Trends in Biotechnology, Vol. 2, No. 6, 1984

RP/057

Why don't plants fix nitrogen?

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The process of biological nitrogen fixation is restricted to a small number of prokaryotic organisms and the majority of the world's crop plants depend on chemical fertilizers to achieve optimum yields. Need this always be the case or have recent advances in the genetic analysis of nitrogen fixation made the construction of nitrogen-fixing plants a realistic goal?

For many years scientists and agronomists have appreciated that in most agricultural areas crop yields are limited by the availability of fixed nitrogen to the plant. Biological nitrogen fixation is found only in a limited, heterogeneous collection of prokaryotic organisms and of these very few are used in agriculture, namely those which form a symbiosis with leguminous crop plants, such as peas, beans and forage legumes, and cyanobacteria (called blue-green algae) which are used directly or in symbiosis as green manure in rice cultivation. Therefore, increases in yield of most of the world's main food crops, wheat, maize, barley and potatoes, depend on the addition of nitrogenous fertilizers. The use of such fertilizers has increased exponentially over the last 75 years whilst the input of biologically-fixed nitrogen has remained more or less constant[1].

Against this background the attraction of developing nitrogen-fixing cereal plants has led some authors to speculate on the possibilities of employing genetic engineering in an attempt to succeed where nature appears to have failed. Two principal approaches to the problem have been suggested. The first involves manipulating existing associations between nitrogen-fixing bacteria and crop plants. These may either involve rhizosphere associations of the type reported between *Azospirillum* and certain tropical grasses or far more complex symbioses such as those between Rhizobia and legumes. Claims for the contribution made by rhizosphere diazotrophs to the nitrogen economy of plants have varied widely and it is presently unclear whether this approach has long-term potential. Modification of the more complex symbioses requires detailed knowledge of the genetic control of the bacterial–plant association. Recent studies of nodulation suggest that it is an extremely complicated process. Not only are the products of the bacterial *nod* and *nif* genes required but estimates suggest that at least 20 plant genes (most of which have yet to be identified) are involved in nodule development and in establishing an effective symbiosis[2]. Consequently, the task of genetically engineering novel symbioses is very complex.

The second approach involves transferring nitrogen fixation (*nif*) genes into the plant genome. Following the transfer of *nif* genes from *Klebsiella pneumoniae* to *Escherichia coli* in 1972[3], a number of hypothetical schemes were proposed for the 'infection' of higher plant cells with *nif* DNA[4,5]. However, when these proposals were made much of the information needed for such a programme was either in a very primitive state or non-existent. The location of the *nif* gene cluster in *K. pneumoniae* had been defined but the number of *nif* genes, their organization, and the function of their products were all unknown. Gene cloning as we now know it was in its infancy and plant molecular biology was yet to make an appearance on the scientific scene.

In the last ten years rapid progress has been made in all these areas of science such that it now seems reasonable to review the situation and ask whether we are now in a position to answer the question: Why don't plants fix nitrogen? Is there a fundamental incompatibility at the genetic, biochemical or physiological level between nitrogen fixation and plant cell biology or do we simply need to speed up the process of evolution by lending a scientific hand?

The *nif* gene cluster and its regulation

The *nif* gene cluster of *K. pneumoniae* has been the model system for genetic analysis of nitrogen fixation for the last fifteen years[6]. The cluster comprises 17 genes arranged in eight operons which cover 23 kb of the genome (Fig. 1). Genes *nifH*, *nifD* and *nifK* encode the three structural polypeptides of nitrogenase; *nifH* determines Kp2, a dimeric iron-sulphur protein, and *nifK* and *nifD* code for the two subunit types of Kp1, a molybdenum- and iron-containing protein which in its native form is an $\alpha_2\beta_2$ tetramer. The *nifF* and *nifJ* gene products are a flavodoxin and a pyruvate:flavodoxin oxidoreductase respectively, which together comprise a specific electron transport pathway to nitrogenase. Kp1 contains an iron-molybdenum cofactor known as FeMoco which is believed to be the site of nitrogen reduction. At least four genes, *nifB*, *nifN*, *nifE* and *nifV*, are known to be involved in the synthesis of cofactor, and *nifS* and *nifU* may also have a role in this process. The *nifQ* product may sequester molybdate for FeMoco synthesis. The functions of the *nifX* and *nifY* products have yet to be determined. Finally, the *nifL* and *nifA* gene products regulate trans-

