of the amount of dinitrogen involved do not seem to have been made. Rates of fixation for a number of Florida lakes were usually at or less than 10 μM-N/m^3 h with some exceptionally high values at 175 μM-N/m^3 h (BREZONIK, 1972). As much as 33% of the N turnover (44 kg/ha y) in Lake George, Uganda, resulted from fixation (HORNE and VINER, 1971), while in Lake Erken, Sweden, blue-green algae, fixing at 30 kg/ha y, were responsible for increasing the annual loading of combined N by 40% (GRANHALL and LUNDGREN, 1971). Low but significant fixation by bacteria, in the depths of lakes and in fresh-water and estuarine sediments, has also been reported (see BREZONIK, 1972). Less seems to be known about fixation in rivers but in well-oxygenated rivers it is thought that fixation is relatively unimportant (RHEINHEIMER, 1962; CURTIS, 1973).

In the treatment of sewage and most industrial waste waters there is no evidence of fixation, though fixers such as *Azotobacter* have often been isolated (DIAS, 1964). However, fixation has been demonstrated in nitrogen-deficient waste-waters such as those from citrus canneries, with or without deliberate inoculation of fixing organisms, but the rate of removal of BOD was lower than when NH_4^+ was added and the organisms which developed were filamentous, giving rise to operational difficulties (McKINNEY *et al*, 1954). An 'activated sludge' grown on a nitrogen-free glucose medium contained *Azotobacter* and fixed about 10 mg N/l d and 7 mg N/g glucose (ALLEN, 1941).

N-deficient chemical and food processing wastes have been successfully treated by use of a maintained 'semi'-pure culture of dinitrogen-fixing bacteria; 35% of COD was removed and about two-thirds reduction in sludge production over conventional treatment was recorded (FINN and TANNAHILL, 1973). Many dinitrogen-fixing species were isolated from paper and pulp mill effluents and high rates of acetylene production were recorded both anaerobically and aerobically (KNOWLES et al, 1974).

NITRIFICATION

Autotrophic nitrification, or simply nitrification, is the name given to the oxidation of ammonia to nitrite and thence to nitrate by autotrophic organisms which derive their energy solely from these oxidations and not from the oxidation of reduced carbon compounds. Heterotrophic nitrification occurs when nitrite and/or nitrate are produced from inorganic or organic compounds by heterotrophic organisms by reactions which are not necessarily oxidations and which are not the sole energy sources for the organism. Two comprehensive reviews of nitrification have appeared recently, dealing mainly with biochemical aspects (WALLACE and NICHOLAS, 1969; ALEEM, 1970).

In Pure Cultures

Autotrophs

Species well recognized for oxidizing ammonia to nitrite are Nitrosomonas europaea and monocella and Nitrosococcus, and for oxidizing nitrite to nitrate are Nitrobacter agilis and winogradskyi and Nitrocystis. Other genera and species have been described from time to time but their status is doubtful; IMSENECKI (1946) thought that apparent symbiotic cultures of Nitrosomonas and heterotrophs could be the reason for the various 'nitrifiers' to be classified as separate genera. However, recent experimental developments may soon help to solve these problems of classification. Using an immuno-fluorescent technique, FLIERMANS et al (1974) have found a clear distinction between Nitrobacter agilis and winogradskyi in spite of claims by WATSON and MANDEL (1971) and PAN (1971) that there was no difference between the two species. All isolates from soil were N. winogradskyi, while those from other sources (oxidation ditch, cave sediments, etc) were N. agilis. Meanwhile, further new genera and species have been reported and others re-isolated. A lobular ammonia-oxidizer, named Nitrosolobus multiformis was isolated from soils from many parts of the world (WATSON et al, 1971) and was said to be an autotroph with a slight heterotrophic potential. Its lobular nature and internally partially compartmentalized cytoplasm distinguished it from other species, though there still appears to be some confusion with what others call

