APPLICATION OF DNA HYBRIDISATION TO THE TAXONOMY OF *RHIZOBIUM TRIFOLII* AND RELATED SPECIES

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ABSTRACT

Rhizobia were grown in Yeast Mannitol broth in the presence and absence of K_2HPO_4. Increasing the concentration of yeast extract in the medium resulted in an increase in growth up to a certain concentration of yeast extract. Growth of rhizobia was inhibited by further increases in yeast extract concentration, especially in the presence of K_2HPO_4. The inhibition is due to glycine present in the yeast extract and is increased by the presence of monovalent cations.

The molecular weight of DNA extracted from rhizobia was determined by centrifugation in alkaline sucrose gradients. It was found that ^{32}P-labelled DNA was more fragile under shear than unlabelled DNA. Unlabelled DNA required 75 seconds and ^{32}P-labelled DNA required 56 seconds sonication to reduce the fragment size to 230,000 Daltons.

Labelled DNA prepared by a phenol-chloroform method was contaminated with polysaccharide and only low homologous hybridisation could be obtained. A hydroxyapatite-urea method of purifying DNA was developed which produced polysaccharide-free DNA. When labelled DNA was prepared by this method homologous hybridisation averaged 72%. Polysaccharide contamination of unlabelled DNA preparations did not effect the % relative reassociation.

Complete reassociation of heterologous DNA required a longer incubation time than did homologous DNA. The homologous reaction was complete after 32 hours (Cot 200) whereas heterologous DNA required an incubation of 40 hours (Cot 250) to achieve maximum reass ociation.

Deoxyribonucleic acid homologies were determined among 27 strains of *Rhizobium trifolii*, 4 strains of *R. leguminosarum* and 4 of *R. phaseoli*. Results from

related strains indicate that DNA homologies correlate with serological relationships and ability to form nodules on legume roots can be lost without detectable change in homology with an independent reference strain. All rhizobia which nodulated effectively on T. repens, T. subterraneum , T. ambiguum, and Vicia hirsuta formed one population with an average relatedness of 70% (range 49-94%) and ΔTm(e) of 0.2-7.5°C with respect to reference strains capable of nodulating the first two clover species. Two strains from African Trifolium species and one from a Japanese species were less closely related. The average relatedness of strains from Phaseolus vulgaris with clover rhizobia was 46% (range 37-50%) and Δ Tm(e) 6.5-10.0°C. Taxonomic revisions consistent with these observations are discussed. It is proposed that R. trifolii and R. leguminosarum should be combined and called Rhizobium leguminosarum Frank. Within this species various biotypes should be designated according to their plant specificity R. phaseoli should be retained as separate species and examined in more detail. The results are discussed in relation to proposed genetic basis for plant specificity.

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INTRODUCTION

1. Significance of the Genus Rhizobium

Although nitrogen is one of the most common elements on earth it is usually growth limiting for plants and animals, because these organisms can only use nitrogen in a bound form. At present 15% of the world supply of nitrogenous fertilisers is synthesised by the Haber-Bosch process (Quispel, 1974) in centralised industrial facilities, using non-renewable resources, such as natural gas, as starting materials. This process may well become uneconomic as costs of production and distribution increase (Hardy, 1974), but an increasing world population requires increased food production from the agricultural sector and additional fixed nitrogen will be required to meet this demand.

Atmospheric nitrogen is fixed biologically by free-living bacteria, blue-green algae, microbial associations in the rhizosphere and phyllosphere and symbiotic associations between microorganisms and plants. Additional fixed nitrogen must come from these sources (Ledeboer, 1978). The symbiotic association between root nodule bacteria and leguminous plants is perhaps the best understood and most widely exploited biological nitrogen-fixing system. It is estimated that 50-70% of world biological nitrogen fixation is carried out by Rhizobium-legume associations. (Quispel, 1974)

In New Zealand the Rhizobium-legume association is quantitatively the most important source of bound nitrogen. MacKinnon et al. (1976) state that biological nitrogen fixation contributes 800,000 tonnes of nitrogen per annum and sales of nitrogenous fertilisers totalled 25,000 tonnes in 1974. On this basis 97% of nitrogen used in New Zealand agriculture is fixed biologically. Approximately $3.0 million is invested in legume seed and $0.4 million in legume seed inoculants annually. Sixty-two per cent of

land sown with inoculated legume seed in New Zealand is sown with clover seeds. Consequently, the relationships between root nodule bacteria and white clover already have a special significance for New Zealand agriculture, and a comparatively minor increase in efficiency would considerably increase the productive capacity of our pastures.

2. Classification by Cross-Inoculation Groups

Fred, Baldwin, and McCoy (1932) listed sixteen cross-inoculation groups consistent with the published data of the time. They defined cross-inoculation groups as "groups of plants within which the root-nodule organisms are mutually interchangeable." They concluded that there was sufficient data available concerning the morphological, cultural, and physiological characteristics of legume root-nodule bacteria to justify the formation of six species in the genus Rhizobium. These six species were related to the agronomically important legume groups: lucerne, clover, pea, bean, lupin and soybean. The remaining ten cross-inoculation groups were designated Rhizobium species and included the cowpea, Lotus, and eight other less well-known groups. The ability of the organisms to cause the formation of nodules upon roots of certain species of the Leguminosae and not upon others, was the principal characteristic chosen to determine the species boundaries. They felt that this characteristic was as fixed and definite as any physiological characteristic of the organism. The six species were Rhizobium meliloti, R. trifolii, R. leguminosarum, R. phaseoli, R. lupinii, and R. japonicum.

The same six species still appear in the eighth edition of Bergey (1974), (Table I). However, Jordan and Allen (1974) recognise that the taxonomy of Rhizobium is controversial and indicate that the present classification can only be regarded as tentative. A classification based on cross-inoculation groups is retained until comprehensive

Table I: Species of Rhizobium and their characteristics (After Jordan and Allen, 1974)

Group	Species	Flagellation	Serum zone in titmus milk	Acid reaction in litmus milk	Growth	Hosts nodulated	Common name of group
I	R. trifolii	Peritichous	+	-	Fast	Trifolium	Clover
I	R. leguniosarum	Peritrichous	+	-	Fast	Pisum, Lens Lathyrus, Vicia	Pea
I	R. phaseoli	Peritrichous	+	-	Fast	Phaseolus vulgaris	French bean
I	R. meliloti	Peritrichous	+	-	Fast	Melilotus, Medicago, Trigonella	Lucerne
II	R. japonicum	Subpolar	-	-	Slow	Glycine max	Soybean
II	R. lupini	Subpolar	-	-	Slow	Lupinus, Ornithopus	Lupin

comparative studies, involving large numbers of bacterial strains from a wide variety of leguminous plants, have been undertaken.

The present classification system has been subject to controversy over a long period of time. Wilson (1944) reported over 500 examples of symbiotic promiscuity. These are instances in which a strain of rhizobium was found to infect plants in more than one of the established cross-inoculation groups. Today only one species, R. meliloti, satisfies the original nodulation criteria, and even here promiscuity has been observed (Graham, 1976). The problem is exacerbated by a report from Trimick (1973) of cowpea rhizobia able to nodulate a non-legume.

Published nodulation data do not give unequivocal support to the cross-inoculation group concept. To date only 8-9% of the 14,000 or so known species of leguminous plants have been examined for nodules and only about 0.3-0.4% studied with respect to their symbiotic relationships with nodule bacteria (Jordan and Allen, 1974). Moreover, nodulation studies generally have been strongly biased toward agriculturally important legumes. This is evident in Table I where the six Rhizobium specific names are derived from only twelve genera of plants. The tendency is to regard all rhizobia associated with previously untested legumes as similar to the slow-growing cowpea organisms (Norris, 1956). While fast-growing organisms isolated from such legumes must always be suspected of contamination with Agrobacterium (Graham, 1974) and be tested accordingly, there are numerous instances where fast-growing rhizobia similar to R. meliloti or R. trifolii have been isolated from root nodules of plants included in the cowpea cross-inoculation group (Norris, 1965; Trimick, 1965).

Rhizobia tend to lose effectiveness after serial cultivation on media containing certain amino acids, especially DL- or D-forms, and after many years of storage

on laboratory media (Jordan and Allen, 1974). Infectiveness is more stable than effectiveness but can still be lost (Labandera and Vincent, 1975). Subsequent identification is then dependent on knowledge of earlier nodulating capability.

An additional reason for questioning the existing classification lies in the possibility that infectiveness and effectiveness are plasmid-borne characteristics. Nuti et al. (1977) and others have unequivocally demonstrated the presence of large plasmids in four different species of Rhizobium. These plasmids comprise up to 3.8% of the chromosomal DNA and are stable. As a result of treatment with agents known to eliminate plasmids (acridine orange and ethidium bromide) Dunican and Cannon (1971) concluded that infectivity and effectivity might be plasmid controlled. Furthermore, Dunican and Tierney (1974) transferred the nitrogen-fixing (nif) genes of R. trifolii T1 to Klebsiella aerogenes. They concluded from indirect evidence that the nif genes were located on a plasmid in R. trifolii. Consequently, basing Rhizobium taxonomy on information carried on a small piece of DNA which may be part of an extrachromosomal element, is unsound.

3. Application of Numerical Taxonomy and Molecular Genetics to the Classification of Rhizobium

Because the existing classification has proved unsatisfactory alternative methods of classifying legume root nodule bacteria have been sought. These have included numerical taxonomy, DNA base ratio studies and DNA hybridisation. Despite differences in sample size and testing procedures the results obtained from such studies have shown a surprising degree of agreement.

Numerical taxonomic studies have been reported on Rhizobium and related groups on four occasions. Graham (1964) studied 100 features of 121 strains of Rhizobium, Agrobacterium, Beijerinckia, Bacillus and Chromobacterium

and concluded:

i) that the present species R. meliloti should continue unchanged.

ii) that the species R. leguminosarum, R. trifolii and R. phaseoli should be combined to form a single species which would be called R. leguminosarum.

iii) that the species Agrobacterium radiobacter and A. tumefaciens should be united and included as R. radiobacter in the genus Rhizobium.

iv) that the species R. japonicum and R. lupini should be combined with organisms of the cowpea miscellany to form a single species. Since these organisms appeared distinct from other rhizobia, further studies were suggested to determine the merits of placing them in a separate genus, Phytomyxa, the species to be known as Phytomyxa japonicum.

A. rhizogenes and A. rubi were not included in this study.

'tMannetje (1967) challenged some of these conclusions. Using a different sorting technique to re-analyse Graham's data, he suggested:

a) that A. tumefaciens and A. radiobacter be combined but not included in the genus Rhizobium until further studies had been made.

b) that the slow-growing root-nodule bacteria be placed in a single species and retained in the genus Rhizobium.

The results of Moffet and Colwells' (1967) and White's (1972) numerical taxonomic studies generally support Graham's conclusions. However, Moffet and Colwell included A. rhizogenes in the revised species R. radiobacter and proposed the retention of R. rubi into which strains presently designated A. rubi were to be placed. A. gypsophilae and A. pseudotsugae were removed from both Rhizobium and Phytomyxa as defined in the study.

De Ley and Rassel (1965) established a correlation between the DNA base composition (% guanine plus cytosine) of 35 strains of Rhizobium and their type of flagellation. They concluded that there were two groups of rhizobia. The peritrichously flagellated, fast-growing organisms contain DNA with a low guanine plus cytosine content in the range 58.6-63.1%; these organisms occur in all cross-inoculation groups investigated. (Lupin, Pea, Cowpea, Clover, Strophostyles, Bean, Lotus, Caragana, Lucerne, Wistaria, Robinia, Soybean). A comparison with Graham's (1964) data indicates that this group is constituted by two subgroups: R. leguminosarum and R. meliloti. The subpolarly flagellated, slow-growing strains have a somewhat higher guanine plus cytosine content in the range 62.8-65.5%; these organisms appear to be specialised mainly for the Lupin, Soybean, Cowpea, Lotus, Wistaria, and Robinia groups. De Ley and Rassel (1965) proposed that only one genetic species was involved, to be called R. japonicum.

Heberlein, De Ley and Tijtgat (1967) used the DNA-agar hybridisation technique to ascertain the relationships between Agrobacterium, Rhizobium and Chromobacterium. Strains of A. tumefaciens, A. radiobacter, A. rubi, A. rhizogenes, R. leguminosarum and R. meliloti exhibited a mean percentage DNA homology greater than 50 with A. tumefaciens and R. leguminosarum reference strains. A. tumefaciens, A. radiobacter, and A. rubi were indistinguishable on the basis of DNA homology, with strain variations for this group involving up to 30% of their base sequences. The remainder of the organisms studied fell into at least six distinct genetic groups:

i) A. rhizogenes which is more homologous to R. leguminosarum than to the A. tumefaciens - A. radiobacter group;
ii) R. leguminosarum;
iii) R. meliloti;
iv) R. japonicum, which had a mean DNA homology of 38-45% with the two reference strains;

v) Chromobacterium, which was as genetically remote from the reference strains as, for example, Pseudomonas; and

vi) A. pseudotsugae strain 180, which had a DNA homology with the reference strains of only about 10%.

The DNA homology results of Heberlein, De Ley and Tijtgat (1967) in association with numerical taxonomy, DNA base composition, and flagellation studies led De Ley (1968) to the species designations, summarised in Table Two, intermediate between those of Graham (1964) and 'Mannetje (1967). A. rubi was incorporated into the enlarged species R. radiobacter and A. rhizogenes was thought to be distinct enough to warrant a separate species R. rhizogenes. A. pseudotsugae, A. gypsophilae, and A. stellulatum were considered to have little relationship to the other strains and were removed from the genus entirely. De Ley (1968) preferred to draw the genus line at 40% so as to include R. japonicum in the genus Rhizobium instead of designating it Phytomyxa japonicum as did Graham (1964). However, he acknowledged that this decision was one of personal preference at that stage rather than a clear demarcation. Further DNA homology results are required to elucidate this point.

