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DINITROGEN FIXATION BY BLUE-GREEN ALGAE FROM PADDY FIELDS

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ABSTRACT

Fluorescence emission spectra at 77°K of isolated heterocysts of Anabaena L-31 do no show F685-695, but show F715-730, thus confirming the absence of photosystem II and the presence of photosystem I. Recent work using radioactive nitrogen has been collated and a scheme is outlined indicating the location of the enzymes and the pathways involved in the assimilation of nitrogen in blue-green algae.

Glutamine synthetase extracted from heterocysts of Anabaena L-31 does not exhibit the adenylylation/deadenylylation phenomenon which is characteristic of the enzyme from bacteria. Our recent experiments suggest that nitrogenase in Anabaena is under dual control by glutamic acid and aspartic acid, the former inhibiting the enzyme and the latter relieving the inhibition. Two extracellular polypeptides have been obtained from this alga, one of which inhibits heterocyst formation whereas the other enhances heterocyst formation and partially relieves the inhibitory effect of the former. An extracellular substance, possibly a glycopeptide, has been obtained from A. torulosa which stimulates sporulation.

Studies with ^{24}Na and ^{22}Na indicate that A. torulosa, an alga from saline habitats, has an active photosynthesis

-linked mechanism for the extrusion of sodium. Sodium is essential for optimum nitrogenase activity and growth.

In field experiments, inoculation with Nostoc 4 resulted in substantial increase in soil nitrogen. Paddy yield was comparable to those plots where 80 kg N/hectare of urea was used.

INTRODUCTION

The blue-green algae of tropical paddy fields are now generally considered (Evans & Barber, 1977) to be the major free-living contributors of microbial dinitrogen (N_2) fixation in agricultural productivity. These algae are unique in that, concomitant to N_2 fixation, they also photoassimilate carbon and evolve oxygen, thereby not only augmenting the nitrogen and carbon status of the soil but also providing oxygen to the water-logged rice paddies. In this paper, I shall collate the evidence and describe some of the work done in our laboratory on the physiological features of blue-green algal N_2 fixation, the pathways of nitrogen assimilation, the regulation of nitrogenase and the control of heterocyst production, the physiology of sporulation and the field application of blue-green algal N_2 fixation. A good deal of information in some of these studies was gained by the use of radioactive isotopes.

SITE OF NITROGENASE ACTIVITY IN BLUE-GREEN ALGAE

Many blue-green algae perform the O_2-evolving mode of photosynthesis while fixing N_2 at the same time. How is the algal nitrogenase protected from inactivation by O_2? Fay (1969) and Wolk and Simon (1969) were the first to report that heterocysts, a cell type characteristic of aerobic filamentous N_2-fixing algae, contain little or no phycocyanin, which is a major pigment component of the O_2-evolving photosystem II (PS II) of blue-green algal photosynthesis. These measurements were made on isolated heterocysts prepared by procedures involving cavitation or high pressures, when the water soluble phycocyanin could have leaked off damaged heterocyst membranes. Using a microspectrophotometric technique which enables measurements of absorption spectra of single cells of intact filaments I showed (Thomas, 1970), that in heterocysts of log phase cultures the entire complement of PS II pigments viz., c-phycocyanin, allophycocyanin, c-phycoerythrin and chlorophyll _a_ 670, are absent or present only in very low amounts. However, in heterocysts of older cultures these pigments reappear (Thomas, 1972). Do such heterocysts have a functional PS II or do all heterocysts irrespective of their age have only a functional PS I ? Tel-Or and Stewart (1975) showed that the quantity of manganese, a key constituent of the O_2-evolving machinery of PS II, is several fold less in

heterocysts than in vegetative cells. O_2 evolution has not been detected in heterocysts (Bradley & Carr, 1971). Various lines of evidence has, however, been obtained to indicate the presence of PS I activity in heterocysts (Fay, 1970;Donze et al 1972; Tel-Or and Stewart, 1976).

