N-methyl-N'-nitro-N-nitrosoguanidine (NTG) is a powerful and widely used mutagen, and its use in Rhizobium japonicum has revealed that it induces a high rate of mutation at low rates of survival. *R. japonicum* appears to be extremely sensitive to NTG. Rate of survival of *R. japonicum* in NTG varied as a function of pH and the suspending medium. Growing organisms in liquid media were found to be more sensitive thereby implying that the lethal action of NTG was more pronounced when growing cultures were exposed. This observation was similar to that observed in *E. coli* (317). Frequency of mutation induced by NTG varied as a function of survival and depended upon the marker selected. Maximum auxotrophs were obtained at 1000μg/ml NTG giving a survival of less than 0.1%. Whereas for the isolation of antibiotic resistance mutants, maximum were obtained when lower concentrations of NTG were used, 100 μg/ml, giving a survival of 2%. Adelberg *et al* (317) found that NTG induces at least one mutation per *E. coli* cell under conditions permitting over 50% survival. However, a high yield of auxotrophs (over 40%) was obtained using 1000 μg/ml NTG with a survival below 5% (317). The optimal conditions for mutation using NTG established in some other systems are as follows:
Dictyostelium discoideum 0.5 mg/ml for 30 min (467), Streptomyces coelicolor 3mg/ml at pH 9.0 for 1 to 1.5 hr with 2% survival (468), Saccharomyces pombe 2% auxotrophs were obtained at 20% survival (469). Moore (470) obtained best results with Coprinus lagopus at 15 µg/ml NTG for 50 to 70 min exposure at 1.85% survival. Therefore survival in NTG and the number of mutations induced varies from organism to organism. This may in fact reflect the susceptibility of the exposed organism to NTG, depending in part, upon surface composition of the organism and the ability of NTG to bind and approach its target site. Freese and Bautz-Freese (471) suggested that NTG being lipophilic could accumulate in the membrane. The ability of NTG to penetrate and reach its site in the membrane and the susceptibility to NTG could also vary from organism to organism. This could be a possible explanation for the differences in survival reported in different organisms including Rhizobium.

The data presented in Chapter IV can be interpreted in terms of NTG inducing mutations at only a restricted number of sites at one time. These sites are presumed to be replication forks. The appearance of peaks of mutation at regular intervals of time during sequential mutagenesis of a synchronous population of R. japonicum clearly
suggests that NTG-induced mutation does not take place at random but occurs at points where replication was in progress at the time of treatment. The five markers selected (rif, str, amp, kan and azi) for mutation in forward direction clearly showed that each peak was distinct from the other four. However, data obtained from sequential mutagenesis is a coarse mapping technique as it permits location of unknown markers in relation to a uni-directional mode of replication. The precision of this technique is dependant on the reproducibility of the synchronization technique. In E. coli, thymine starvation (356) and release of inhibition of DNA synthesis after treatment with nalidixic acid (378) and rifampicin (389) have been some of the methods employed to induce synchrony prior to mutagenesis with NTG. Synchronization of the population results in the appearance of sharp well defined mutation frequency peaks (378, 389, 472). The sharp peaks of mutation achieved for all the five markers in R. japonicum indicates that phenethylalcohol (PEA) is able to induce synchrony in Rhizobium. More detailed experiments involving the tracing of DNA synthesis during and after the synchronising procedure would prove useful to further improve this technique in Rhizobium. It will also enable a more accurate mapping of markers.
Guerola et al. (357) have shown that NTG induces a high frequency of closely linked multiple mutations. They have observed that the index of closeness is the ratio of the frequency of double mutants in the population selected for one of them to the frequency of the other in an unselected population (357). We made use of this technique to further improve the mapping positions obtained by sequential mutagenesis. The data obtained for six markers in two strains of R. japonicum are summarised in Fig. 1. This data is in agreement with a map (Fig. 2) that assumes that chromosomal replication is bidirectional, with two replication forks starting in opposite directions from the origin; the origin of replication being located somewhere between kan and amp, close to the latter. The selection after NTG mutagenesis leads to the enrichment of only those mutants which co-mutate with the marker selected. For example, str selection leads to enrichment of kan (Fig. 1D) and vice versa (Fig. 1B), rif is enriched on amp and azi selection (Fig. 1A), amp is enriched on rif selection (Fig. 1C), azi on amp, rif and pea selection (Fig. 1E) and pea on azi selection (Fig. 1F). Markers closely linked or situated at a position in which it would be simultaneously replicated show high frequency of double mutation. This procedure of examining double mutants induced by NTG, complements the previous technique of
Marker selected after MTG mutagenesis

Fig. 1: Double mutation analysis of *R. japonicum* D211 and *R. japonicum* 61A76 with NTG

- □ strain D211
- ■ strain 61A76
mapping with a synchronized culture. The precision is much greater and it can even be used to distinguish between a uni- or bi-directional mode of replication (389). This technique can be used for fine mapping in conjunction with the coarse mapping provided by sequential mutagenesis of synchronized cultures. It also enables mapping of the origin of replication (383). The exact measurement of distances between the various markers (Fig. 2) is not possible with the present data, as DNA synthesis will have to be studied to measure the speed of the replication fork movement, the length of the replication region, the time required for one round of replication and the occurrence of reinitiation of DNA synthesis. Also it will have to be determined if the movement of the replication forks in either direction is uniform.