More recently Gibbins and Gregory (1972) compared 20 strains of Rhizobium and Agrobacterium by three methods of nucleic acid hybridisation. They confirmed the close relationships between R. leguminosarum and R. trifolii finding these to be indistinguishable from each other. They also found a close relationship between R. lupini and R. japonicum and with less certainty between R. meliloti and R. phaseoli. DNA from the three Agrobacterium strains used hybridised as well with DNA from the R. lupini and R. leguminosarum reference strains as did DNA from several Rhizobium strains. They clearly differed from Heberlein et al. (1967) in that they found R. phaseoli to be more related to R. meliloti than to R. leguminosarum and R. trifolii. They examined Graham's (1964) data and

Table II: Species of Rhizobium and their characteristics (obtained from Graham, 1976; as proposed by De Ley, 1968)

Species	Relationship to species of Jordan and Allen (1974)	Flagellation	%GC	Serum zone in litmus milk	Acid reaction in litmus milk	Growth rate	Nodule forming characteristics, special features
R.leguminosarum	R.phaseoli+ R.trifolii+ R.leguminosarum	peritrichous	59.0-63.5	+	-	Fast	Forms nodules on one or more of Trifolium, P. vulgaris, Vicia, Pisum, Lathyrus, Lens
R.meliloti	unchanged	peritrichous	62.0-63.5	+	+	Fast	Forms nodules on Melilotus, Medicago, Trigonella.
R.rhizogenes	A.rhizogenes	peritrichous	61.0-63.0	+	-	Fast	Causes hairy root disease of apples and other plants.
R.radiobacter	A.tumefaciens+ A.radiobacter+ A.rubi	peritrichous	59.5-63.0	+	-	Fast	Frequently produce galls on angiosperms. Produce 3-ketoglycosides.

/...

R. japonicum	*R. japonicum* + *R. lupini* + Cowpea miscellany	sub polar	59.5-65.5	-	-	Slow	Nodulates many different legumes including one or more of *Vigna*, *Glycine*, *Lupinus*, *Ornithopus*, *Centrosema*, etc.

pointed out that his evidence for merging R. phaseoli with the other two species was not compelling since one of his five strains of R. phaseoli had a higher percentage similarity with R. meliloti and A. tumefaciens than with R. leguminosarum and R. trifolii strains, and another strain had a higher percentage similarity with R. japonicum strains.

The numerical taxonomy and molecular genetics results are all at variance with those of Jordan and Allen (1974) based on the cross-inoculation group concept. A closer relationship exists between strains of Agrobacterium and the fast-growing rhizobia than between fast-growing and slow-growing rhizobia. Secondly, some strains separated into different species on the basis of host-infectivity data are more closely related to one another than to other strains with the same host infectivity. In view of this additional evidence, De Ley's (1968) classification (Table II) seems more acceptable than that of Jordan and Allen (1974) (Table I).

4. Determination of Base Sequence Homology

A shortcoming of conventional microbial taxonomy is that a uniform battery of tests is not applied to all bacteria. A test applied to one genus may not even be considered in another taxonomic group (Colwell, 1973). Tests indicative of a few phenotypic characters, indirectly representing the bacterial genome, are weighted to produce the taxonomy of microbial species.

Numerical taxonomy is based on a large number of phenotypic characters and weighting is not desirable (Sokal and Sneath, 1963). However, it can only utilise characters determined by a small proportion of the genome and the interpretation of the results depends partly upon the characters selected and partly upon the mathematical treatment applied to them. This latter point is illustrated

by the discrepancy between Graham's (1964) and 't Mannetje's (1967) conclusions.

The ideal classification system should give an indication of the sequence of bases in the DNA of the organisms being classified, since it is the expression of these sequences which give an organism its phenotypic characteristics. Genetic mapping of Rhizobium strains has recently been undertaken (Doctor and Modi, 1976; Meade and Signer, 1977; Kandorosi et al., 1977; Beringer and Hopwood, 1976) but it is too laborious for the taxonometric analysis of a large number of bacterial strains. The percentage guanine plus cytosine in DNA has been used in the classification of Rhizobium (De Ley and Rassel, 1965; Gibbins and Gregory, 1972) but it gives no indication of the base sequences in the DNA and indeed organisms with a similar percentage guanine plus cytosine need not be closely related.

Nucleic acid hybridisation permits a quantitative measurement of the proportion of DNA from two differnnt sources which contain base sequences sufficiently similar to reassociate under defined conditions. It has been used successfully in the classification of a wide variety of organisms (Hontebeyrie and Gasser, 1977; Brenner et al., 1978; Seki et al., 1978; Owens and Snell, 1976; Brenner et al., 1972). There are four basic methods of observing DNA hybridisation. In the earliest procedure a DNA/DNA hybrid was prepared between unlabelled DNA and DNA labelled with heavy isotopes (^{15}N, ^{2}H). The three kinds of DNA present after hybridisation can be separated on a CsCl density gradient by analytical ultracentrifugation (Schildkraut, Marmur and Doty, 1961). This technique has rarely been used. A much more convenient procedure consists of fixing single-stranded, unlabelled, DNA from each of the bacteria to be compared onto a solid support, such as an agar gel (Bolton and McCarthy, 1962) or a membrane filter (Nygaard and Hall, 1963; Denhardt, 1966; Warnaar and Cohen, 1966; Legault-Démare et al., 1967).

Radioactively labelled, single-stranded DNA of low molecular weight is incubated with unlabelled DNA from each organism and the extent to which it becomes bound, measured. The DNA-agar technique was used successfully on Rhizobium by Heberlein et al. (1967) and the DNA-filter technique by Gibbins and Gregory (1972). De Ley, Cattoir and Reynaerts (1970) proposed a spectrophotometric method, in which the initial rate with which single-stranded DNA from differing sources renatures, was followed by observing the decrease in optical density with time. Comparison of the rates at which homologous DNA renatures, with the rate at which a corresponding heterologous sample renatures, permits the calculation of the percent homology between two samples of DNA. This technique was used by Gibbins and Gregory (1972) on Rhizobium and was found to have a four-fold greater standard deviation than the DNA-filter technique and was also time consuming. A theoretical method for predicting maximal homology between two bacterial strains has also been described by De Ley (1969) and used by Elkan (1971) to study R. japonicum.

Bernadi (1965) and Miyazawa and Thomas (1965) reported separation of reassociated DNA by specific absorption onto hydroxyapatite, a calcium phosphate gel. Briefly, labelled, sheared, single-stranded DNA is reassociated with unlabelled fragments similarly prepared. The solution mixture is allowed to incubate until completion of the reaction and the solution is passed through a column of hydroxyapatite. The reassociated DNA specifically absorbs onto the surface of the hydroxyapatite and the single-stranded fragments pass through the column under the conditions used. The percent homology is obtained by expressing the radioactivity associated with double-stranded DNA as a percentage of the total radioactivity in the sample.

This method has the following advantages:

i) it is not necessary to immobilise the unlabelled DNA, and one need not be concerned with reassociated (labelled with unlabelled) DNA leaching out of agar or from a filter during thermal elution studies.

ii) the binding of labelled DNA fragments to unlabelled DNA from the same source is routinely 20-40% in agar (Brenner, Martin and Hoyer, 1967), as high as 70% on filters (Johnson and Ordal, 1968), and from 75-95% in free solution (Brenner and Cowie, 1968). The more complete reassociation obtained in free solution virtually rules out the possibility that binding is not representative of the entire DNA molecule.

iii) since in the hydroxyapatite method, unlabelled DNA is not immobilised, its optical density can be assayed. The optical density measurement provides an internal control for the ability of the unlabelled DNA to reassociate.

iv) the kinetics of DNA reassociation in free solution are typical of a second-order reaction and the rate of reassociation is inversely proportional to the size of the bacterial genome (Britten and Kohne, 1966) whereas reassociation kinetics in agar (McCarthy and Bolton, 1964) and on filters (Brenner et al., 1969) are more complex.

v) the precision of the hydroxyapatite method may be in the order of 1-2% of the mean value which is more reproducible than the other methods (Staley and Colwell, 1973).

The major disadvantage of column hydroxyapatite chromotography is that it is time consuming and only one or two columns can be handled simultaneously, whereas ten or more samples may be simultaneously processed using agar or filters. Because a large number of comparisons are required in a taxonomic study, speed of operations is important.

In 1969, Brenner et al. described a batchwise hydroxyapatite thermal elution method that allowed simultaneous handling of up to ten samples. The sensitivity and reproducibility of this centrifuge hydroxyapatite assay were identical to those observed in the column assay. This method has been extensively used in microbial taxonomy, especially by Brenner and his coworkers, and is the method chosen here as the most suitable.

5. Aim of the Investigation

The DNA hybridisation on Rhizobia to date have been concerned with its relationships to other genera, especially the Agrobacterium, and the relationships between different species of Rhizobium. The present study was undertaken to determine the intraspecific relationships of the agronomically important species R. trifolii and its relationship to R. leguminosarum and R. phaseoli.

MATERIALS AND METHODS

1. Microbiological Methods

1.1 Media

The Yeast Mannitol (YM) medium (Vincent, 1970) which was normally used had the following composition (g/litre): K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; mannitol, 10; yeast extract (Difco Laboratories, Detroit, Michigan, U.S.A.), 0.4. The medium was autoclaved at 121°C for 15 minutes. When solid medium was required 15g/litre of agar was added. ^{32}P-labelled DNA was prepared from cells grown in YM broth without K_2HPO_4.

1.2 Microorganisms

The bacterial strains included in this investigation are listed in Table III. The 35 strains of rhizobia were obtained from Mr R.M. Greenwood, Applied Biochemistry Division, D.S.I.R., Palmerston North, New Zealand, as slope cultures on YM agar. The method of Moustafa and Greenwood (1967) was used by Mr Greenwood to test the nodulation ability and effectiveness of each strain. All *R. trifolii* strains were tested on white clover (*Trifolium repens*) and on subterraneum clover (*T. subterraneum* c.v. Tallarook). A few strains were also tested on subterraneum clover c.v. Woogenellup. Tests were also made with *T. ambiguum*, *T. semipilosum* and *T. africanum* using the corresponding *R. trifolii* strains. The *R. leguminosarum* strains were tested on *Vicia hirsuta*, subterraneum and white clovers, and the *R. phaseoli* strains on *Phaseolus vulgaris*.

1.3 Cultivation

Stock cultures were maintained on YM agar slopes at 5° C and also as freeze-dried ampoules. Bacteria were transferred to fresh slopes at two month intervals. Before each use stock cultures were checked for purity by observing the colony morphology, Gram staining reaction of cultures grown on YM streak plates, and their growth on Brain Heart Infusion (BHI) agar (Difco).

Mass cultures for DNA preparation were obtained by transferring a well isolated colony on YM agar to YM broth (2 x 100cm^3) and incubating on a gyratory shaker for 2 days at 30° C. These cultures were used to inoculate YM broth (2 x 1400cm^3) which was incubated a further two days at 30° C, by which time it had reached the stationary phase of growth. The cells were harvested with a Sorvall RC-2B centrifuge fitted with a GSA head. Centrifugation at 10,400g for 15 minutes at 0-5° C gave a firm pellet of cells which was readily resuspended. When the cells were harvested the cultures were again streaked on YM and BHI agar and examined microscopically to detect contaminants. This method was also used to prepare radioactively-labelled cells, but K_2HPO_4 was omitted from the liquid media, and 20mc ^{32}P (as a sterile solution of orthophosphate in dilute hydrochloric acid) was added to the final culture (1 litre) after 4 hours incubation at 30° C. The ^{32}P was obtained from the Radiochemical Chemical Centre Ltd. (Amersham, Buckinghamshire, England) and the Australian Atomic Energy Commission (Lucas Heights, Sydney, Australia).

1.4 Growth Curves

Inocula for the experimental determination of growth rates from turbidity measurements were prepared in YM broth and in YM broth without K_2HPO_4. Volumes of each medium (20cm^3) were sterilised in 50 cm^3 flasks fitted with side arms which could be inserted into a Klett-Summerson colourimeter (Klett MFG Co., N.Y., U.S.A.). Each set of flasks was inoculated with culture (2.0cm^3) which had been

TABLE III Strains of *Rhizobium* included in this study and their ability to nodulate and fix atmospheric nitrogen (effectiveness) on two species of *Trifolium*

Strain Number	Geographical Origin[a]	Effectiveness on[b] *Trifolium repens*	Effectiveness on[b] *Trifolium subterraneum* cv Tallarook	Remarks
Rhizobium trifolii				
cc275e	Australia	E	E(d)	New Zealand standard inoculant strain
TA1	Tasmania, Australia	E	E	Australian & New Zealand standard inoculant strain
TA2	Tasmania, Australia	E	E	serologically related to, but not identical with TA1
WU95	West Australia	SE	E	Australian standard inoculant strain
WU290	West Australia	O	O	serologically identical with WU290iii
WU290iii	West Australia	O	O	non-nodulating variant of WU290
cc2480a	Greece	I	I(d)	ineffective variant of Australian standard inoculant strain for subterranean clover
TLN3	Japan	I	I	isolated in Japan from *T. lupinaster*
CB782	Kenya	O	O	a non-nodulating variant of the standard inoculant strain for *T. semipilosum*
K8	Netherlands	E	SE	reported to be good on acid soils

1/6	New Zealand	E	R	previous N.Z. standard inoculant strain
UNZ29	New Zealand	E	E	previous Australian standard strain, same origin as NZP1/6
514/1	Te Anau, N.Z.	E	E	-
514	Te Anau, N.Z.	O	O	non-nodulating variant of 514/1
540 (PDDCC2666)	Otago, N.Z.	E	E	reisolated from a seedling inoculated with PDDCC2153 and serologically identical with this strain
549	Palmerston North, N.Z.	SE	E	isolated from *T. uniflorum*
554	Palmerston North, N.Z.	ME	E	isolated from *T. pallescens*
560 (PDDCC2153)	Otago, N.Z.	E	E(c)	New Zealand standard inoculant strain
5039	Palmerston North, N.Z.	E	I	)isolated from ineffective nodules on
5117	Waikaremoana, N.Z.	E	I	)*Clianthus punicus* but nodulates)*Trifolium* spp.
SA3	South Africa	I	I	effective on *T. africanum*
cc227	Turkey	I	I	effective on *T. ambiguum*
cc229	Turkey	O	O	non-nodulating variant of a strain isolated from *T. ambiguum*
cc321a	Turkey	I	I	effective on *T. ambiguum*
550/2	U.S.A.	E	E	isolated from a mixed culture inoculant for *T. vesiculosum* from U.S.A.
SU202(Coryn)	Wales, U.K.	I	I	standard ineffective strain
cc277a	U.S.A. via Australia	I	I	isolated from *T. dasyphyllum* a U.S. alpine species

Rhizobium leguminosarum				
SU391	Australia	O	I	Australian standard inoculant strain for peas. Effective on *Vicia hirsuta*
TA101	Tasmania, Australia	O	I	Australian & New Zealand standard inoculant strain for peas & vetches. Effective on *Vicia hirsuta*
CB596	England	O	I	from Rothamstead Experimental Station. Effective on *Vicia hirsuta*
5225	Palmerston North, N.Z.	O	I	effective on *Vicia hirsuta*
Rhizobium phaseoli				
cc511	Australia	-	-	non-nodulating variant of Australian & New Zealand standard inoculant strain for *Phaseolus vulgaris*
5097	Lincoln, N.Z.	-	-	non-nodulating variant of strain isolated from *Phaseolus vulgaris*
5459	Lincoln, N.Z.	-	-	effective on *Phaseolus vulgaris*
CB971	Phillipines	-	-	non-nodulating variant of Australian & New Zealand standard inoculant strain for *Phaseolus vulgaris*

FOOTNOTES:

a. Alternative designations for the same strain are given in brackets. Strain numbers not prefixed by letters are N.Z.P. numbers.

b. Effectiveness ratings: effective, E; moderately effective, ME; slightly effective, SE; ineffective, I; no nodules, O.

c. Moderately effective (ME) on subterranean clover cv. Woogenellup.

d. Slightly effective (SE) on subterranean clover cv. Woogenellup.

prepared in the same medium and incubated at 30°C in a shaker water bath for 74 hours. Turbidity was measured every two hours in a colourimeter fitted with a blue filter (400-465nm) for 56 hours. After 74 hours the cultures were inspected for purity by Gram's stain and streak-plated on BHI and YM agar.