Fluorescence excitation and emission analysis is a major tool in examining PS I and II activities. Detailed fluorescence emission analysis show that F685 and F696, the fluorescence peaks characteristic of PS II activity, are absent in all heterocysts preparations (Thomas, 1977) including those of older heterocysts where the entire PS II pigment system reappears. In contrast, F715-730, the PS I fluorescence peak, is always present. Polarographic measurements have confirmed that heterocysts, irrespective of their age, do not evolve O_2. Significantly, there was light-induced enhancement of O_2 consumption (Fig. 1) in heterocysts. The light-induced O_2 consumption may have some bearing on the light requirement for nitrogenase activity in isolated heterocyst suspensions (Thomas et al, 1977) even when all the known cofactors for nitrogenase activity are provided exogenously. This phenomenon may be an additional mechanism for ensuring the anaerobic milieu essential for nitrogenase activity.

Although a great deal of indirect evidence (Fay et al, 1968; Flemming & Haselkorn, 1973; Stewart & Rowell, 1975; Thomas & David, 1972; Van Gorkom & Donze, 1971; Wolk and Wojciuch, 1971) has indicated that heterocysts are the sites of N_2 fixation, in no case was N_2 shown to be reduced in isolated heterocysts. The main hurdles were the lack of procedures for isolating heterocysts which retain substantial nitrogenase activity and the absence of a sensitive isotopic technique. Recently Peterson & Burris (1976) isolated heterocyst preparations with high specific activity for acetylene reduction (1840 nmol/mg chl/min). However, the heterocysts were processed over a gas phase which included hydrogen and thus would inhibit N_2 reduction. Active heterocysts were isolated by Thomas et al (1977) after incubating filaments of Anabaena cylindrica for 45 min under argon gas in a solution containing 2-5 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] buffer, 6.5 mM sodium dithionite, 26 mM Na_2EDTA [(ethylene dinitrilo)tetracetic acid] and 650 mM D-mannitol, followed by cavitation for 6 s/ml. These heterocysts reduced 1560 nmol C_2H_2/mg chl/min.

On exposure to the radioactive nitrogen gas, $^{13}N_2$, heterocysts made $^{13}NH_4$. Formation of $^{13}NH_4$ was inhibited nearly completely in the presence of acetylene, confirming that nitrogenase-mediated NH_4 formation occurred.

USE OF RADIOACTIVE NITROGEN (^{13}N) IN NITROGEN FIXATION RESEARCH

As indicated above, the use of $^{13}N_2$ resulted in the first demonstration of the reduction of dinitrogen in isolated heterocysts. $^{13}N_2$ and $^{13}NH_4$ have been remarkably effective in critical experiments designed to establish the pathways of assimilation of ammonia in blue-green algae.

Enzymological studies on heterotrophic nitrogen-fixing bacteria have shown that the glutamine synthetase/glutamate synthase pathway is the main route of assimilation of N_2-derived ammonia (Nagatani et al, 1971). However, in blue-green algae such investigations had implicated glutamic acid dehydrogenase and alanine dehydrogenase (Batt & Brown, 1974; Haystead et al 1973; Neilson & Doudoroff, 1973; Pearce et al, 1969; Scott & Fay, 1972) or glutamine synthetase (Dharmawardene et al 1973) as the enzyme(s) involved in the primary assimilation of ammonia. Use of ^{15}N to trace the pathway of metabolism of nitrogen in free-living and symbiotic bacteria (Burma & Burris, 1957; Kennedy, 1966 a, b) and in blue-green algae (Stewart et al 1975) did not yield conclusive results.

A main reason for not using the radioactive nitrogen, ^{13}N, in nitrogen fixation research is its very short half life (10 min). Conventional methods for processing the assimilation

products would need several hours. Methods were developed by which 10 amino acids (likely early products of ammonia assimilation) could be separated and identified in two dimensional electrophoresis/chromatography in about 35 min (Thomas et al 1975; Wolk et al 1976). Using $^{13}N_2$ labelling, coupled with the above and a variety of other procedures including pulse chase experiments and the use of inhibitors which preferentially repress particular enzyme activity, the glutamine synthetase/glutamate synthase pathway has been shown to be the main in vivo route of ammonia assimilation in blue-green algae (Thomas et al 1975, Wolk et al 1976). A method was also developed by which upto 18 mCi $^{13}NH_4$ could be produced rapidly (Thomas et al 1977). Using $^{13}NH_4$, several fold higher incorporation of radioactivity could be achieved and the above results were confirmed. Further, it was shown that the same pathway operates whether A. cylindrica is grown with N_2 or NH_4 as the nitrogen source (Meeks et al 1977). These experiments clearly indicated that glutamate dehydrogenase and alanine dehydrogenase have only a minor role in the assimilation of ammonia in this alga.