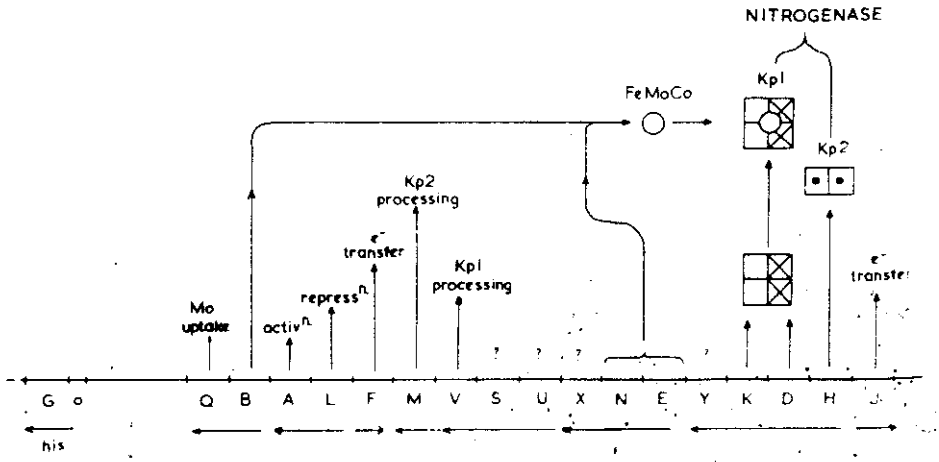

Fig. 1. The nif gene cluster of Klebsiella pneumoniae.

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cription of the *nif* operons as described below.

In *K. pneumoniae* nitrogenase synthesis is repressed in strains grown on ammonia or in the presence of oxygen and is derepressed under anaerobic nitrogen-limiting conditions. This pattern of regulation is controlled by a two-tier cascade involving three nitrogen control genes *ntrA*, *ntrB* and *ntrC* which coordinate general nitrogen metabolism in the cell, and the *nif*-specific genes *nifL* and *nifA*.

In derepressing conditions the *ntrA* and *ntrC* products are required to activate transcription of the *nifLA* operon. Then the *nifA* product together with the *ntrA* product activate transcription of the other *nif* operons (Fig. 2). The requirement for *ntrC* product can be overcome by linking the *nifA* gene to a constitutive promoter thus allowing *nif* expression in an *ntrC*$^-$ strain[7]. Predictably, however, strains carrying a constitutive *nifA* gene still require *ntrA* for *nif* transcription[8,9]. Repression by fixed nitrogen is mediated by the *nifL* gene product which inactivates *nifA* product, and by the *ntrB* product which in turn inactivates the *ntrC* product. The *nifL* product also appears to mediate oxygen repression by inactivating *nifA* product (Fig. 2). DNA sequence analysis of the *nif* promoter regions and of the promoters of other genes subject to *ntr* control has demonstrated that they do not have the usual canonical RNA polymerase recognition sequences found in promotors of enteric bacteria[10–12]. Consequently, it has been proposed that the *ntrA* product may be a novel sigma-like protein which modifies RNA polymerase to recognize *ntr*-controlled promoters. This proposition has important consequences for genetic manipulation of *nif*, which will be discussed later.

Potential locations for the *nif* genes

The genome of the plant cell is complex and there are theoretically three potential locations for introducing foreign genes; the nucleus, the mitochondrion or the chloroplast. In choosing the best location for a foreign gene, consideration should be given both to the problems of achieving gene expression (i.e. transcription and translation) and to the subsequent question of whether the gene product synthesized will function as required. In this sections; the biochemical or physiological problems will be mentioned later.