To determine the viable cell count in each culture, 0.1cm^3 of an appropriate dilution in sterile distilled water was aseptically spread over the surface of a YM agar plate. Duplicate dilutions were plated in duplicate. After incubating for 5 days at 30°C, the colonies were counted, and the average of the four counts was expressed as viable cells per cm^3 of original culture.

1.5 Cell Yield Experiments

The inocula for these experiments were prepared from 3-day cultures in YM broths. The culture (5cm^3) was placed in a sterile screw-capped Kimax tube and centrifuged at setting 80 in Sorvall Type A angle centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.) for 30 minutes, washed twice with 5cm^3 of sterile distilled water and resuspended in 5cm^3 sterile distilled water. The cell suspension was cultured on YM and BHI agar to detect any contaminants introduced during the washing process. Washed cells (0.5cm^3) were used to inoculate flasks containing 100cm^3 of both YM broth and YM broth without K_2HPO_4. Cultures were incubated on a shaker for 72 hours at 30°C.

To determine cell dry weight in a culture, 40cm^3 of culture were harvested by centrifugation at 16,300g for 30 minutes, washed twice in 40cm^3 of deionised water, resuspended in 2cm^3 of deionised water and transferred quantitatively to a tared glass tube. The centrifuge tube was further rinsed with 8cm^3 of deionised water in consecutive 4cm^3 washes. The cells were pelleted in a Sorvall

bench centrifuge fitted with a Type A rotor, at setting 80 for 30 minutes, and the supernate was removed. The tubes were dried at 75°C for 24 hours, cooled in a dessicator over concentrated H_2SO_4, and reweighed. The cell dry weight was expressed in mg/cm^3 of original medium.

To determine the cellular protein concentration in a culture, 10cm^3 of culture were harvested by centrifugation at 27,000g for 30 minutes, washed twice with deionised water and resuspended in 5cm^3 of deionised water. The cells were sonicated for 10 minutes to release intracellular proteins and the protein concentration of the resulting suspension was assayed by the protein dye-binding method of Bradford (1976). The protein concentration was expressed in μg/cm^3 of original medium.

2. DNA Preparation

2.1 Preparation of Unlabelled DNA by a Modification of the Method of Brenner et al. (1969)

2.1.1 Lysis of cells: the cells from 3 litres of YM broth were resuspended in 200cm^3 of lysing solution which had the following composition: 0.05M EDTA, pH 8.0, 0.05M Tris-HCl buffer, pH 8.0; 0.10M NaCl. Pronase solution[a.] and 25% w/v Sodium Lauryl Sulphate (SLS) were added to a final concentration of 50μg/cm^3 and 10mg/cm^3 respectively, and the suspension incubated overnight at 37°C.

Footnote: a. A 10mg/cm^3 Pronase (Calbiochem, P.O. Box 12087, San Diego, CA92112, U.S.A.) solution was prepared, incubated for one hour at 37°C to allow self digestion of impurities, and stored at -16°C in 5cm^3 aliquots.

2.1.2 Extraction of DNA: an equal volume of freshly prepared tris-saturated phenol was added to cells in lysing solution. This mixture was shaken vigorously until homogeneous and then centrifuged at 5,900g for 10 minutes. The upper aqueous phase was carefully removed. Sufficient 5M sodium perchlorate was added to make the aqueous phase 1M w.r.t. perchlorate. An equal volume of chloroform was added and the mixture shaken until homogeneous. After a further 3 minutes the mixture was centrifuged and the upper aqueous layer removed. To this were added two volumes of cold 95% ethanol. The two layers were slowly mixed with a $1cm^3$ pipette and precipitated DNA was collected on the pipette. This DNA was placed in $20cm^3$ of deionised water and shaken intermittently until it dissolved.

2.1.3 Purification of DNA: two further ethanol precipitations followed in the prescence of 0.1M NaCl. The DNA solutions were then made 0.1M w.r.t. NaCl, 0.05M w.r.t. EDTA, and 0.05M w.r.t. Tris-HCl buffer. Sufficient RNase[b.] solution was added to give $50\mu g/cm^3$ and the mixture was incubated at 60° C for one hour. After cooling, Pronase solution was added to give a final concentration of $50\mu g/cm^3$ and SLS to give 1% SLS, and the mixture was further incubated at 37° C for two hours. The DNA was re-extracted with phenol, washed twice with chloroform, precipitated with ethanol and dissolved in deionised water. Aqueous solutions of DNA were preserved by the addition of a drop of chloroform. Typical yields of DNA from 3 litres were 5-15mg.

Footnote: b. A $2mg/cm^3$ pancreatic ribonuclease (RNase) (Sigma Chemical Company, P.O. Box 14508, St Louis, MO 63178, U.S.A.) solution was prepared, incubated for 10 minutes at 90° C to destroy deoxyribonucleases, and stored at -16° C in $5cm^3$ aliquots.

2.1.4 Routine measurement of purity of DNA: this was assessed from absorption at wavelenghts between 210 and 300nm. The following spectral ratios were regarded as satisfactory: 258/230nm 1.8-2.3, and 258/280 nm 1.8-2.0. Absorption minimum and maximum were regarded as satisfactory when within 5nm of 230nm and 258nm respectively. If the purity was unsatisfactory further purification was undertaken.

2.1.5 Concentration of DNA: this was determined from the extinction at 258nm. DNA concentrations of 1mg/cm^3 have an extinction of 20.0 unsheared and 24.0 sheared (Brenner and Falkow, 1971).

2.1.6 Shearing of DNA: solutions (5cm^3) containing 200-1000$\mu g/cm^3$ (mean 400$\mu g/cm^3$) DNA in deionised water were sheared by sonication for 75 seconds at 0-5°C with a 100 watt ultrasonic disintegrator (Measuring and Scientific Equipment Ltd, Buckingham Gate, London SW 1, England) fitted with a 19nm probe and tuned for maximum output.

2.2 Preparation of Labelled DNA

2.2.1 Lysis and Extraction of DNA: the cells from 1 litre of YM broth without K_2HPO_4 were lysed under the same conditions as unlabelled cells but lysis was continued for only 4 hours and 50$\mu g/cm^3$ RNase was added to the lysing solution. After 3 hours at 37°C the quantities of Pronase, RNAse, and SLS were increased to 75$\mu g/cm^3$, 75$\mu g/cm^3$, and 15mg/cm^3 respectively. At the end of the lysing period a phenol extraction was carried out in the same way as for unlabelled DNA preparations.

2.2.2 Preparation of Hydroxyapatite: Hydroxyapatite (Bio-Rad Laboratories, Richmond, California, U.S.A.) was prepared in 0.0014M sodium phosphate buffer. ph 6.8

(PB) by adding 600cm^3 of 0.0014M PB to 100g of hydroxyapatite (HA). The suspension was allowed to settle and the supernatant decanted. A further 300cm^3 of 0.0014M PB were added, the suspension allowed to settle and the supernatant decanted. The HA suspension was completed by adding 300cm^3 of 0.0014M PB.

2.2.3 Hydroxyapatite Purification of DNA: DNA labelled with ^{32}P was purified by a modification of the hydroxyapatite-urea method (Britten et al., 1970). HA suspension (40cm^3) was washed once on a centrifuge (6,000g/2 minutes) with 8.0M urea in 0.14M PB and the supernatant discarded. Solid urea was added to the phenol-extracted DNA to make the solution 8.0M w.r.t. urea, 1.4M PB was added to make the solution 0.14M w.r.t. PB, and the mixture was stirred for 5-10 minutes until the urea dissolved.

The HA was suspended in this solution and separated by centrifugation. The supernatant containing protein and RNA was discarded and replaced with 100cm^3 8.0M urea in 0.14M PB. In this way, HA and bound DNA were washed 10 times on the centrifuge. Urea was then removed by washing the HA five times with 100cm^3 0.014M PB. The DNA was then released from the HA by washing it with 10cm^3 0.66M PB and then with four further 10cm^3 washes of 0.4M PB. The first wash was 0.66M PB as Britten et al. (1970) suggested that the volume of the HA pellet must be taken into account as a diluent. Most of the DNA was contained in the first two fractions and these were pooled and dialysed against two litres of 0.14M PB for two hours. Dialysis against two litres of 0.14M PB was repeated once. Typical yields were between 1-4mg in the first two fractions.

2.2.4 Shearing of DNA: once the DNA satisfied the purity criteria outlined for unlabelled DNA (Methods 2.1.4) it was sheared. Solutions (5cm^3) of labelled DNA, containing 71-137μg/cm^3 (mean 98μg/cm^3) in 0.14M PB, were sheared by sonication for 56 seconds at 0-5°C with a 100

watt ultrasonic disintegrator (M.S.E.), fitted with a 19mm probe and tuned for maximum output.

2.2.5 Stripping of DNA: this process was carried out to decrease non-specific binding to HA (Brenner et al., 1969). Sonicated, labelled DNA was placed in a boiling waterbath for 10 minutes and immediately cooled to 0°C in an ice bath. The resulting single-stranded DNA was then passed through a water-jacketted column containing $15cm^3$ HA suspension. The column was equilibrated with 0.14M PB + 0.4% SLS and held at 60°C. Five $10cm^3$ aliquots of 0.14M PB + 0.4% SLS were eluted from this column. The first two were pooled, made up to 0.28M PB, and used in reassociation.

3. Methods of Assessing the Suitability of DNA for Hybridisation Experiments

3.1 Determination of Melting Temperature of Double-Stranded DNA

A modification of the method of Mandel and Marmur (1968) was used. Reassociated DNA ($3cm^3$) in 0.28M PB was placed in a quartz cuvette. The absorbance at 258nm was read at 25°C in a Unicam SP 1800 (Pye Unicam Ltd, York St., Cambridge, CBI 2PX, England) spectrophotometer fitted with a temperature-controlled cell holder and an SP 876 Series 2 temperature programme controller. The temperature was raised to 60°C, the cuvettes removed and tapped gently to remove gas bubbles which stuck to the sides, and replaced in the cell holder. The temperature was raised in 5°C increments to 80°C and then in 1°C increments to 100°C. The absorbance at 258nm was read after equilibrium had been reached at each temperature. Absorbance values were not corrected for solvent expansion at the higher temperatures but were divided by the initial absorbance at 25°C and plotted against the temperature of the solution. The temperature corresponding to half the final increase in relative absorbance was the melting temperature, (Tm).