LOCATION OF THE ENZYMES OF NITROGEN ASSIMILATION

In isolated heterocysts $^{13}N_2$ is converted to $^{13}NH_4$ and ^{13}N-glutamine (Thomas et al 1977). Acetylene virtually

blocks the formation of $^{13}NH_4$ and ^{13}N-glutamine indicating that nitrogenase and glutamine synthetase (GS) are coupled in heterocysts. Isolated heterocysts also formed ^{13}N-glutamine from added $^{13}NH_4$ and glutamate and ^{14}C-glutamine from added ^{14}C-glutamate and ammonia. The formation of glutamine was strongly inhibited by methionine sulfoximine. Even under a variety of favourable assay conditions ^{13}N-glutamate was not formed in heterocysts either from added $^{13}N_2$, $^{13}NH_4$ or amide-labelled ^{13}N-glutamine. Related experiments on the kinetics of solubilisation of glutamate synthase activity during cavitation of A. cylindrica suspensions indicated that this enzyme is located in vegetative cells (Thomas et al 1977). Moreover, these studies confirmed that GS is present also in vegetative cells. Interestingly, isolated heterocysts formed ^{14}C-glutamate rapidly from α-(^{14}C) ketoglutarate, and various stable amino acids. These reactions were severely inhibited by aminooxyacetate, but not by azaserine or MSX, indicating high rates of aminotransferase activity in heterocysts.

From the foregoing it is clear that nitrogenase, GS, and aminotransferases are present in heterocysts, while glutamate synthase and GS are present in vegetative cells. The aminotransferases in vegetative cells were not examined separately. Is there a possibility that nitrogenase occurs

also in the vegetative cells of aerobic cultures as some data (Wolk _et al_ 1974) suggest? The kinetic experiments with $^{13}N_2$ and $^{13}NH_4$ (Wolk _et al_ 1976; Thomas _et al_ 1977; Meeks _et al_ 1977) clearly afford a different interpretation. The time course of appearance of ^{13}N in glutamine and in glutamate differ extensively depending on whether $^{13}N_2$ or $^{13}NH_4$ was added as tracer (Meeks _et al_, 1977). During $^{13}N_2$ fixation the ratio of ^{13}N in glutamate to ^{13}N in glutamine is greater than unity after 90 s. Conversely, during $^{13}NH_4$ assimilation this ratio is less than 0.25 in both NH_4-grown and N_2-grown cultures. The simplest interpretation of this difference is that $^{13}N_2$ is reduced to $^{13}NH_4$ only in heterocysts where heterocyst glutamine synthetase mediates the formation of ^{13}N-glutamine. This glutamine needs to be transported to vegetative cells for amide transfer (glutamate synthase reaction) to form ^{13}N-glutamate. The delay of 90 s, before glutamate becomes as radioactive as glutamine, corresponds to the time needed for these sequential and spatially separated reactions to take place. Conversely, when exogenous $^{13}NH_4$ is used the ^{13}N-glutamine formation would be mediated mostly by the GS located in vegetative cells resulting in large pools of glutamine and the observed low ratio of glutamate to glutamine.

This finding is perhaps the strongest evidence to-date for the view that nitrogenase in aerobic cultures of filamentous blue-green algae is restricted to heterocysts.

The definitive experiments using $^{13}N_2$, $^{13}NH_4$, ^{14}C-glutamate and α-(^{14}C)ketoglutarate have enabled the formulation of a scheme for the pathways and distribution of enzymes of nitrogen assimilation in A. cylindrica (Fig. 2). According to the scheme N_2-derived ammonia and glutamine formation occurs only in heterocysts. Most of the glutamine is transported to vegetative cells where it is metabolised to glutamate and various other amino acids respectively by glutamate synthase and aminotransferases located in vegetative cells. Part of the glutamine could be converted to glutamate directly in the heterocysts by the aminotransferase located there. The glutamate thus produced may function as a substrate for sustaining more glutamine formation. Glutamate could also be transported to heterocysts from vegetative cells either directly or as aspartate which could be transaminated in heterocysts. Glutamate dehydrogenase and alanine dehydrogenase have only minor roles in the primary assimilation of ammonia.