The mechanisms controlling gene expression in eukaryotic nuclei are markedly different from those in prokaryotes. However, by using plant-vector systems based on the *Agrobacterium tumefaciens* Ti plasmid, combined with some elegant genetic engineering, it has recently been

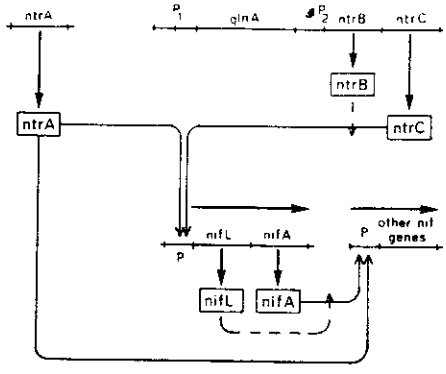

Fig. 2. Cascade model for regulation of the nif genes. Solid lines indicate positive regulatory circuits and broken lines indicate negative regulatory circuits. Horizontal arrows show transcriptional organization.

possible to express single prokaryotic genes in the nuclei of higher plants[13,14]. Nevertheless, because of the complexity of the *nif* gene cluster, expression of the *nif* genes in a plant nucleus would necessitate individual manipulation of at least 15 genes.

Recent advances in mitochondrial genetics have demonstrated that mitochondria in animals, fungi and higher plants all employ DNA codes slightly different from that found almost universally in bacteria and in the nuclear DNA of higher organisms[15]. Hence ensuring that the faithful translation of the prokaryotic *nif* genes in a mitochondrion is not a realistic proposition.

By comparison with mitochondria, chloroplast genes appear to use the universal genetic code and DNA sequencing studies have demonstrated that the signals for transcription and translation in chloroplasts closely resemble those of enteric bacteria[16]. Consequently, the chloroplast appears to provide the most suitable environment for expression of the *nif* gene cluster in a plant cell and it is possible that the *nif* genes could be expressed in a chloroplast without extensive genetic manipulation, provided that the appropriate regulator-

Chloroplast molecular biology

Chloroplast DNA ranges in size from 85 to more than 190 kb. Maps of the chloroplast genome from a number of plant species are available and chloroplast rRNA and tRNA genes show considerable homology with those of *E. coli*. Comparison of the upstream non-coding regions of several chloroplast genes shows a short sequence near to the initiator codon which is complementary to the chloroplast 16S rRNA and is therefore analogous to the prokaryotic Shine–Dalgarno sequence. Chloroplasts contain their own DNA-dependent RNA polymerase which is distinct in some respects from bacterial polymerases. However, sequences preceding the transcription initiation sites of chloroplast genes closely resemble the −10 and −35 regions required for polymerase recognition and transcriptional control in bacteria, although the distance of these sequences from the proposed transcription initiation site is rather variable. Finally the 3′ untranslated regions of several chloroplast genes determine a corresponding mRNA which can be folded into a characteristic stem-loop structure strongly resembling prokaryotic rho-independent transcription termination sites.

The apparent prokaryotic nature of transcription and translation in chloroplasts has been further highlighted by demonstrations that chloroplast genes for the large subunit of ribulose bisphosphate carboxylase (*rbcL*) can function to produce a product of correct size in *E. coli*[17]. Furthermore, *E. coli* RNA polymerase can apparently recognize the chloroplast transcription initiation sites[18].