Fig. 2: Genetic alignment in R. japonicum.

The possibility that the double mutants induced by NTG could be due to pleiotropic effects of the markers themselves was ruled out because neither spontaneous azi
and str mutants showed any double mutation nor the azi and str mutants isolated after U.V. mutagenesis showed any double mutation. This clearly indicated that the double mutations induced were due to the property of NTG itself and not due to a pleiotropic effect of the resistance markers themselves.

The experiments on the pre-treatment of R. japonicum with ampicillin and penicillin (Chapter III) resulted in marked lysis of the cells following the addition of lysozyme. It appears that pre-treatment with the antibiotic increased permeability of the cell or disorganized the cell-wall structure and hence rendered the mureopeptide substrate accessible to the action of the enzyme. These results give some insight into the structure of the R. japonicum cell wall. The inability of lysozyme alone to cause lysis could be due to the fact that the mureopeptide layer was not accessible. The reasons for the inability of bactericidal concentrations of penicillin and ampicillin to cause lysis of the cells could be due to the differences in the lipid, protein and carbohydrate components of the cell wall of these bacteria. Electron microscopy has shown that three layers are distinguishable in the cell wall of gram negative bacteria in general (364-365), the murein layer being sandwitched between the inner membrane and outer layer (366-368). Recent election
micrographic evidence suggests that the trilaminar structure of the wall exists in *R. japonicum* (137).

Bacteriophage that lyse capsulated bacteria often give rise to plaques surrounded by a transluscent halo (421,424,473) caused by the diffusion of a polysaccharide depolymerase. Phage M-l gave such a plaques and an exo-polysaccharide depolymerase was found to be associated with its infection of *R. japonicum* D211. Phages infecting several species provided with capsules have been found to display capsular depolymerases (421,473-477). The enzyme from phage M-l could be isolated from the phage infected cell lysate. The substrate curve showed that the enzyme was regulated by the substrate and the double reciprocal plots gave non-linear kinetics. However, a similar effect could be brought about if the substrate was complex and contained inhibitors which resulted in inhibition at high substrate concentration. Although the substrate was extensively purified of proteins, lipids and nucleic acids the possibility of the presence of an inhibitor cannot be entirely rules out. The rather long initial lag in the V vs S plot may or may not mean an allosteric effect and may be a gross effect of a large, complex substrate molecule. No kinetic information is available from other
systems on the regulation of phage depolymerases by their substrates although in the case of \(P_{22}\) it has been shown that products can reduce the reaction rate (430).

Phage M-1 was found to recognize the exopolysaccharide (EPS) as well as its constituents (glucose, galactose, glucuronic acid and glucosamine). The close similarity in the properties of adsorption and those of the EPS depolymerase viz, requirement for divalent cation and inhibition with EDTA indicate that this enzyme may well be a part of phage M-1 itself. Many capsular phages are known to induce so called polysaccharide depolymerases (421,474,478-481). In most cases, these enzymes have been shown to be associated with the phage particles, and correspond to the base-plate part (420,430). For *Aerobacter* and *Klebsiella* phages too, this has been suggested (474, 481). The aminoacid composition of \(P_{22}\) base plate parts (430) resembles that of some polysaccharide depolymerases (474,481), \(T_4\) tail fiber (482) and spike proteins of phage \(\varnothing x 174\) (483) which are all responsible for attachment of phage to the cell surface. Phage M-1 was shown to contain a base plate; electron micrographs showed (299) a distinct knob like structure at the end of the tail.

Characterization of the receptor revealed that the phage recognised the pyranose moiety, and the C-6, C-1
and C-2 positions were important in the recognition process. Glucuronic acid appears to be the sugar which is specifically recognised in the EPS during adsorption. The inhibition of Phage M-1 with glucosamine was very severe and indicated that it could cause irreversible inactivation of the phage. Glutamine also gave a strong inhibition. The presence of glucosamine in the membrane could perhaps be a site for the irreversible adsorption of phage M-1. Although the titrability of phage M-1 is lost in the presence of glucosamine even after dialysis, it is not known if it causes ejection of phage DNA.

Intracellular replication of phage M-1 in the host *R. japonicum* has revealed that it is dependent on the physiological status of the host. Some host functions are involved in the intracellular replication of Phage M-1. Although the phage has a virulent mode of replication it appears that some of the host genes play an important role in the lytic cycle of phage M-1. Mutation in the azi region of *R. japonicum* D211 exerts a strong influence on the replication of phage M-1 and lysogeny is preferred in these hosts rather than lysis. Lysogeny may be preferred because of the membrane alterations associated with mutation in this region. The azi region consists of closely linked genes involved probably in the structure/function of
maintenance of some hitherto unidentified membrane component involved in the lytic cycle of phage M-1.