Nitrosocystis coccoides. WATSON (1971) has also re-isolated from soils Nitrosospira briensis, originally described by the Winogradskys in 1933; this isolation is apparently only the third since the original. Two new obligate marine nitrite-oxidizing species have been isolated by WATSON and WATERBURY (1971); Nitrococcus mobilis is a large motile coccus with unique tubular cyto-membranes, while Nitrospira gracilis is a long slender rod lacking an extensive membrane system. In a survey of soils, Nitrosomonas europaea was detected only in soils treated with dung or other organic fertilizers; Nitrosocystis coccoides and Nitrosospira spp. were found in other soils (SORIANO and WALKER, 1973).

Heterotrophs

Some 104 varied species and a further number of unnamed Gram-negative rods were cited as forming low concentrations of nitrite from ammonia (CUTLER and CRUMP, 1933; FISHER et al, 1956). Organic compounds containing nitrogen, e.g. pyruvic oxime, can also be converted to nitrite and nitrate by such organisms as Nocardia, Alcaligenes and Agrobacter and are not inhibited by substances such as thiourea and methionine which inhibit autotrophic nitrite-formers. Aspergillus flavus produces β-nitropropionic acid which is converted to nitrate without apparently passing through the nitrite stage (BECKER and SCHMIDT, 1964), while in cultures of another strain of the same fungus, nitrite and bound forms of hydroxylamine were also formed (MARSHALL and ALEXANDER, 1962; MOLINA and ALEXANDER, 1971). In contrast, completely inorganic pathways were reported for ammonia oxidation by Aspergillus wentii (ALEEM et al, 1964) and Arthrobacter globiformis (GUNNER, 1963). However, Arthrobacter sp. isolated from sewage produced nitrite and nitrate from ammonia (only in the presence of an organic substrate) via free and bound hydroxylamine, an hydroxamic acid and a primary nitro compound (VERSTRAETE and ALEXANDER, 1972). The same authors have made the first demonstration that heterotrophic nitrification occurs in nature - in soils, sewage treatment, river and lake waters (VERSTRAETE and ALEXANDER, 1973); 1-nitroso-ethanol, a potentially toxic compound, was also identified as a product of Arthrobacter-like nitrification (up to 10 mg N/l in amended samples).

Although in general the rate of formation and amount of oxidized nitrogen by these processes is low, some authors feel that heterotrophic nitrification plays an important role in nitrogen metabolism. As opposed to this view, selective inhibitors of autotrophic nitrification when added to soil and activated sludge usually inhibit nitrification completely.

The question of autotrophy

It has long been known that Nitrosomonas and especially Nitrobacter could assimilate organic compounds (e.g. acetate and some amino acids) and that in some cases growth could be stimulated (e.g. DELWICHE and FINSTEIN, 1965). Formic acid could be used in place of nitrite by Nitrobacter to fix CO_2, but at a much lower rate (VAN GOOL and LAUDELOUT, 1966). Also the 'heterotrophic' enzyme content of nitrite-oxidizing species (WILLIAMS and WATSON, 1968), their similar ultra-structure after growing heterotrophically and autotrophically (PAN et al, 1969) and their ability to form and utilize 'heterotrophic' reserve polymers, poly-β-hydroxybutyric acid, glycogen, polyphosphate (VAN GOOL et al, 1971) suggested that they should at least be considered as pseudo- or facultative-autotrophs. Nitrosomonas lacked certain key enzymes so was regarded as an obligate autotroph (HOOPER, 1969). Recently, assuming that growth on organic compounds was prevented by the formation of toxic products, PAN and UMBREIT (1972) have succeeded in growing a number of species previously regarded as obligate autotrophs on glucose in an apparatus using dialysis to remove any toxic products. Nitrosomonas europaea grew faster not only on glucose, if dialysed, than conventionally on ammonia but also on ammonia if dialysed. The toxic substance(s) was not identified for N. europaea, but for Nitrobacter agilis it was probably pyruvic acid, at about 5×10^{-5}M (4.4 mg/l). The reported absence of key enzymes in N. europaea (HOOPER, 1969) may have been due to faulty technique; an improved method which released the intact membrane-envelope-complex (HOOPER et al, 1972) may lead to a reversal of the earlier negative findings.