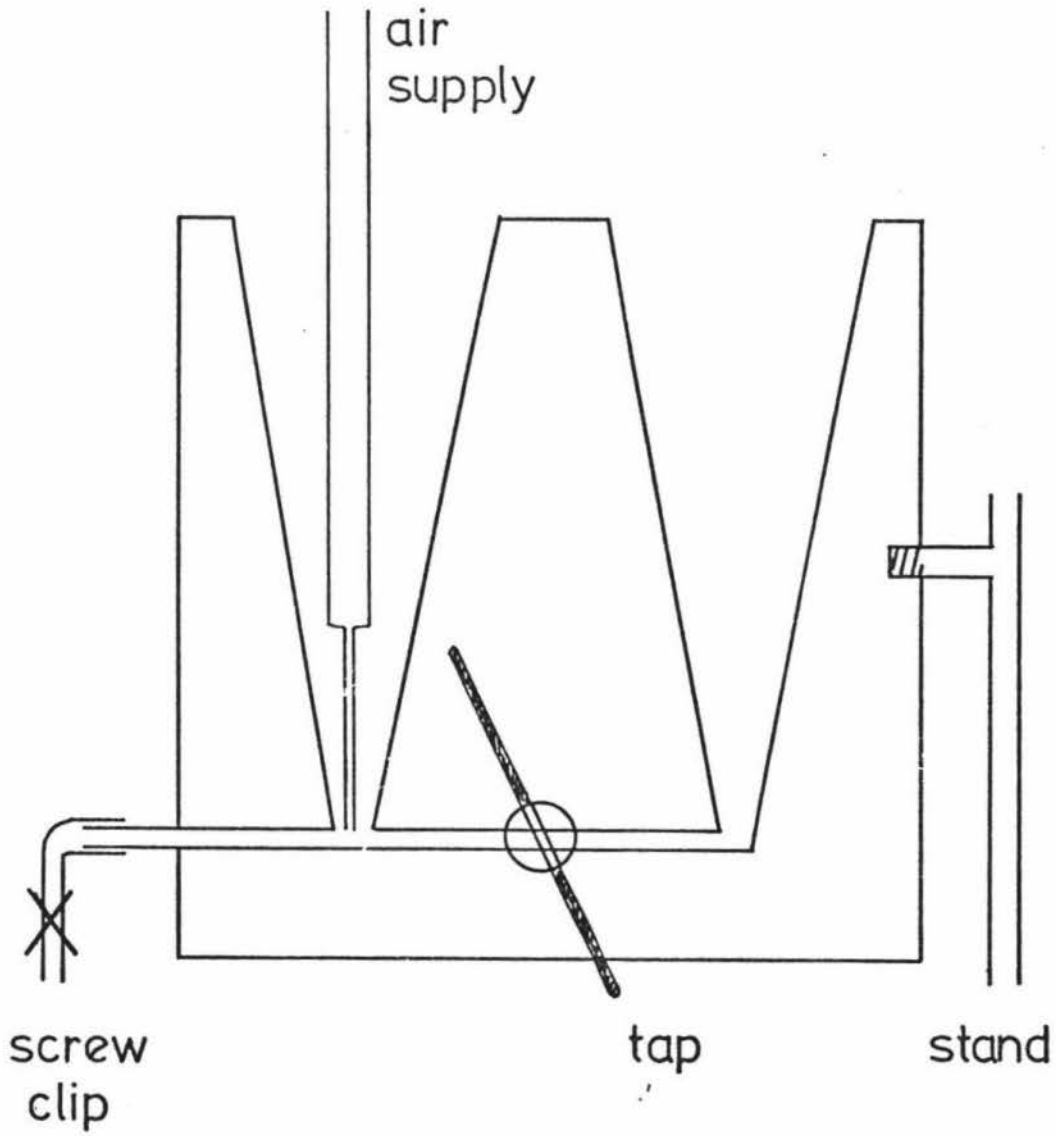


Figure 1. Apparatus for the preparation of linear sucrose gradients.

3.2 Molecular Weight Determination

The proceduredescribed by Abelson and Thomas (1966) was used to determine the molecular weight of DNA. The DNA was sonicated in 0.14M PB, made 0.9M w.r.t. NaCl, 0.1M w.r.t. NaOH, and 100$\mu g/cm^3$ w.r.t. DNA. A $5cm^3$, linear, alkaline, sucrose gradient in an SW 39L polyallomer tube was prepared using the apparatus in Figure 1. To the left-hand well was added $2.5cm^3$ of 20% (w/v) Analar sucrose in 0.9M NaCl and 0.1M NaOH. To the right-hand well was added $2.5cm^3$ of 5% (w/v) Analar sucrose in 0.9M NaCl and 0.1M NaOH. The air supply produced a constant stream of bubbles to allow mixing of the two sucrose solutions once the tap was opened. The tap and the screw clip were opened simultaneously and the gradient was formed in the tube with the alkaline sucrose being applied gently to the meniscus. The DNA sample ($0.1cm^3$ containing 10μg) was added to the gradient with a tuberculin syringe fitted with a 22-gauge needle. Three balanced tubes were centrifuged at 20°C in a SW 39L rotor in a Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California, U.S.A.) at $134,200g_{max}$. The time of centrifugation was varied for different samples to allow the DNA to migrate approximately halfway down the tube. Deceleration occurred without the brake being applied. The density gradients were unloaded by injecting a sucrose solution of greater density (30%) through the bottom of the tube, and displacing the gradient upward through an Isco Model 640 Fractionator (Instrumentation Specialties Company, Lincoln, Nebraska, U.S.A.). The gradient then passed through an Isco Type 6 Optical Unit attached to an Isco UA-5 Absorbance Monitor. An example of the results recorded on the monitor is shown in Figure 12.

The volume of sucrose solution required to displace the gradient to the DNA peak was recorded on the chart produced by the absorbance monitor. This volume was equal to

$\pi.r^2.h$ when r^2 = the square of the radius of the tube, and
h = the distance the sample moved in the gradient in cm. The volume divided by $\pi.r^2$ (1.13) was numerically equal to the distance moved in the gradient by the sample. This value was inserted in the formula proposed by Abelson and Thomas (1966) to obtain the sedimentation coefficient corrected to the viscosity and density of water at 20° C. The equation is:

$$S_{20,w} = \frac{0.00662\ (rf-ri)}{w^2.t}$$

where rf-ri = h = the distance the sample moved in the gradient in cm.
w = revolutions per minute
t = time of centrifugation in hours
The sedimentation coefficient was expressed in Svedberg units when multiplied by 10^{13}. The molecular weight (M) of the DNA was calculated from a formula proposed by Burgi and Hershey (1963): $S^{\circ}_{20,w} = 0.080M^{0.35}$

3.3 Colourimetric Determination of DNA Concentration

The sensitive diphenylamine assay of Burton (1956) was used. Diphenylamine reagent was prepared by adding glacial acetic acid ($100cm^3$) and concentrated H_2SO_4 ($1.5cm^3$) to 1.5g of diphenylamine. On the day of use $0.1cm^3$ of aqueous acetaldehyde ($1cm^3$ acetaldehyde in $50cm^3$ of distilled water) was added to each $20cm^3$ of reagent. A stock solution of calf thymus DNA ($0.2mg/cm^3$) was made by dissolving DNA in 5mM NaOH. A working standard ($0.1mg/cm^3$) was prepared by adding an equal volume of 1M perchloric acid ($HClO_4$) and heating at 70° C for 15 minutes. Unknown DNA solutions were prepared by adding 1.0M $HClO_4$ ($0.5cm^3$) to the DNA solution ($0.5cm^3$) and heating at 70°C for 15 minutes. The reaction was carried out by adding the DNA solution ($1cm^3$) to the diphenylamine reagent ($2cm^3$) and incubating at 30° C for 16 hours. The absorbance at 600nm was determined on a Unicam SP1800 spectrophotometer. The DNA concentrations reported are the means of duplicated determinations. Figure 2 shows a typical standard curve

obtained with this procedure.

3.4 Colourimetric Determination of Protein Concentration

3.4.1 The Method of Bradford (1976) was generally used but some protein determinations were carried out using the method of Lowry et al. (1951). The dye-binding reagent was prepared by adding 85% (w/v) phosphoric acid (H_3PO_4) ($100cm^3$) to Coomassie Brilliant Blue G-250 dissolved in 95% ethanol ($50cm^3$). Distilled water was added to a final volume of one litre. A bovine serum albumin protein standard was prepared gravimetrically and adjusted to $1mg/cm^3$ so the absorbance of the solution at 280nm was 0.66 (Kirschenbaum, 1970). Five cm^3 of the dye-binding reagent were added to $0.1cm^3$ sample containing 10-100μg of protein, and the contents mixed by vortexing. The absorbance at 595nm was measured after two minutes and before one hour against a reagent blank. The protein concentrations reported are means of duplicate determinations. Figure 3a shows a typical standard curve obtained with this procedure.

3.4.2 The Method of Lowry et al. (1951) using a bovine serum albumin standard was also used to determine protein concentration. Reagent A was prepared by adding 2.0% sodium tartrate ($1.0cm^3$) to a mixture of 2.0% Na_2CO_3 in 0.1M NaOH ($100cm^3$) and 1.0% $CuSO_4.5H_2O$ ($1.0cm^3$). Two volumes of distilled water were added to one volume of Folin reagent. Five cm^3 of freshly prepared reagent A was added to $1.0cm^3$ of sample containing 10-500μg of protein. The mixture was vortexed and allowed to stand for 10 minutes at room temperature. Then $0.5cm^3$ of diluted Folin reagent were added, and the contents were mixed and allowed to stand for 30 minutes at room temperature. The absorbance was determined on a Unicam SP 1800 spectrophotometer. The protein concentrations reported are means of duplicate determinations. Figure 3b shows a typical

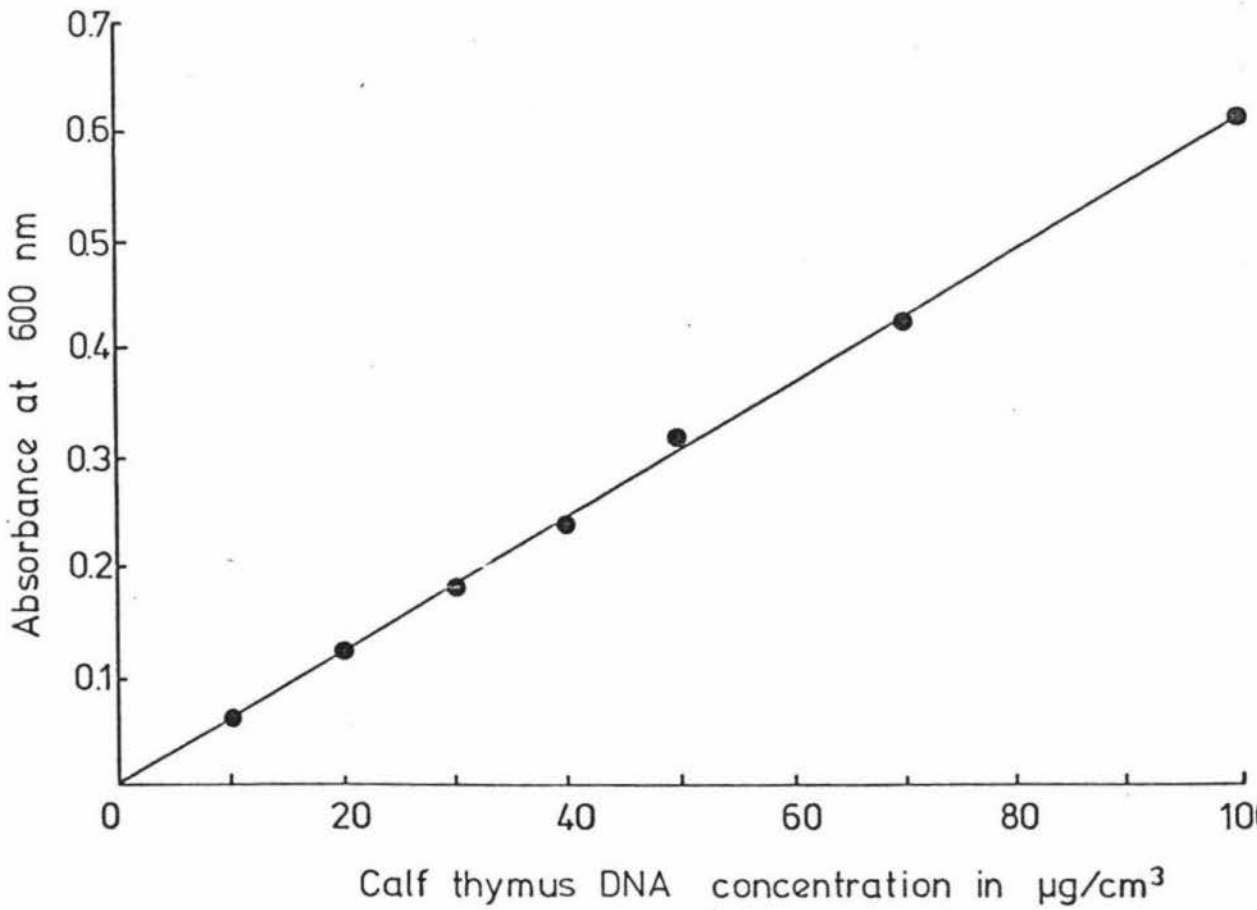


Figure 2. Colourimetric determination of DNA concentration by the method of Burton (1956)

standard curve obtained with this procedure.

3.5 Colourimetric Determination of Polysaccharide Concentration

3.5.1 The Method of Trevelyn and Harrison (1952) was initially used to determine the concentration of polysaccharide. Dilute H_2SO_4 was prepared by adding concentrated H_2SO_4 ($500cm^3$) to distilled water ($200cm^3$) and allowed to cool. Fresh anthrone reagent was prepared daily by adding anthrone (0.2g) to dilute acid ($100cm^3$) which was then cooled and filtered through a grade 4 fritted glass filter to obtain a clear solution. The standard solution was $100mg/cm^3$ glucose. The sample ($1cm^3$) containing 10-100μg glucose or other carbohydrate was added to ice-cold anthrone reagent ($5cm^3$). Tubes were vortexed and heated in a vigorously boiling waterbath for 10 minutes and then placed in cool running water. The absorbance was determined on a Unicam SP 1800 spectrophotometer at 620nm. The polysaccharide concentrations reported are means of duplicate determinations. Figure 4a shows a typical standard curve obtained with this procedure.

3.5.2 The Method of Bailey (1967) was found to be more reproducible. Concentrated H_2SO_4 ($70cm^3$) was added to distilled water ($30cm^3$) and the mixture cooled. Anthrone (0.1g) was added to dilute acid ($100cm^3$), allowed to age for at least two hours at $0^\circ C$ and discarded after 24 hours. To a one cm^3 sample in a cold water bath, anthrone reagent was added slowly. The tubes were mixed by inversion and transferred to a vigorously boiling waterbath for 7 minutes and then placed in an ice-water bath in the dark for 30 minutes. The absorbance was determined in a Unicam SP 1800 spectrophotometer at 625nm against a reagent blank. The polysaccharide concentrations reported are means of duplicate determinations. Figure 4b shows a typical standard curve obtained with this procedure.

3.6 Colourimetric Determination of Ribonucleic Acid Concentration

The orcinol reaction of Ashwell (1957) using a guanidine monophosphoric acid (100$\mu g/cm^3$) standard was used to determine the RNA concentration. To hydrolyse the RNA in the samples 1.0M KOH ($2.5cm^3$) was added to the DNA samples ($2.5cm^3$). The pH was then brought to 2.0 with 30% $HClO_4$ and the sample was centrifuged (Sorvall Type A, setting 70 for two minutes) to remove the precipitate containing protein, DNA and potassium permanganate. The pH was raised to 3.5 with 1.0M KOH to remove excess $HClO_4$, and the sample centrifuged to recover the supernatant. The volume of the neutralised hydrolysate was measured in order to calculate the dilution factor on hydrolysis. To $1.5cm^3$ of neutralised hydrolysate were added $3.0cm^3$ acid reagent ($0.5cm^3$ 10% $FeCl_3.6H_2O$ in $100cm^3$ concentrated HCl) and $0.2cm^3$ of freshly prepared 6% orcinol solution in 95% ethanol. The mixture was heated in a boiling water bath for 20 minutes, cooled and the absorbance determined in a Unicam SP 1800 spectrophotometer at 665nm against a reagent blank. The RNA concentrations reported are means of duplicate determinations. Figure 5 shows a typical standard curve obtained with this procedure.