REGULATION OF NITROGENASE IN BLUE-GREEN ALGAE

Glutamine synthetase is known to exist in two forms in many bacteria. Under low levels of ammonia, GS exists in

the biosynthetically active unadenylylated state, whereas high levels of ammonia result in the inactive adenylylated state (Ginsburg & Stadtman, 1973; Wohlhueter et al, 1973). The unadenylylated GS has been shown to be a positive transcriptional activator of the nitrogenase operon (Streicher et al 1974; Tubb 1974). Experiments conducted at Stewart's laboratory (Dharmwardene et al, 1973) have shown that the adenylylation/deadenylylation control may not be operative in blue-green algae. We have shown that nitrogenase is coupled to GS in heterocysts (Thomas et al, 1977). There is the possibility that GS from heterocysts may behave differently from the GS isolated from whole filaments. However, we find that the GS extracted from isolated heterocysts of Anabaena L-31 also does not exhibit adenylylation/deadenylylation characteristics (Tuli & Thomas, unpublished results).

It has been shown (Stewart & Rowell, 1975; Wolk et al, 1976), that although NH_4^+ accumulates in the presence of MSX, inhibition of nitrogenase activity or heterocyst production does not occur in A. cylindrica. This suggests that, rather than NH_4^+, glutamine or a product of its metabolism is involved in nitrogenase regulation. Our results indicate (Thomas et al, 1977) that most of the glutamine synthesised is quickly lost

from heterocysts and will thus help nitrogenase to remain derepressed. This, to my mind, may be a major way of regulation of the enzyme activity.

In the above context, we have examined some of the amino acids which have been shown to be early products of glutamine metabolism in blue-green algae (Thomas et al, 1975). The effects of glutamic acid and aspartic acid were found to be interesting. Unlike many other amino acids tested, exogenous glutamic acid inhibited N_2-supported growth, but not NO_3-supported growth, suggesting that it prevents growth by inhibiting nitrogenase (David and Thomas, unpublished results). Experiments on the time course of inhibition of nitrogenase indicate that glutamic acid inhibits the synthesis of new nitrogenase protein. Relatively small amounts of aspartic acid relieve the inhibition of nitrogenase and normal growth ensues. These results suggest that the ratio in vivo of glutamic acid to aspartic acid may be a major factor in the regulation of blue-green algal nitrogenase. Recently Shanmugham and Morandi (1976) found that a mixture of amino acids repressed nitrogenase of Klebsiella pneumoniae and suggested that amino acids play an important role as regulators of nitrogen fixation.

REGULATION OF HETEROCYST FORMATION

Current knowledge indicates that sources of nitrogen such as nitrate and ammonia inhibit the formation of heterocysts

(Fogg, 1949; Thomas & David, 1971). However, when ammonia accumulates in the presence of MSX heterocyst differentiation is not affected (Stewart & Rowell, 1975). It has been proposed that heterocysts inhibit nearby vegetative cells from becoming heterocysts (Wolk, 1967; Wilcox, 1973). This inhibition, thought to be mediated by a diffusible substance produced in heterocysts, has been shown to be relieved by rifampicin (Wolk & Quine, 1975).

We have partially characterised and compared the role in heterocyst production of nitrogenous substances excreted by Anabaena L-31 during N_2-supported and NO_3 supported growth. Under both situations most of the excreted nitrogen is in the form of polypeptides. The polypeptide from either condition has a molecular weight near 5000 daltons but has distinctly different amino acid composition. When added to a culture, the peptide from N_2-fixing condition enhances the formation of heterocysts whereas the peptide from non-N_2 fixing condition inhibits their formation (Thomas, 1977; David & Thomas, unpublished results). The inhibitory effect of the latter is relieved significantly by the addition of the former suggesting that the relative proportion of "inducer" and "inhibitor" substances regulates heterocyst differentiation in blue-green algae.