Requirements

Growth of nitrifiers is slow by comparison with most heterotrophs and the yield of cells is low. Also until 1955 it had been thought that suspended particles, usually calcium carbonate, were necessary for growth. For these reasons, the nutritional requirements of these organisms have still to be established in detail. In 1955 GOLDBERG and GAINEY showed that Nitrobacter could be grown on clear media and in 1958 ENGEL and ALEXANDER did the same for Nitrosomonas.

Besides carbon dioxide, carbonate or bicarbonate, and ammonia or nitrite, a minimum concentration of dissolved oxygen is an absolute requirement for growth. Phosphate, magnesium, iron and copper (at 0.03 mg/l for Nitrosomonas) are required by both Nitrobacter and Nitrosomonas, and calcium has been shown to be required by Nitrosomonas. The optimal phosphate concentration - about 310 mg P/l - was much higher for both organisms (VAN DROOGENBROECK and

LAUDELOUT, 1967) than that reported by other workers e.g. 5 mg/l for Nitrobacter (e.g. ALEEM, 1959); the explanation could lie in carry-over of the element in inocula and/or the effect of phosphate on the pH of the medium which is a very important factor in the growth of the nitrifiers. Stimulation of growth of Nitrobacter was observed on the addition of zinc at 1 mg/l and by molybdenum at 0.1 µg/l (ALEEM, 1959; FINSTEIN and DELWICHE, 1965).

The concentration of sodium salts is important for the growth of Nitrosomonas, although it is not clear whether the effects are specific to sodium or could be produced by similar elements. Two terrestrial species isolated from activated sludge grew best in concentrations of sodium chloride or sulphate of about 0.2% (as Na) (LOVELESS and PAINTER, 1968) while marine species were most active at about 0.5% Na (VARGUES and BRISOU, 1963). However, other freshwater species were not stimulated by marine salts and some marine species were not dependent on marine salts for growth (FINSTEIN and BITZSKY, 1972).

The nutrition of nitrifiers cannot yet be considered complete since the addition of EDTA (5 mg/l) to media considered to be complete gave increased growth rates of Nitrosomonas (LOVELESS and PAINTER, 1968), although the higher rate of autotrophic growth in a dialysis apparatus (PAN and UMBREIT, 1972) suggests that inhibition by a metabolite also plays a part. Also, on growing the organism at relatively high concentrations deficiencies in media become more apparent; dissolved oxygen had probably become limiting in the experiments of SKINNER and WALKER (1961) and when air was used as a source of CO_2 and ammonium hydroxide was used to neutralize the nitrous acid formed, the carbon source became limiting (LOVELESS and PAINTER, 1968). By using ammonium carbonate for neutralization, higher concentrations of Nitrosomonas and of nitrite - up to 2500 mg N/l - were obtained.

As mentioned earlier, some organic compounds stimulated the growth of the nitrifiers. Biotin (FUNK et al, 1964) and compounds such as acetate stimulated Nitrobacter (DELWICHE and FINSTEIN, 1965) and some amino acids gave increased production of nitrite and of protein by Nitrosomonas, although carbon dioxide was still necessary for growth (CLARK and SCHMIDT, 1967). Using impure culture, COOPER and CATCHPOLE (1973) reported apparent increases in the ease with which nitrification occurred by the addition of glucose, p-aminobenzoic acid or other substances (at 5 mg/l) which formed pyruvic acid during the activated-sludge treatment of ammonium carbamate and of carbonization liquors. Once started, nitrification sometimes proceeded at the increased rate when the 'growth factor' was withdrawn. The effect, which remains to be tested on pure