We postulate that mutation at a locus designated hcl was responsible for lysogeny in azi hosts. hcl co-mutates with a high frequency with azi and pea on mutagenesis with NTG. The frequency of the azi hcl double mutation is very low among spontaneous and U, V. induced azi clones, indicating that mutation with NTG increases the incidence of double mutation in this region. Although the spontaneous and U.V. induced mutation data of azi region indicates that azi pea and hcl are separate genes, perhaps under same control, the possibility of pea and hcl being pleiotropic effects of the azi mutation cannot be entirely ruled out. However, two questions still remain: 1) Does phage M-1 lysogenise these azi mutants of R. japonicum? 2) If so, how does the hcl mutation cause lysogeny of phage M-1.

It is not known whether phage M-1 lysogenises in the classical sense. Phage M-1 can quite possibly enter a 'carrier state' in R. japonicum. The state in which bacterial cultures produce phage without lysing and during which the bacteria are resistant to super infection, has been called 'carrier state' (484) or viral persistance (485); alternatively phage M-1 could form 'lasting
complexes' as described by Fraser for T₃ (486). However, Carrier State or lasting complexes are not stable and phage-free sensitive clones can usually be isolated on growth in the presence of phage antiserium, or by serial subcultures of single colonies (48,486). The lysogenic state established by phage M-1 in these altered hosts was stable even after five serial subcultures of single colonies, and were also found to be inducible on irradiation with U.V. Whether the genome of phage M-1 integrates into the host chromosome or remains extrachromosomal is not known. A small titre of spontaneous release of phage was detected in lysogenic clones and could be due to reversion of the azi hcl loci which does show a high frequency of reversion and mutation.

The altered physiological state created by mutation in the azi pea hcl region could cause lysogeny by affecting DNA synthesis. Although we do not have any evidence for this in our culture, it has been reported that the azi pea region in E. coli is involved in the control of DNA replication (454,487-489). And recently Steinberg and Gough (490) showed that phage P₂₂ lysogenises at high efficiency when the DNA synthesising capacity of S. typhimurium is limited. Alternatively, the azi hcl mutants may be incapable of supporting vegetative
replication of the phage as has been found for the maf-l mutant in \textit{E. coli}. This mutation maps at 1 min on the \textit{E. coli} chromosome near azi (491) and prevented attachment of the F factor to the membrane, a step necessary for DNA replication.

The experiments presented in Chapter VII demonstrate that the competent physiological state is acquired by rhizobial cells through the mediation of a macromolecular cell product, a protein. Whether this factor is an enzyme or a complex factor needed for a more complex reaction is not known yet. It is clear, however, that the dramatic emergence of competence during growth can be explained by the production of this factor (CF). The CF could be isolated from the competent phase only and not from cultures at any other phase of growth. Secondaly, the inhibition of competence by chloramphenicol indicated that protein synthesis was required for the formation of competence. It is possible that the CF may be synthesised in this phase. Osmotic shock treatment of competent cells indicated that the CF may be located in some compartment near the cell surface, probably the periplasmic region of the cell. In \textit{E. coli} this procedure has been shown to release, cyclic phosphodiesterase, acid hexose phosphatase, and 5'-nucleotidase, three typical periplasmic enzymes (492).
The release of CF during the competence phase appears to be important for the development of competence. The culture at this stage is in the early log phase of growth when active cell wall synthesis would be occurring. Therefore, the release of CF from a compartment below the cell surface can be easily envisaged. In other transformation systems the topographic location for the uptake of DNA appears to be the equitorial region where nacent cell wall synthesis usually takes place (236,238,239).

The CF from *R. japonicum* is comparable to those isolated in other species of bacteria like *Streptococcus* (187,194), *Pneumococcus* (195) and *B. subtilis* (194,196); however the main difference exists in the size. Although most other CF's are of low molecular weight (464), the CF from *Rhizobium* appears to be large with an approximate mol. wt. of 82,000. However more studies on this protein will be required before any explanation can be given for this observation. We do not know if the *Rhizobium* CF contains subunits.

The extremely fast onset and spread of competence throughout the majority of the cells after a lag period is one of the most striking features of the competence phase of *R. japonicum*, whereas the dramatic spread of competence can be explained by the release of CF at that
stage. The data on varying the inoculum size on the development of competence clearly indicated the physiological incompetence of stationary and late log phase cells. This could be due either to the intrinsic incapacity of these cells to react with the activator (CF) or to the presence of an inhibitor, as was shown for pneumococci (188).

The observation that the competence of cultures does not stay constant but actually decays after 5 hr of growth in competence medium clearly demonstrates the inherent lability of the competent state itself. It may be concluded that the acquisition of competence is primarily a function of the cell concentration and probably reflects a particular growth stage of the bacteria in which they are capable of taking up DNA.