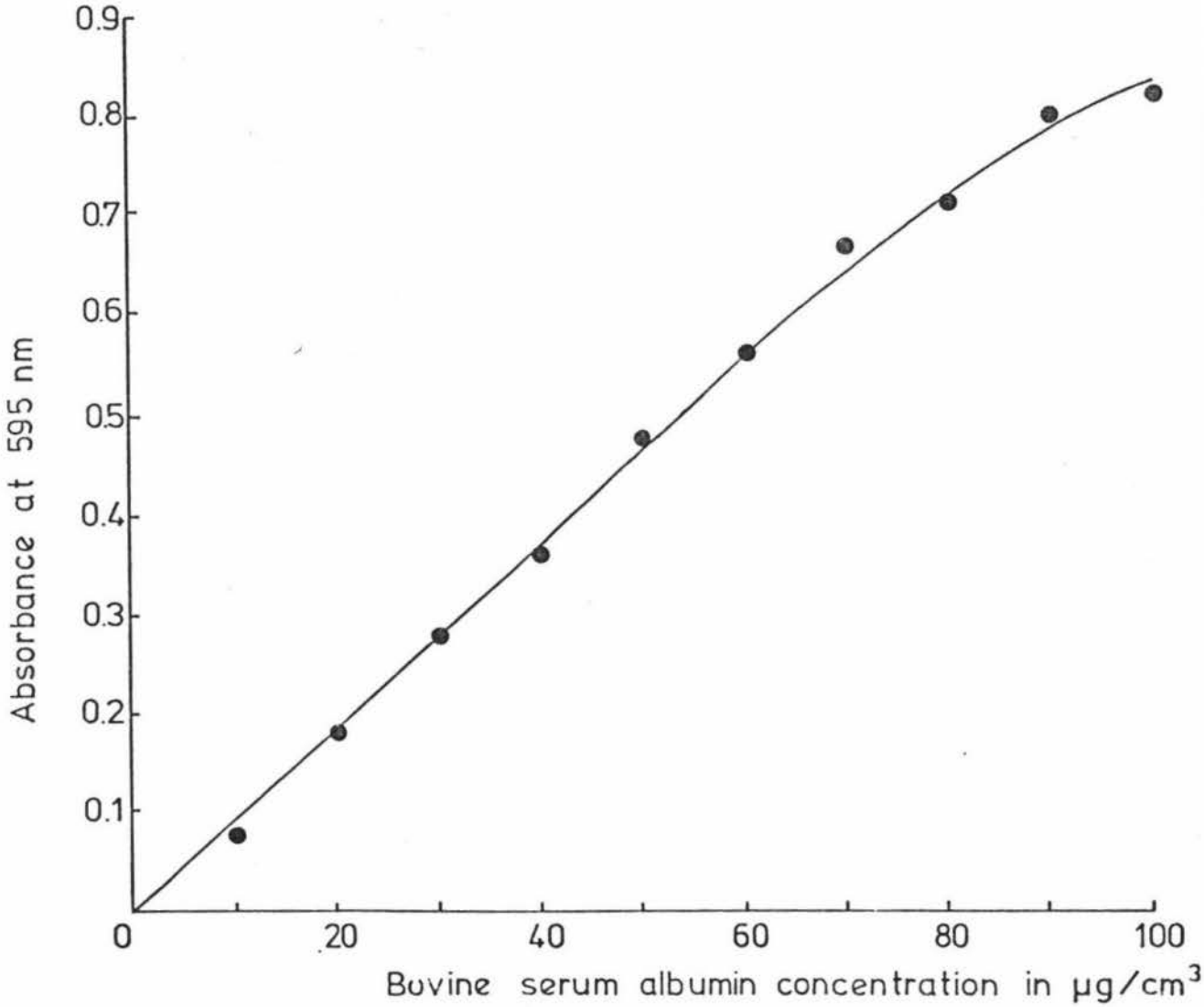
4. Base Sequence Analysis

4.1 Reassociation of DNA

Reassociation was carried out at 65°C in screw-capped Kimax tubes (1 x 10cm) and at 80°C in Quickfit glass-stoppered tubes (1.5 x 15cm). Each tube contained 150μg of unlabelled DNA in 0.28M PB and 0.1μg labelled DNA in 0.28M PB. The tubes were placed in a boiling water bath for 10 minutes then immediately quenched in ice-water to stop renaturation. They were transferred to a water bath at 65°C or 80°C for 40 hours to reassociate. After incubation the tubes were quenched in ice-water and diluted to 0.14M PB. These conditions gave an equivalent Cot

Figure 3. Colourimetric determination of protein,
a) by the method of Bradford (1976).

b) by the method of Lowry *et al*. (1976).


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance at 595 nm
0
20
40
60
80
100
Bovine serum albumin concentration in µg/cm³

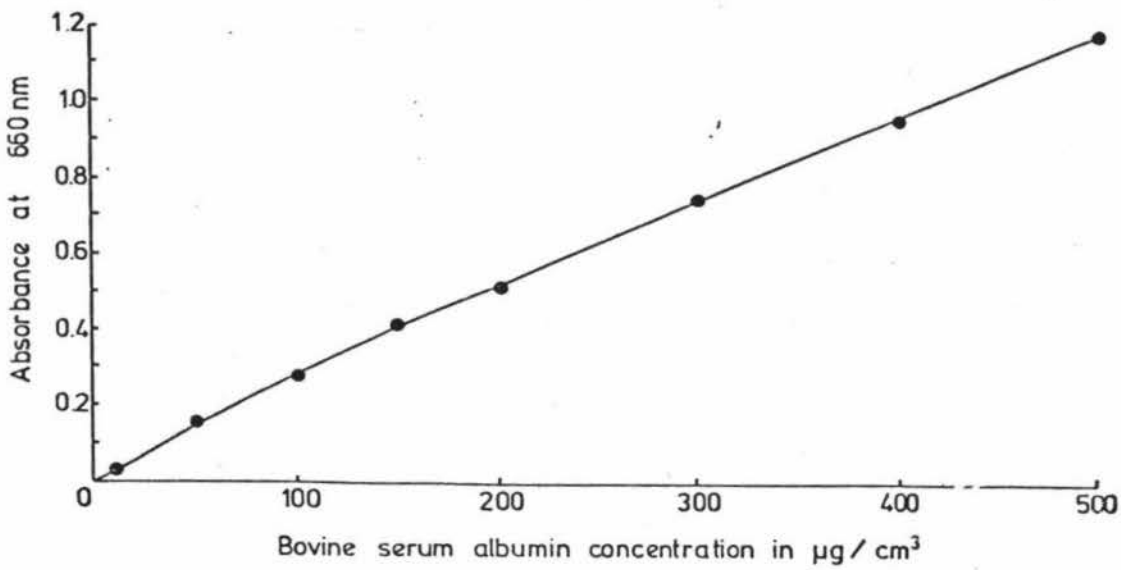
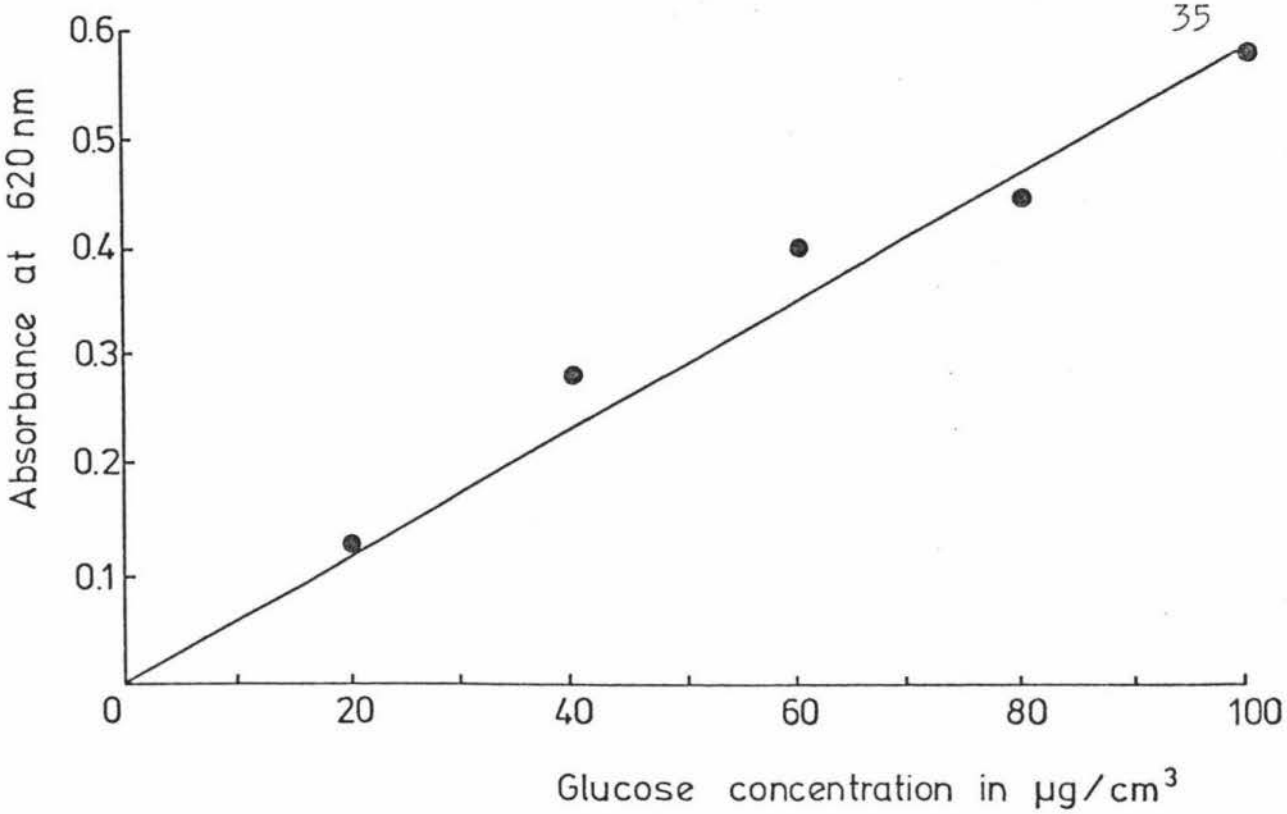

1.2
1.0
0.8
0.6
0.4
0.2
Absorbance at 660 nm
0
100
200
300
400
500
Bovine serum albumin concentration in µg / cm³

Figure 4. Colourimetric determination of polysaccharide concentration,

a) by the method of Trevelyn and Harrison (1952)

b) by the method of Bailey (1967)


Absorbance at 620 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
Glucose concentration in µg/cm³