Engineering the *nif* genes

Given that the chloroplast is the location of choice for introducing the *nif* genes into the plant genome, we can ask how the *nif* gene cluster might be modified to obtain gene expression in the chloroplast. Introducing an unmodified *nif* cluster into the chloroplast would not be expected to result in *nif* expression because the *ntrC* and *ntrA* products are needed to activate *nif* transcription. However, as described earlier, the constitutive synthesis of *nifA* product removes the requirement for *ntrC* product. Therefore, if the *ntrA* and *nifA* gene products could be synthesized in a chloroplast, it is pos-

achieved by the chloroplast machinery without recourse to further genetic manipulation. Such an approach has the attraction of maintaining the inherent regulatory features of the *nif* DNA sequences intact and causing minimal alteration to this finely balanced gene complex. To obtain optimal expression of *nifA* and *ntrA* products these genes would probably need to be fused to suitable chloroplast promoter sequences. These promoters could be chosen so as to determine the conditions under which the regulatory genes are expressed. Hence, the *nif* regulatory genes could be engineered to express only in certain developmental types of plastid or under certain physiological conditions. This point will be considered in more detail later. A hypothetical *nif* gene package for introduction into the chloroplast is presented in Fig. 3.

Our model of *nif* regulation in *K. pneumoniae* requires the regulatory proteins to interact in some way with the bacterial RNA polymerase. It would therefore be necessary for the *ntrA* and *nifA* gene products to interact with chloroplast RNA polymerase in order to activate transcription of the *nif* genes. *E. coli* RNA polymerase has been shown to transcribe chloroplast genes *in vivo* and *in vitro* and conversely maize plastid RNA polymerase will transcribe bacterial DNA *in vitro*. However, in the presence of a 27 kDa polypeptide designated S factor, the maize enzyme shows preferential *in vitro* transcription of maize plastid DNA[19]. Thus, it is not possible to predict how effectively chloroplast RNA polymerase would transcribe bacterial genes, in particular *nif* genes, if they were introduced into a chloroplast. Assuming that the *nifA* and *ntrA* products could function effectively in concert with chloroplast RNA polymerase, regulation of *nif* gene transcription would effectively be controlled by the activity of the chloroplast promoters to which the regulatory genes were fused. If *ntr*-activatable promotors could not be recognized in the chloroplast using this approach, it would be necessary to substitute chloroplast promoters for each of the *nif* promoters. Such an approach, whilst complex, still only requires modification of 6 or 7 promoters as opposed to the modification of 15 genes required for expression of this system in the plant nucleus.

Having considered how the *nif* genes might be engineered for expression in the chloroplast, it is necessary to consider ways in which such a gene package might be introduced into this organelle. There is at present no system for transforming cloned genes back into chloroplast. Plant nuclear gene transformation has been demonstrated in two systems. One uses the Ti plasmid, which has so far only been shown to insert into nuclear DNA of higher plants[13,14] and a second system has been described in the unicellular alga *Chlamydomonas rheinhardii* using a plasmid carrying a yeast replication origin to transform a cell wall-deficient mutant[20]. *Ch. rheinhardii* could also provide a potential model system for development of plastid transformation. The organism has a single large chloroplast, the genome of which has been extensively characterized. Autonomously replicating sequences have been isolated from the chloroplast genome and these could provide the basis of a vector system[21]. Such a system would also require selectable markers which are only expressed in the chloroplast. The recent demonstration that a herbicide-resistant mutant of *Ch. rheinhardii* carries a mutation in a chloroplast gene coding for a protein of photosystem II[22] suggests that such markers can probably be obtained readily.

Finally, methods must be developed for introducing chloroplast vectors back into the organelle genome. Three possible methods could be considered: (1) the method used to achieve nuclear transformation as described above; (2) a two-step transformation in which a plasmid with both nucleus- and chloroplast-specific markers is first transformed into the cytoplasm and, having established replication within the organism, selection is made for the chloroplast-specific marker on the plasmid which could only be expressed in the plastid; and (3) a microinjection method to introduce cloned genes directly into the chloroplast. The large chloroplast of *Ch. rheinhardii* makes it a very suitable organism for such an approach.

At this point we have described the main considerations in constructing a *nif* gene package for expression in a plant chloroplast and possible approaches for introducing this package into the plastid. There are however post-translational problems to be overcome in order to synthesize an active nitrogenase enzyme which will fix nitrogen in this novel environment.