CONTROL OF SPORULATION IN BLUE-GREEN ALGA

Spores of blue-green algae withstand adverse conditions and start vegetative growth once favourable conditions are restored. Sporulating strains are therefore better for field application. Results obtained by Fisher & Wolk (1976) indicated the secretion of a substance which stimulates sporulation in Cylindrospermum licheniforme. We have found extracellular substances which stimulate sporulation in A. torulosa (Thomas, 1977). The substances resolved as two separate peaks on elution from Sephadex G-25 column (Fernandes & Thomas, unpublished work). Comparison of the elution pattern with standard biomolecules indicates that both substances have a molecular weight near 5000. Preliminary analyses suggest that both substances are glycopeptides. Addition of either substance induces early sporulation and a doubling of the number of spores (Fig. 3).

SODIUM TRANSPORT AND METABOLISM IN BLUE-GREEN ALGAE

Sodium is known to be required for the growth of blue-green algae (Allen & Arnon, 1955; Brownwell & Nichclas, 1967). Many blue-green algae thrive in saline terrestrial habitats (where sodium is the main element causing salinity) and some have been reported (Singh, 1950) to be efficacious in reclaiming such areas. However, very little is known about the

transport of sodium (Na^+) and its role in the metabolism of these organisms.

Using the radioactive isotopes $^{24}Na^+$ and $^{22}Na^+$ we have examined (Apte & Thomas, 1974; Apte & Thomas, unpublished results) the uptake and extrusion of Na^+ in a fresh water blue-green alga *Anabaena* L-31 and in *Anabaena torulosa*, normally prevalent in moderately saline habitats. In the absence of added potassium (K^+) or in the presence of low amounts of it, Na^+ uptake increased with increasing external Na^+ concentration. This increase was triphasic suggesting that at least three sites, with different affinity for Na^+, are operative in the uptake of the ion. Low levels of K^+ stimulated Na^+ uptake. At equimolar concentrations of Na^+ and K^+ or at higher concentrations of Na^+, uptake of Na^+ was little affected by K^+. But at low concentrations of Na^+, potassium severely inhibited Na^+ uptake.

Uptake of Na^+ in *Anabaena* L-31 was inhibited in the dark or in the presence of DCMU/3-(3,4-dichlorophenyl)-1,1-dimethylurea/ in light, whereas in *A. torulosa* uptake was enhanced under these conditions. The results indicate that differential mechanisms of Na^+ transport exist in these species: active processes facilitating uptake in the fresh

water alga Anabaena L-31, as opposed to active processes facilitating extrusion of Na^+ in the saline water alga A. torulosa.

In a simplified mineral medium A. torulosa did not grow in the absence of combined nitrogen and showed low acetylene reduction activity even when ample molybdenum was provided. Provision of 0.25 mM Na^+ more than doubled acetylene reduction and ensured normal growth of the alga. In Anabaena L-31 added Na^+ was less effective in enhancing acetylene reduction. Work in progress in our laboratory is aimed at further elucidating the role of Na^+ in the metabolism of these organisms.

The work on long-term sodium uptake has indicated that blue-green algae accumulate sodium. After a period of growth on soil the thin layer of top soil with algae may be removed leaving behind soil with reduced salinity. This procedure may also help in augmenting the nitrogen content of the soil. In laboratory experiments on the reclamation of saline kharland soils, after 5 weeks growth of A. torulosa, there was substantial decrease in salinity (Fig. 4) and considerable enhancement in nitrogen content of the soil.

FIELD APPLICATION OF BLUE-GREEN ALGAE

The role of blue-green algae in the nitrogen economy of tropical paddy fields has been recognised for long

(Singh, 1961) and many field experiments were conducted especially in India (Subrahmanyan, 1972), Japan (Watanabe, 1960) and Phillippines[1]. Another approach in India has been to exploit the additive effect of blue-green algae (Venkataraman, 1975) when applied along with the normal quantity of nitrogen fertiliser. This effect however, cannot be attributed to N_2 fixation. An old practice common in some south-east Asian countries involving the use of the water fern Azolla is currently receiving considerable international attention (Evans & Barber, 1977). Azolla, which harbours Anabaena azollae (Peters, 1974) endophytically in its fronds, grows profusely in shallow ponds and can be used as green manure.