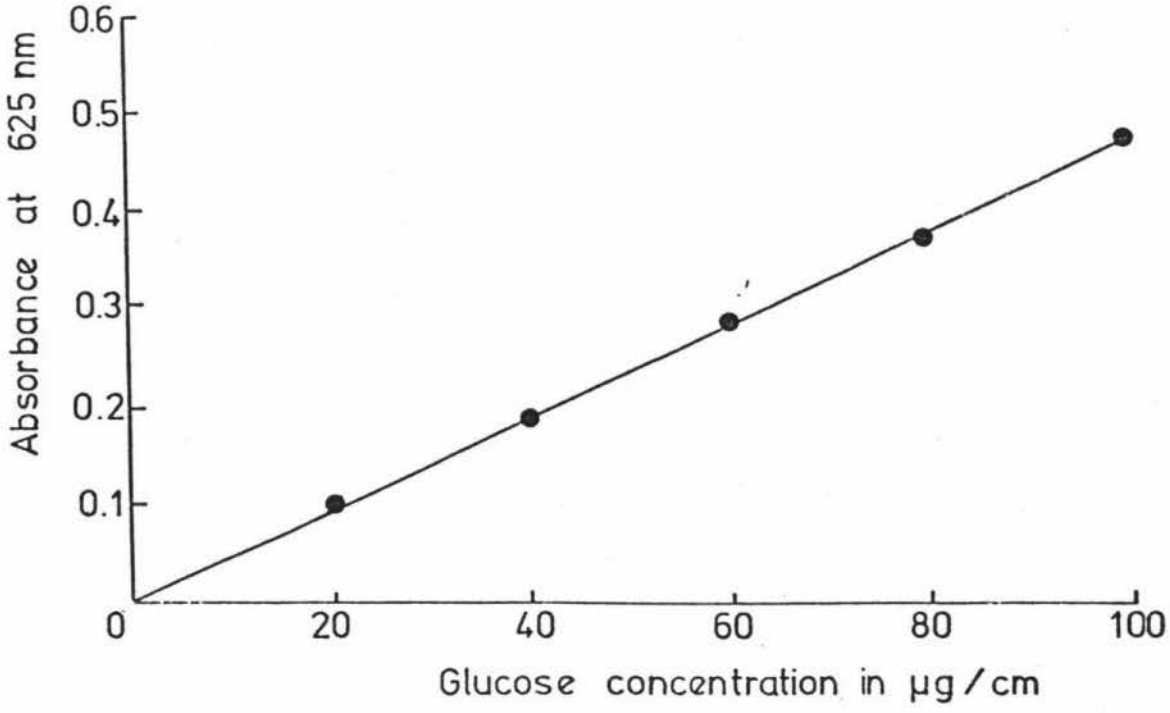
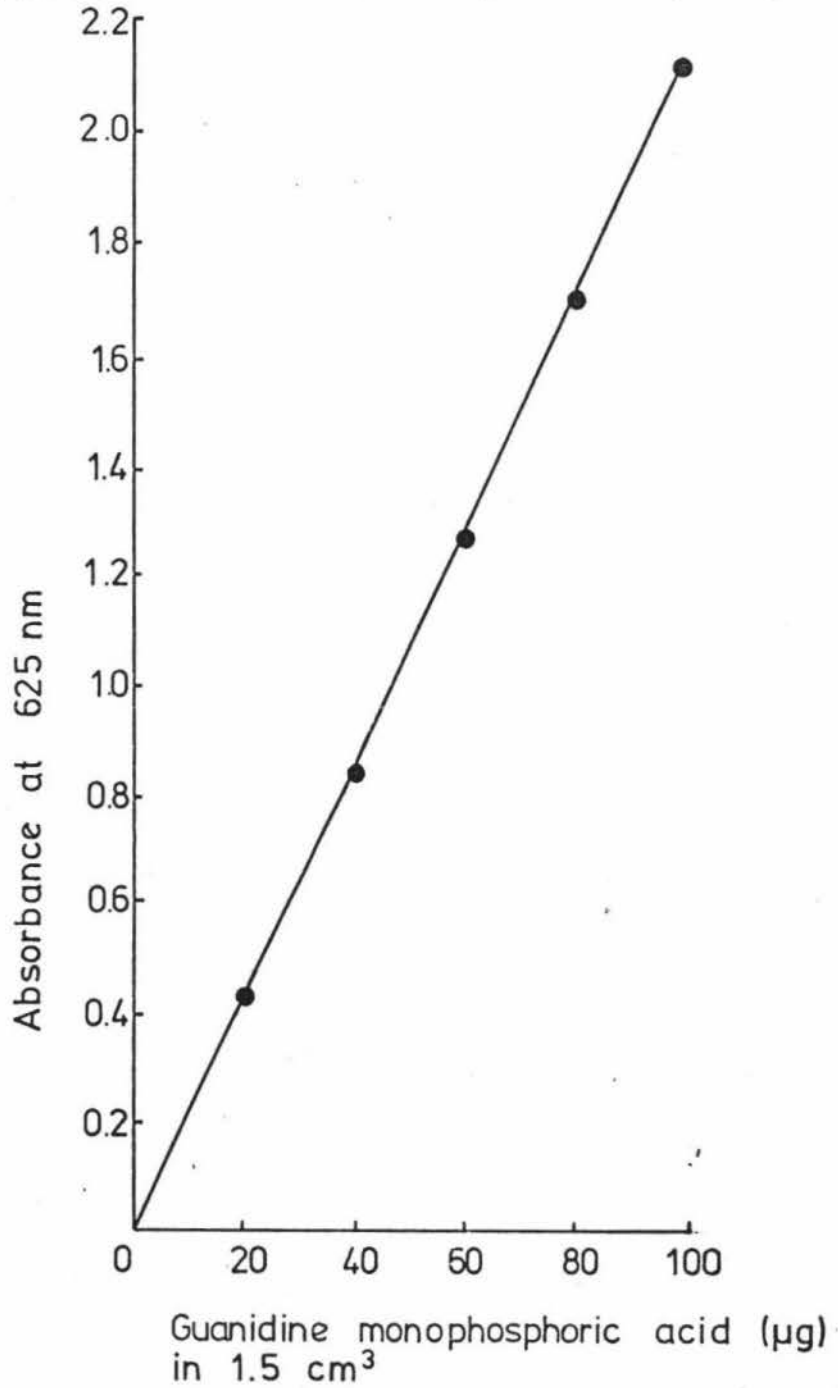

Absorbance at 625 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
Glucose concentration in µg/cm

Figure 5. Colourimetric determination of RNA concentration by the method of Ashwell (1957)



(Results 3.4.2) in 0.12M PB of 250 for unlabelled DNA and 0.063 for labelled DNA (Britten and Kohne, 1967).

4.2 Separation of Single and Double-Stranded DNA on Hydroxyapatite

The procedure was basically that described by Brenner et al. (1969). Tubes containing 5 cm^3 HA suspension (Methods 2.2.2) were centrifuged to separate the HA and the HA was resuspended in PB of suitable molarity containing 0.4% SLS. It was necessary to establish the correct molarity with each new batch of HA. The tubes were recentrifuged and the supernatant buffer discarded. Diluted reassociated DNA solution (0.5cm^3) was added followed by 9.5cm^3 PB. The tubes were stirred with an overhead stirrer (Multispeed, Anderman and Co. Ltd, Central Ave., E. Molsey, Surrey, England), raised to the required temperature in a circulating water bath (Haake Type F, Haake Circulators, Berlin, Germany) and immediately placed in a Sorvall bench centrifuge fitted with a Type A rotor held at 65°C in an incubator. The tubes were centrifuged for one minute at setting 70, slowed for 30 seconds, and stopped quickly by hand. Each supernatant was immediately poured onto a scintillation vial. Further buffer was added and the process repeated. Four low PB + 0.4% SLS washes eluted the unbound single-stranded DNA and these were followed by four 0.4M PB washes which removed the double-stranded DNA. The radioactivity in each vial was measured in a Packard liquid scintillation spectrophotometer at tritium settings by Cerenko counting (Clausen, 1968).

Usually 23 samples of unlabelled DNA at a time were compared with a labelled reference DNA. These included a sample of unlabelled homologous DNA and DNA from Escherichia coli B. A further tube contained only the reference DNA. The relative percentage hybridisation was obtained by subtracting the apparent self-hybridisation of reference DNA from the percentage hybridisation of reference DNA with each unlabelled DNA. The percentage hybridisation of labelled reference DNA with unlabelled homologous DNA was

adjusted to 100% and the same factor was applied to hybridisations with heterologous DNAs. Eachhybridisation was separated in duplicate and at least two independent hybridisations were carried out between a reference DNA and every unlabelled DNA sample. Where the results did not agree within 5% the procedure was repeated until four concordant results were obtained.

4.3 Thermal Stability of Reassociated Duplexes

This was determined by a modification of the above procedure. After reassociation at 65°C for 40 hours, residual single-stranded DNA was removed by washing four times at 65°C with PB of low molarity containing 0.4% SLS. The HA was then resuspended in $10cm^3$ of the same molarity which did not contain SLS and raised to a temperature of 70°C before the buffer was separated from the HA by centrifugation at 65°C and collected in a scintillation vial. The process was repeated six times with the elution temperature raised in 5°C steps to 100°C. The residual DNA bound to HA was eluted with o.4M PB. Each supernatant was collected separately and assayed by Cerenkov counting. The temperature at which 50% of the radioactivity associated with double-stranded DNA is released is called the thermal melting point and designated Tm(e). The difference between Tm(e) for homologous and heterologous DNA species is designated ΔTm(e) (Brenner et al., 1972). This value provides an index of sequence divergence in reassociated DNA fragments.

4.4 Statistical Analysis of Reassociation Data

The methods proposed by Gibbins and Gregory (1972) were followed. The mean for each relative percent hybridisation between the heterologous unlabelled DNA and a given reference DNA and the standard error of each mean was calculated. Duncan's (1955) new multiple range test was used to determine whether the differences between samples were significant at the 5% level. The procedure was as

follows: ($s\bar{x}$) was determined by, $s\bar{x} = \sqrt{(\text{error mean square})/r}$, where r is the number of replicates. The significant studentised ranges (SSR) were determined for the appropriate error degrees of freedom by consulting Duncan's table. The least significant range (LSR) was determined by multiplying $s\bar{x}$ by SSR. The means were ranked, highest to lowest and the differences tested. With only one exception, a difference is declared significant if it exceeds the corresponding LSR. The sole exception is that no difference between two means can be declared significant if the two means concerned are contained in a larger subset with a nonsignificant range. Results are recorded in Tables IV, V and VI by arranging the strains so that lines can be used to indicate those whose relative hybridisation values with the reference strain , are not significantly different. (Refer to Duncan (1955) and Steel and Torrie (1960) for further details).

4.5 Regeneration of Hydroxyapatite

The method of preparing HA described by Bernadi (1971) was adapted to regenerate HA which had been used in hybridisation experiments. To 500cm^3 of wet packed HA two litres of 0.1M NaOH were added, mixed by swirling, left to stand for 25 minutes, and the supernatant decanted. After repeating this step the HA was washed once with 4 litres of deionised water, allowed to settle and decanted. Four litres of 0.01M PB were added, mixed, allowed to settle and decanted. This step was repeated but the suspension was stirred continuously and brought to the boil by placing the flask in a water bath at 100°C. This step was repeated but the suspension was held at 100°C for 5 minutes and then repeated twice, the contents of the flask being held at 100°C for 15 minutes on each occasion. Finally, the HA was resuspended in 4 litres 0.001M PB, held at 100°C for 15 minutes, allowed to settle, decanted and then stored in 0.001M PB at 4°C until required.

RESULTS

1. Growth of Rhizobium

1.1 Growth Curves

The growth of three strains of Rhizobium in Yeast Mannitol (YM) broth with and without K_2HPO_4, was followed for three days as described in the methods (Method 1.4). There were minor differences between the growth curves of the three strains (Figure 6), however, in all cases the lag phase was complete in 12 hours and the exponential phase complete in 40 hours. Consequently, it was decided to incubate all cultures for DNA preparation for 48 hours to achieve the stationary phase and to minimise its extent.

Deletion of K_2HPO_4 from the medium resulted in a reduction in the maximum growth for all three strains as measured by turbidity. Viable cell counts after three days incubation in YM plus K_2HPO_4 averaged 4.05×10^8 Rhizobium cells per cm^3, whereas the broth minus K_2HPO_4 supported only 1.75×10^8 Rhizobium cells per cm^3. The length of time required for each strain to reach the stationary phase was not affected by the presence or absence of K_2HPO_4.

1.2 Improvements in Cell Yield

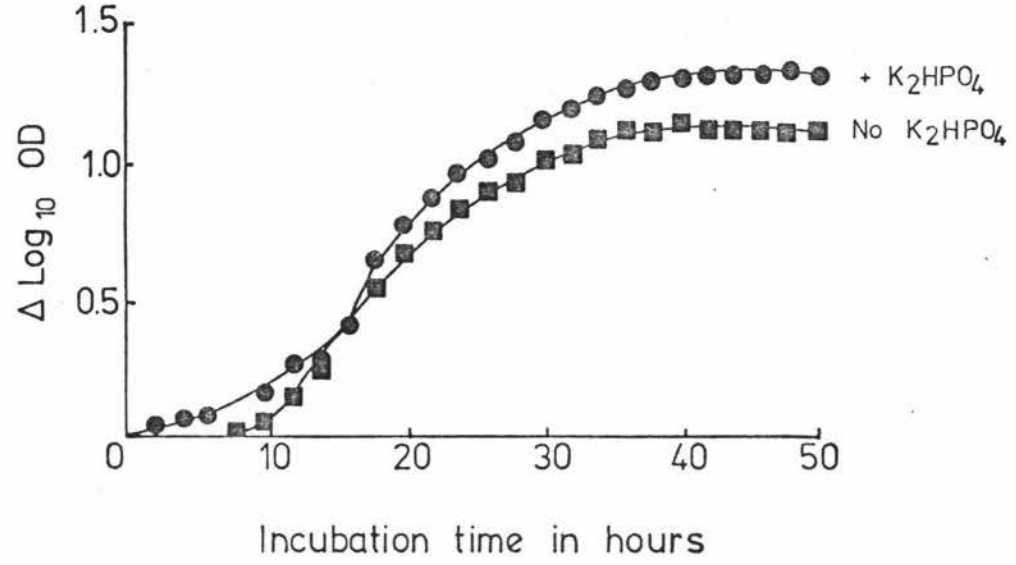
Initially this work was undertaken to increase the yield of cells containing the labelled DNA. To reduce competition for uptake between ^{32}P and phosphate ions (PO_4^{3-}) in the media, K_2HPO_4 was deleted. The reduction in growth that resulted (Results 1.1) was undesirable since DNA of high specific activity was required in large enough amounts for successful precipitation with ethanol. The pH of YM broth without K_2HPO_4 dropped from 7.0 to an average of 6.0 after growth of these rhizobia. It was postulated that the reduction in growth, and consequently in DNA yield, was due to the loss of buffering activity on the deletion of K_2HPO_4

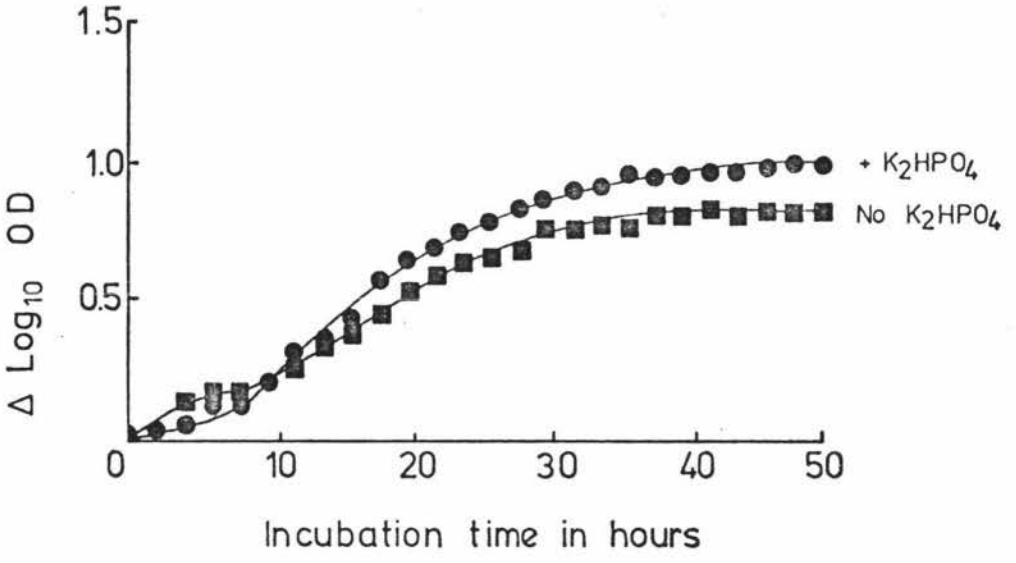
Figure 6. Growth of Rhizobium in yeast mannitol broth with and without dipotassium hydrogen phosphate

a) Rhizobium trifolii strain TA1

b) Rhizobium trifolii strain WU290

c) Rhizobium phaseoli strain TA101


1.5
1.0
0.5
0
10
20
30
40
50
Δ Log10 OD
+ K2HPO4
No K2HPO4
Incubation time in hours