Post-translation problems

Both components of the active nitrogenase enzyme contain iron-sulphur clusters and, in addition, Kp1 contains the iron-molybdenum cofactor, FeMoco. Hence, the active enzyme requires adequate supplies of inorganic iron, sulphur and molybdenum, all of which should be available in the plant cytosol.

In *K. pneumoniae* electrons are transferred to nitrogenase by a *nif*-specific electron transport chain comprising the products of *nifJ* and *nifF* (a flavodoxin). However, component 2 of nitrogenase can be reduced *in vitro* by sodium dithionite or reduced dyes such as methyl viologen and in some organisms, such as *Clostridium pasteurianum*, by a ferredoxin. A variety of electron donors are therefore capable of functioning with nitrogenase and indeed an *Azotobacter* ferredoxin has been used to mediate electron transfer between illuminated spinach chloroplasts and *Azotobacter vinelandii* nitrogenase[23]. Hence there should be no problem in using reducing power generated by chloroplasts to feed into the *nif*-specific electron transport chain.

Nitrogenase consumes up to 15 moles of ATP per mole of nitrogen reduced and therefore the provision of a high concentration of ATP is another requirement for active nitrogen fixa-

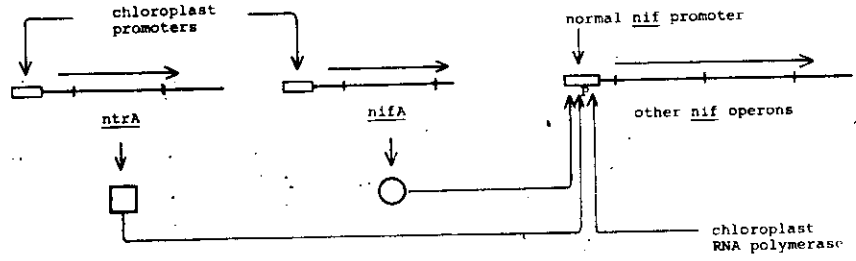

Fig. 3. Proposed manipulation of nif regulatory genes to engineer nif gene expression in a plant chloroplast.

tion. Carbon dioxide fixation by the enzymes of the Calvin cycle also uses large amounts of ATP and, as these reactions take place in the chloroplast, this organelle is well supplied with energy.

The ammonia produced as a consequence of nitrogen fixation is normally assimilated by the glutamine synthetase/glutamate synthase pathway. Since both these enzymes are found in plastids[24] there is no apparent barrier to assimilation of fixed nitrogen in the chloroplast.

Finally, we need to consider the problem of the oxygen sensitivity of the nitrogenase enzyme. Both component proteins of nitrogenase are irreversibly inactivated by oxygen. This problem has been solved in a variety of ways by nitrogen-fixing bacteria. In *K. pneumoniae*, a facultative anaerobe, the *nifL* gene product acts to repress *nif* gene transcription in the presence of oxygen; whilst *Azotobacter* uses both increased respiration and the synthesis of a protective protein which can complex with nitogenase, to protect the enzyme from oxygen damage[25].

The nitrogen-fixing cyanobacteria provide the closest analogy to a nitrogen-fixing plant cell as they have the additional problem of protecting nitrogenase from their own, photosynthetically produced, oxygen[26]. Of those cyanobacteria which fix nitrogen aerobically, most possess heterocysts. These differentiated cells lack photosystem II of photosynthesis and therefore provide an environment in which nitrogen fixation is spatially separated from photosynthetic oxygen evolution. In non-heterocystous cyanobacteria, such as *Gleocapsa*, temporal separation is used so that most nitrogen fixation takes place in the dark.

Plastids occur in all tissues of higher plants although the photosynthetically-active chloroplast is by far the most widely studied form of this organelle. Little is known at present about factors controlling differentiation of the plastid but in the future it should be possible to identify promoters in the plastid which are subject to different developmental controls. Consequently, the choice of appropriate promoters for the *nif* regulatory genes should allow control of the conditions under which *nif* is expressed in a plant.