The advent of the acetylene reduction technique stimulated considerable field work involving rhizobial N_2 fixation and in associative symbiosis. However, except for the work of Balandreau et al (1975) and the recent annual reports of the International Rice Research Institute, Phillippines, no published studies are available of such work, in other laboratories, on tropical rice paddies.

The pitfalls of using acetylene reduction as a measure of N_2 fixation has been recently brought out in

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[1] International Rice Research Institute 1976. Annual Report for 1975, Los Banos, Phillippines.

the work of David & Fay (1977) who showed that long periods (over one hour) of incubation with acetylene led to a multifold increase of acetylene reducing activity in blue-green algae and in N_2-fixing photosynthetic and heterotrophic bacteria.

In field experiments, which we have been conducting during the last few years, nitrogen fixation was examined by 30 min assays of <u>in situ</u> acetylene reducing activity and determinations of soil nitrogen levels after 2-3 weeks of algal growth. Algae selected on the basis of laboratory experiments were examined for their field performance, initially using sterile soil in pots. Acetylene reduction activity was determined at 4 day intervals after algal inoculation and at 4 hr intervals on specific days (David & Thomas, unpublished work). Maximum acetylene reduction activity occurred 8 days after inoculation in three of the algal species tested and their mixtures. The hours of optimum acetylene reduction activity varied with the species used, one alga preferring the cooler morning and evening hours while the others showed higher activities during the warmer and brighter hours of the day. These algae were then examined for their capacity for survival on nonsterile soils. <u>A. torulosa</u> and <u>Nostoc</u> 4 routinely survived on such soils and were used to inoculate field plots.

In field trials normally conducted in paddy soils blue-green algae are broadcast after transplantation of rice seedlings. Although the algae fix N_2 and grow, very little of the fixed nitrogen would become available to the rice plant. The algal organic nitrogen needs to be degraded to yield ammonia or nitrate, compounds which are easily assimilated by plants. We therefore took a different approach. Plots (20 m^2) were flooded and inoculated with algae three weeks before transplantation. Rates of acetylene reduction were several fold higher in these plots. Twenty days after inoculation the soil was ploughed over and kept in a semi-dry condition for 3 days to facilitate degradation of organic nitrogen. Our results show that flooding the plots for 3 weeks prior to transplantation by itself increases the soil nitrogen level (Thomas, 1977). However, increase in soil nitrogen was substantially higher in the plots inoculated with Nostoc 4 and in those where a mixture of this alga and A. torulosa was inoculated. The level of soil nitrogen after algal growth corresponded to 980-1520 kg total N/hectare whereas the initial value was 375-405 kg total N/hectare. Grain and straw yield were comparable to that obtained when 80 kg N/hectare of urea was used.

These results hold promise for developing suitable strains and practices for large scale applications. Both _A. torulosa_ and _Nostoc_ 4 sporulate readily and have therefore better chance of survival and ececis in the field. _A. torulosa_ is amenable to large scale fermentor cultures and may be manipulated to obtain extensive and early sporulation by using the sporulating substances which we have isolated. This alga, as I have indicated earlier, has the additional advantage of growing well under moderately saline situations and could be employed in certain areas in India where paddy is grown in such conditions. _Nostoc_ 4 grows rapidly in moist or flooded soil and can be easily cultivated in shallow ponds or tanks prior to broadcasting on larger areas.

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Fig. 1. Light-induced enhancement of O_2 consumption by heterocysts isolated from _Anabaena_ L-31. Shaded portions indicate dark O_2 uptake and unshaded portions indicate enhancement, over the dark rate, of O_2 uptake in light. Subsequent bars in an experiment show the decline in O_2 uptake in sequential light dark regimens using the same heterocyst suspension.