1.5
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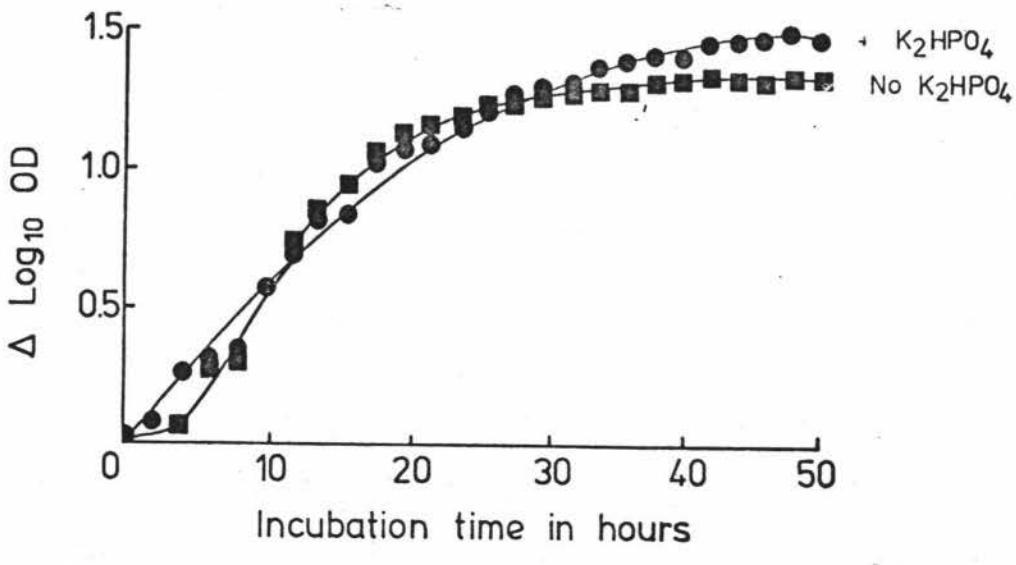
Incubation time in hours


1.5
1.0
0.5
0
10
20
30
40
50
Δ Log10 OD
+ K2HPO4
No K2HPO4
Incubation time in hours

from the media. Two alternative buffers, 0.05M Sorenson's Citrate II (Bates and Paayo, 1970) and 0.5M Tris-Acetic Acid, inhibited the growth of R. trifolii strain 561 entirely. The use of expensive buffers, such as Hepes (Sigma), could not be contemplated in large amounts of media.

The phosphate concentration of YM broth without K_2HPO_4 was measured by the phosphomolybdate method. The medium contained 0.4g/litre yeast extract (Difco) and the phosphate ion concentration was 0.21μg/cm^3. Consequently, Difco yeast extract (YE) adds negligible amounts of PO_4^{3-} compared with the K_2HPO_4 normally included in YM broth (0.5g/litre). Five strains of Rhizobium were grown in YM broth at various YE concentrations and in all cases growth was improved by the addition of YE in excess of 0.4 g/litre. Figure 7 shows the effects of increased YE concentration and the addition and deletion of K_2HPO_4 on the growth of three strains of Rhizobium, as measured by cell dry weight. When growth was measured by cellular protein concentration similar curves were obtained. The presence of K_2HPO_4 in YM broth inhibited the growth of four strains at 3.2 g/litre YE. In the absence of K_2HPO_4 growth of these four strains was increasing. The exception was R. trifolii strain TA_1.

Figure 8 shows the effects of increased YE concentration on the growth of R. trifolii strain TA_1, as measured by both cell dry weight and cellular protein concentration. This strain gave exceptionally poor growth with the first jar of YE that was also used for the other strains, but a freshly opened jar of a different batch gave a dramatic increase in yield, comparable to that obtained by the other strains. Inhibition of the growth of R. trifolii strain TA_1 was achieved in the presence and in the absence of K_2HPO_4. Inhibition of growth was achieved at 1.6g/litre and 3.2g/litre in the presence and absence of K_2HPO_4 respectively, for the first jar of YE. For the second jar of YE, inhibition of growth occurred at 6.4g/litre for both media.

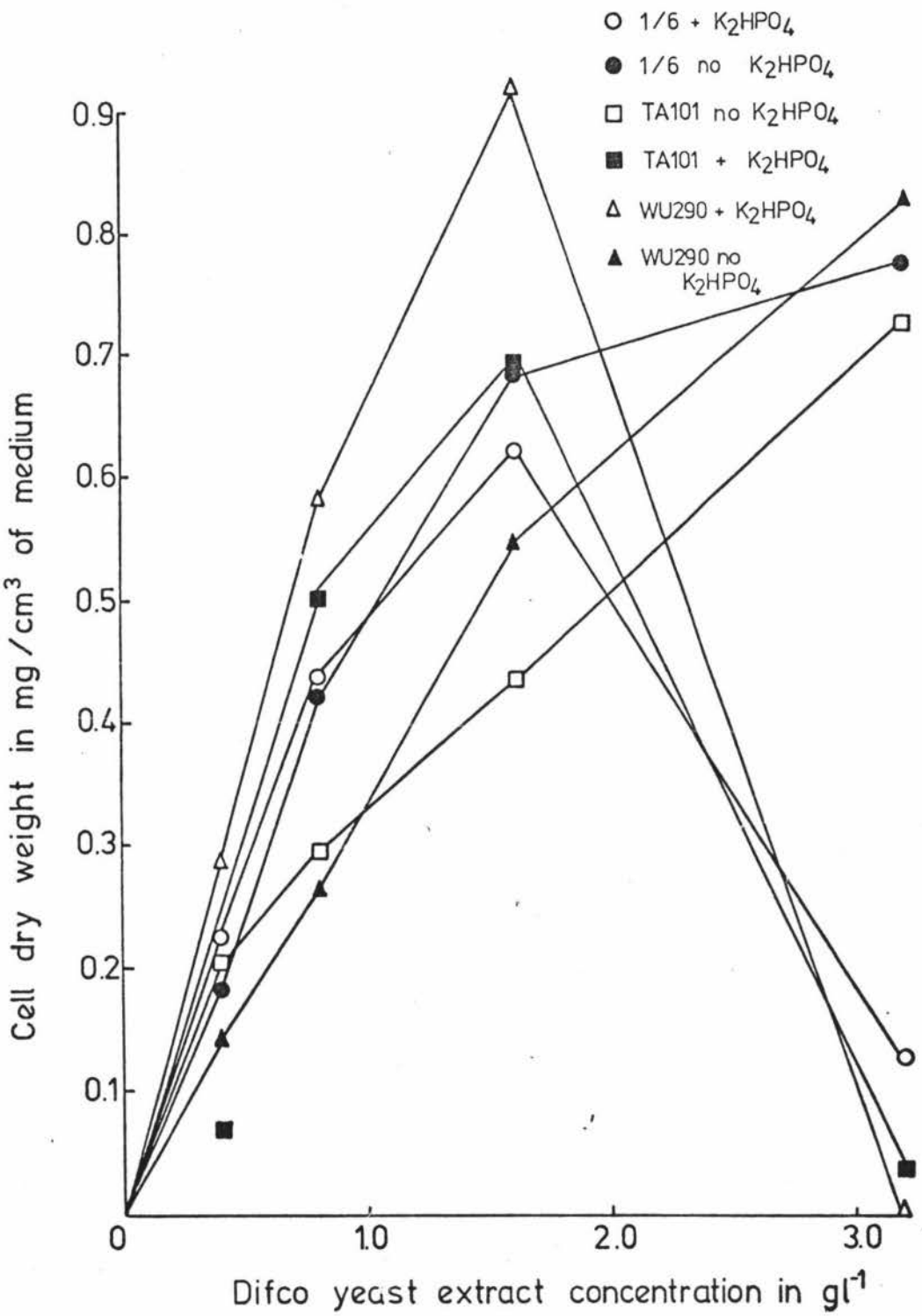
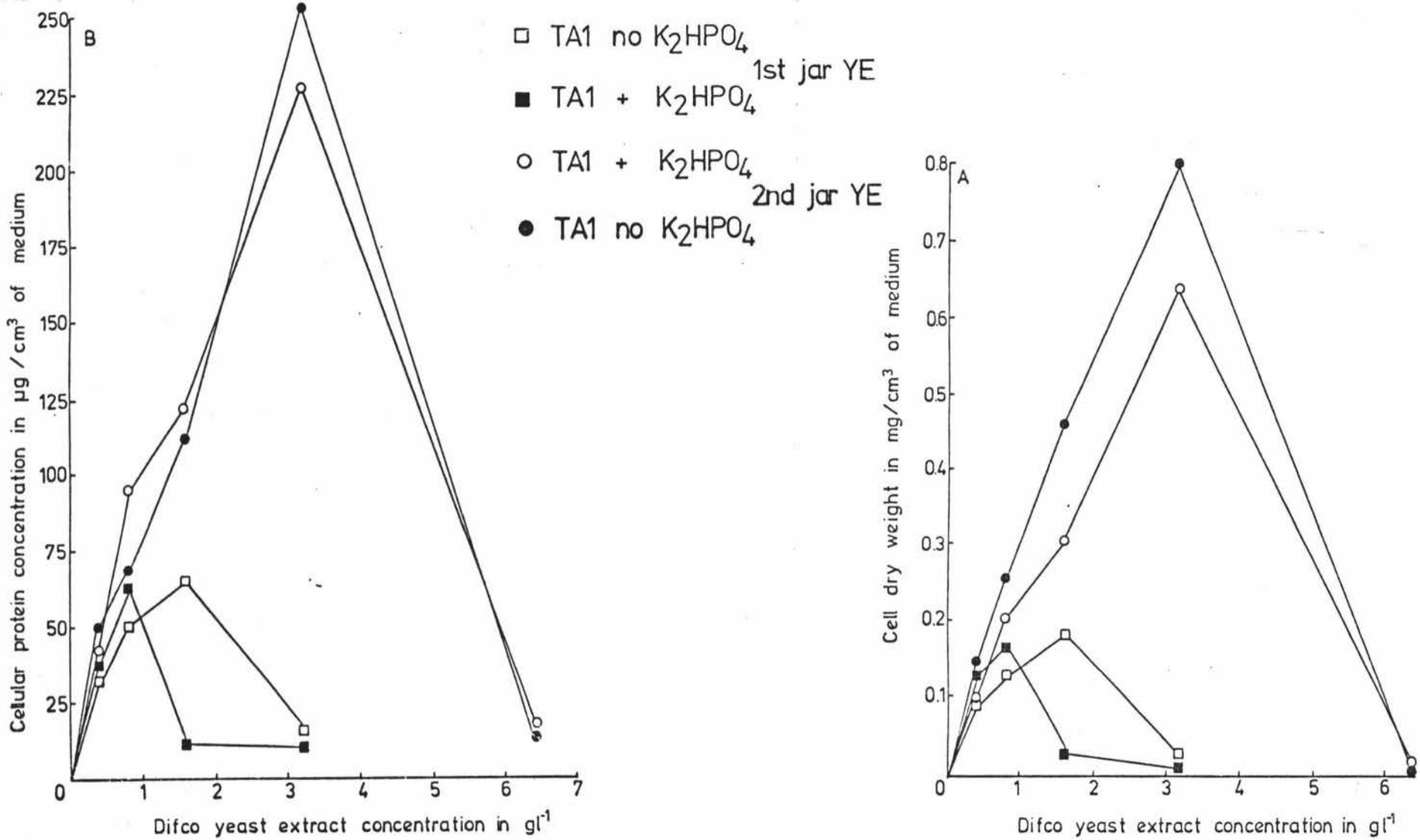


Figure 7. Effect of increasing yeast extract concentration on the growth of Rhizobium trifolii strains 1/6, WU290, and Rhizobium phaseoli strain TA101.

Figure 8. Effect of different batches of yeast extract on the growth of Rhizobium trifolii strain TA1:

a) as measured by cellular protein concentration.

b) as measured by cell dry weight.


B
A
□ TA1 no K_2HPO_4 1st jar YE
■ TA1 + K_2HPO_4
○ TA1 + K_2HPO_4 2nd jar YE
● TA1 no K_2HPO_4
Cellular protein concentration in µg / cm³ of medium
Cell dry weight in mg/cm³ of medium
Difco yeast extract concentration in gl⁻¹
Difco yeast extract concentration in gl⁻¹

2. Molecular Weight Determination of DNA

2.1 Molecular Weight of Unsonicated DNA

Unsonicated DNA had varying molecular weights depending on the nature of the DNA and the purification procedure adopted. Unlabelled DNA purified by the phenol-chloroform method (Method 2.1) had a molecular weight of 71×10^6 Daltons; unlabelled DNA purified by the hydroxyapatite-urea method (Method 2.2) had a molecular weight of 8×10^6 Daltons and labelled DNA purified by the hydroxyapatite-urea method had a molecular weight of 4×10^6 Daltons.

2.2 Molecular Weight of Sonicated DNA

Sonication of the DNA resulted in a reduction of the molecular weight. Figure 9 shows the effects of different probe diameters on the sonication of DNA. A plateau was reached with both probes after which longer sonication times had little effect on the size of the DNA fragments produced. However, the larger probe was able to impart more energy to the solution and reduced the DNA to fragments of smaller molecular weight. Figure 10 compares sonication of labelled DNA with unlabelled DNA. Labelled DNA appeared to require less sonication time to reach a suitable molecular weight than did unlabelled DNA. The molecular weight of unlabelled DNA was found to be largely independent of DNA concentration between 82 and 650 $\mu g/cm^3$; a range which included most of the DNA preparations used.