With these considerations in mind it

those employed by other aerobic diazotrophs in avoiding oxygen damage to nitrogenase. Spatial separation might be achieved by regulating *nif* so as to be expressed only in non-photosynthetic root tissue. This would create a situation analogous to that in the legume symbiosis and would locate the *nif* genes in that part of the plant where nitrogen assimilation normally occurs. Alternatively, in certain tissues (notably the bundle sheath cells of C4 plants such as maize) oxygen evolution by photosystem II does not occur, and these cells should provide a relatively 'low oxygen' environment, comparable to that found in the heterocyst. Studies on expression of the gene for the large subunit of ribulose bisphosphate carboxylase (*rbcL*) have shown that transcripts of this gene in maize are confined to bundle sheath cells and are not present in photosynthetic mesophyll cells[27]. Consequently, the tissue-specific expression of *nif* genes in the bundle sheath cells of a C4 plant might be achieved by linking the *nif* regulatory genes to the *rbcL* promoter. Finally, it may be possible to regulate the genes temporally so that they are expressed only in the dark; a situation analogous to that found in *Gleocapsa*. Plastid genes subject to light-induction, 'photogenes', have already been described[28] but a converse class of 'dark-induced' genes has not yet been identified.

Answering the question

In this article we have outlined possible strategies for developing nitrogen-fixing plants. We have concentrated on one particular approach, namely the introduction of bacterial *nif* genes into the plastid genome, because the state of scientific knowledge is such that this is the only strategy which can be approached systematically at present. It is possible that future analyses of the *Rhizobium*/legume symbiosis will make the genetic manipulation of the plant symbiotic genes and the development of new symbioses a feasible alternative. Either of these approaches could potentially result in a significant disturbance of the metabolic balance of the new host plant and as such it is not possible to state categorically whether either is likely to succeed. Likewise, it is impossible to give a time-scale for a genetic engineering project of this

plant molecular biology culminating in the expression of prokaryotic genes in the plant nucleus. Comparable advances in our understanding of biological nitrogen fixation allow us to be optimistic about the prospects for demonstrating transcription of *nif* genes in higher plants, although the synthesis of an active nitrogenase enzyme is likely to be a far more complex task.

Regarding the question posed in the title of this article, we are not aware of any definitive reason why plants should not fix their own nitrogen and it may well be that biological nitrogen fixation is such a recent process in evolutionary terms that the evolution of nitrogen-fixing higher plants has not yet occurred. The experimental approaches described here may reveal barriers to expression of nitrogen fixation in higher plants. However, the effective co-existence between photosynthesis and nitrogen fixation in the cyanobacteria suggests to us that there is a potential for the construction of diazotrophic crop plants.

Acknowledgement

The authors wish to thank J. R. Postgate for his comments on the manuscript.

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Biotechnology of ergot alkaloids

Zdeněk Řeháček

The ergot alkaloids are a group of pharmacologically active compounds produced primarily by species of *Claviceps*, a fungal parasite of grasses and cereals.

Ergot is of historical interest: medieval midwives collected the fungus from naturally infected plants and used it in the induction of childbirth and in the control of postpartum bleeding. Many additional uses of the ergot alkaloids have been discovered since then, and yet the method of production has persisted to this day basically unchanged.

However, submerged culture of *Claviceps* is starting to supersede the use of systematically infected rye and a number of new opportunities, including the development of high yielding fungal strains and the production of semi-synthetic alkaloids, have become apparent.