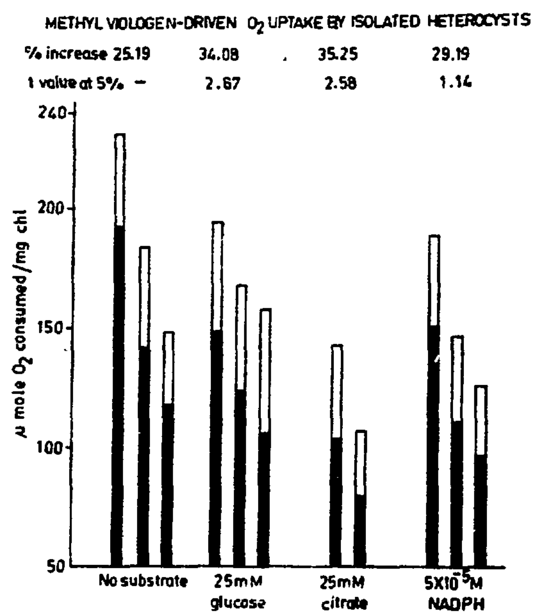

Fig. 2. A tentative scheme for the initial pathways and distribution of the enzymes of nitrogen assimilation in *Anabaena cylindrica*. The major pathway and its secondary routes are indicated by heavy and light lines respectively. Minor pathways are represented by dashed lines. Question mark (?) indicates uncertain pathway or source (vegetative cell or heterocyst) of metabolite.

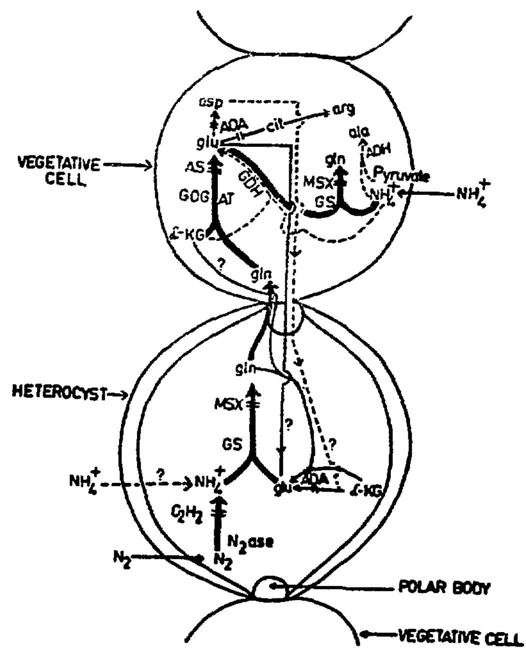

Fig. 3. Stimulation of sporulation in *Anabaena torulosa* by extracellular glycopeptide. Per cent of total heterocysts with attached spores (■—■), with two spores (□—□) and more than two spores (O—O) per heterocyst when a partially purified glycopeptide was added to culture. Dashed lines indicate respective control values.

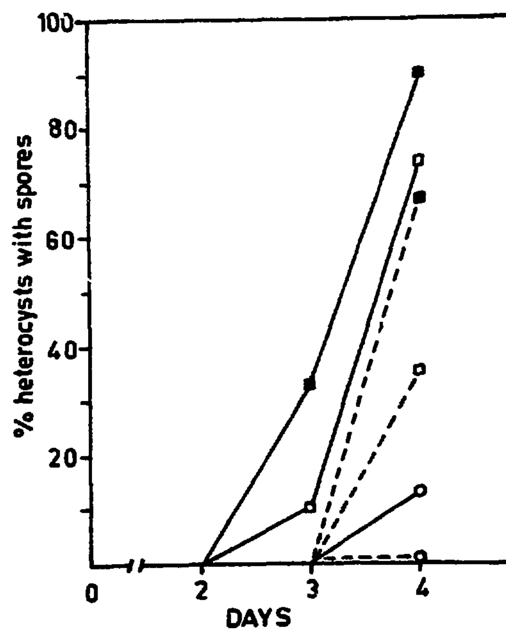

Fig. 4. Effect on saline soil by inoculation of *Anabaena torulosa*. Saline soils 1, 2 and 3 represent paddy field soil mixed with *kharland* saline soil in the ratio 3:1, 2:1 and 1:1 (wt/wt) respectively.