Chemical structures

The common part of the chemical structure of ergot alkaloids is the tetracyclic ergoline ring system (Fig. 1a). In the naturally occurring alkaloids, the basic ergoline system is methylated at the nitrogen atom in position 6 and carries a further C atom at position 8. In most cases there is a double bond in the 8–9 or 9–10 positions. Typical examples are the clavine alkaloids, lysergic acid, amides of lysergic acid (Fig. 1b, c, d) and the peptide alkaloids, ergopeptines (Fig. 2). The pharmacologically active laevorotary lysergic acid series and the inactive dextrorotary lysergic acid series differ only in configuration at C-8. The ergopeptines are characterized by a modified tripeptide structure containing L-proline, another amino acid and an *α*-hydroxy-*α*-amino acid which form cyclols with a carbon from the proline carboxyl group[2]. Ergopeptines are the only natural substances shown to have cyclol structure. Ergopeptams[3,4] represent a group of noncyclol peptide alkaloids. Ergot alkaloids can be investigated at concentrations as low as 1 p.p.m. by techniques such as high-performance liquid chromatography[5–7] and mass spectrometry[8]. HPLC can be used for separation and quantitative analysis of a mixture of ergot alkaloids as well as for the study of the kinetics of isomerization reactions and degradation reactions.

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Biological activities and therapeutic applications

The ergot alkaloids cannot be regarded as a simple pharmacological entity. Their diversity and wide range of biological effects may be explained by assuming[9] that: (1) the ergot compounds act on many different receptor sites, including receptors for noradrenaline, serotonin and dopamine; (2) many of them exert both agonistic and antagonistic actions on the same receptor; and (3) in some cases one and the same compound acts on all three types of receptor but in different ways, i.e. as agonist and/or antagonist.

Cytostatically active clavines (Table 1) interfere with DNA synthesis in mammalian cells. Agroclavine and elymoclavine possess antibiotic activity[11]. Some ergot derivatives influence the secretion of several hormones and act at the central nervous system level. Some of the semi-synthetic ergot alkaloids are currently used in medicine or clinical tests.

The alkaloids of ergot find application in the treatment of a variety of clinical conditions the most important of which are postpartum hemorrhage, migraine and other vascular headaches, venostasis, orthostatic hypotension, senile cerebral insuffiency, conditions associated with hyperprolactinemia, and Parkinson's disease. Stimulation of uterine motor activity is the oldest therapeutic use of the group of drugs.

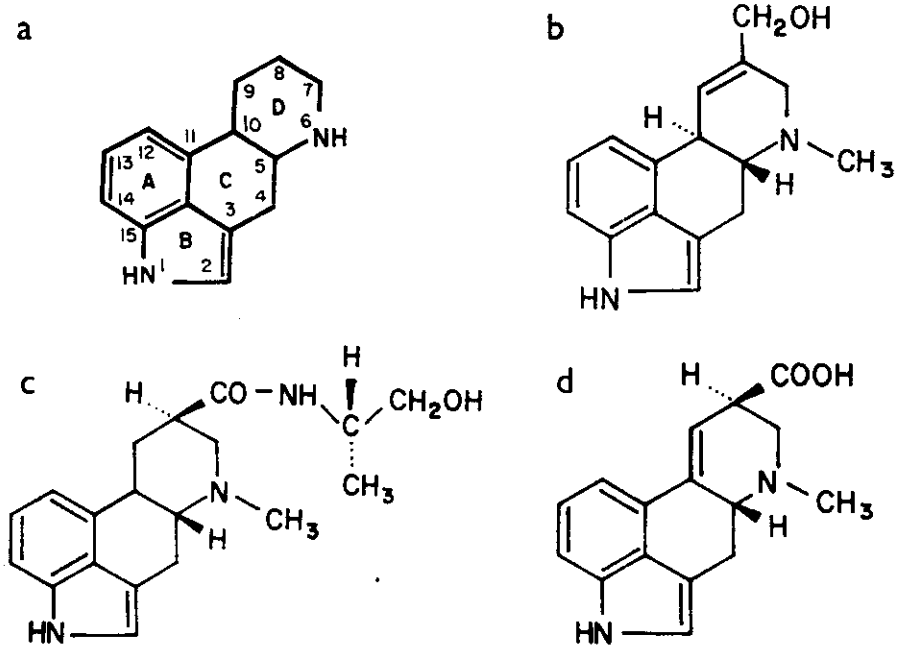

Fig. 1. The ergoline ring system and some derivatives (a) Ergoline, (b) Elymoclavine, (c) Ergometrine and (d) D-Lysergic acid.