3. Difficulties Encountered in Hybridisation

3.1 First Preparation of Labelled DNA

3.1.1 Attempted Hybridisation: labelled DNA was prepared by the phenol-chloroform method (Method 2.1) from

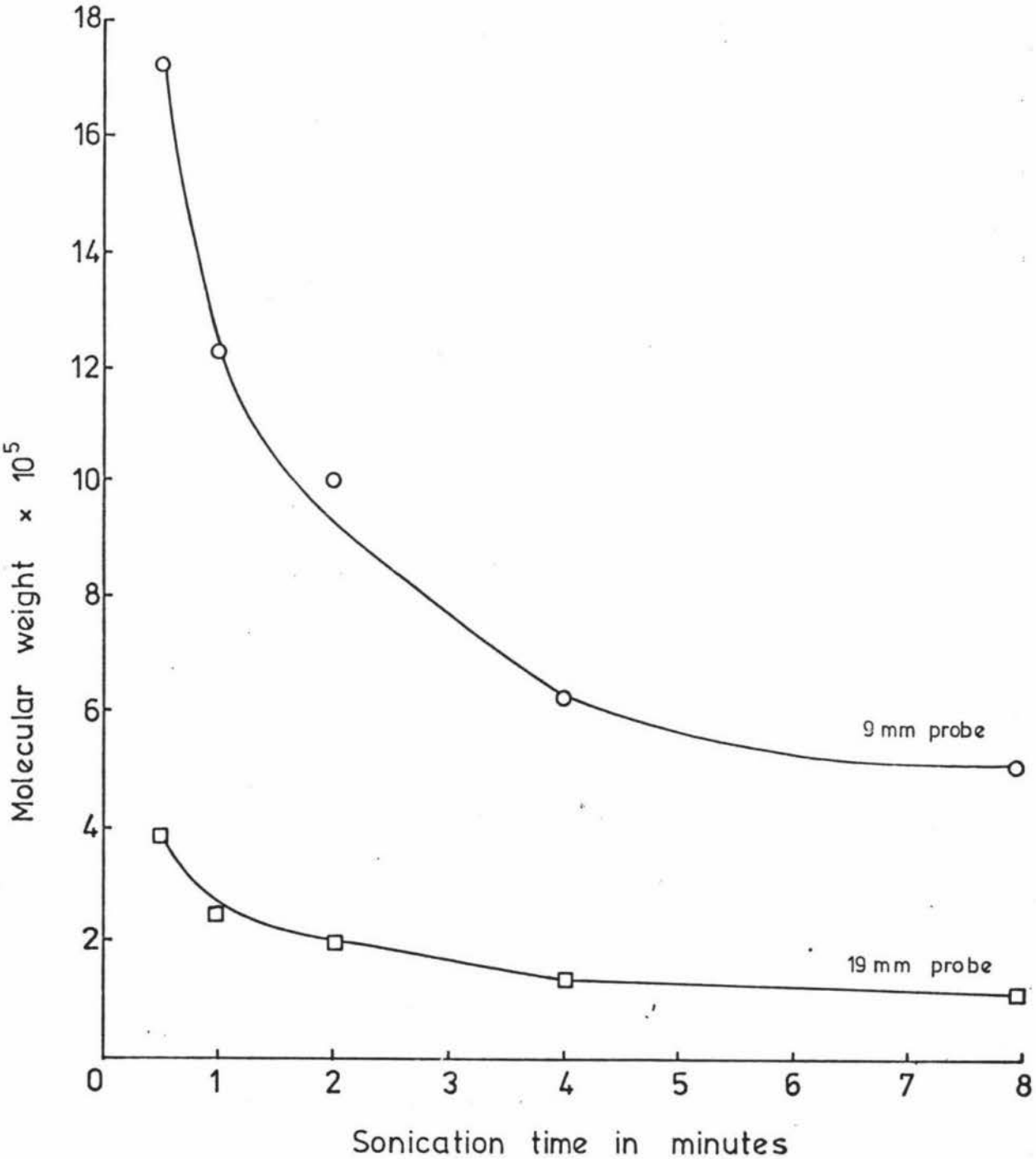


Figure 9. The effect of probe diameter on the sonication of DNA.

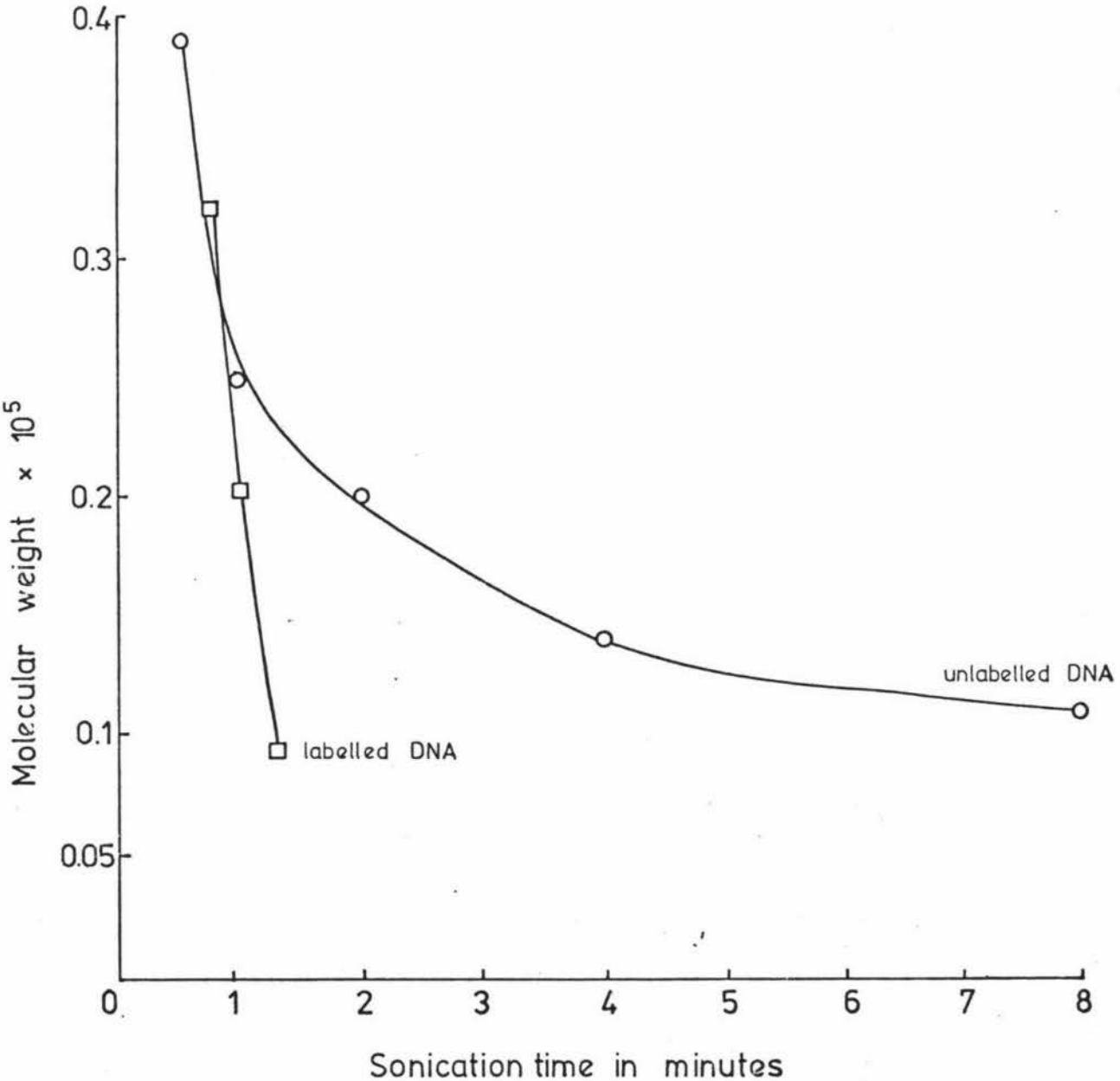


Figure 10. The effect of ^{32}P-labelling on the molecular weight of sonicated DNA

R. trifolii strain cc275e. Hybridisation with homologous DNA was attempted with equivelent Cot values of up to 611 but less than 1% of DNA was detected as double-stranded.

3.1.2 Absorbance Spectrum: the absorbance spectrum between 200 and 300nm (Method 2.1.5) was displaced toward higher wavelengths and the ratios were low (258:230nm, 1.39; 258:280nm, 1.59). After stripping (Method 2.2.5) the ratios decreased (258:230nm, 1.13; 258:280, 1.48). These ratios indicate that the DNA preparation was contaminated with protein and possibly with other materials.

3.1.3 Thermal Melting Curve of Reassociated DNA: single-stranded labelled DNA was incubated with homologous single-stranded unlabelled DNA to determine whether reassociation could occur under the incubation conditions provided. The melting profile of the incubated DNA was determined spectrophotometrically (Method 3.1) (Figure 11). Considerable hyperchromicity (26%) was observed, this is consistent with the dissociation of reassociated DNA. It was concluded that reassociation occurred between unlabelled strands, but reassociation was not detected between labelled and unlabelled strands (Result 3.1.1).

3.1.4 Separation of DNA: it was possible that the low level of reassociation detected was due to the failure of the hydroxyapatite procedure (Method 4.2) to separate single-stranded and double-stranded DNA. Sonicated calf thymus DNA (600μg) was mixed with hydroxyapatite suspension (10cm^3) and eluted with 4cm^3 volumes of 0.14M phosphate buffer (PB) + 0.4% sodium lauryl sulphate (SLS) and with 0.4M PB. DNA in the eluants was assayed by the diphenylamine method (Method 3.3). The results showed that 100% of the DNA was recovered and the separation technique was capable of distinguishing between double-stranded and single-stranded DNA.

3.1.5 Assay of Potential Contaminants in Labelled and Unlabelled DNA: the conditions used permitted reassociation and the subsequent separation of single-stranded and double-stranded DNA. It follows that failure to observe reassociation between homologous DNAs was due to the impurity of the labelled DNA preparation. The results of assays for protein (Method 3.4.1), polysaccharide (Method 3.5.2), and RNA (Method 3.6) in labelled and unlabelled DNA preparations are summarised in Table IV. The first labelled preparation from strain cc275e was contaminated with protein and polysaccharide. Unlabelled DNAs from cc275e and 514/1 had satisfactory spectral ratios but protein was detectable in some samples and all samples appeared badly contaminated with polysaccharide. The orcinol reaction indicated that RNA was present in some unlabelled DNA samples, but 100$\mu g/cm^3$ RNA-free calf thymus DNA had an apparent RNA content of 7$\mu g/cm^3$ using this reaction. It was concluded that the specificity of the reaction was not absolute at the DNA concentrations involved, and this accounts for part of the apparent RNA content of the DNA. The results in Table IV indicate that the failure of labelled DNA to hybridise with unlabelled DNA was due to contamination of the labelled preparation with protein and polysaccharide.

3.2 Second Preparation of Labelled DNA

3.2.1 Absorbance Spectra and Attempted Hybridisation: the phenol-chloroform procedure (Method 2.1) was applied with vigorous shaking to ensure more complete extraction of contaminants. The absorbance profile between 200 and 300nm of the resulting DNA was still displaced towards higher wavelengths (maximum 262nm), but the absorbance ratios at 258:230nm and 258:280nm were 1.95 and 1.80 respectively. These values are usually regarded as acceptable (Method 2.1.4).

This solution of labelled DNA was divided in half,

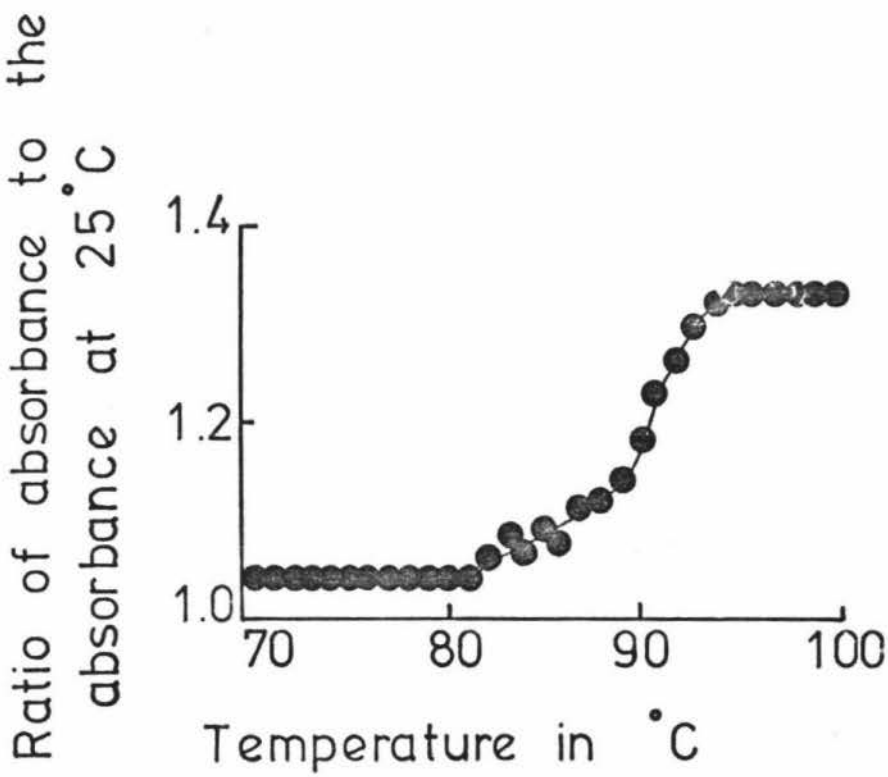


Figure 11. Melting curve of incubated homologous DNA from Rhizobium trifolii strain cc275e.

one half was stripped (Method 2.2.5) to reduce zero time binding with hydroxyapatite (HA) and the other stored frozen. After stripping, the absorbance ratios decreased (258:230nm, 1.23; 258:280nm, 1.55) and hybridisation with homologous DNA was 14%. The slight improvement in percentage hybridisation appeared to be associated with the improved absorbance spectrum of the labelled DNA preparation.

The second portion of this DNA was repurified by the phenol-chloroform procedure but the absorbance ratios were not improved by this treatment and hybridisation with homologous DNA decreased to 5%.

3.2.2 Assay of Potential Contaminants in the Second Preparation of Labelled DNA: the results in Table IV indicate that this DNA solution contained no protein but that polysaccharide was present. The proportion of polysaccharide to DNA could not be decreased by repeating the purification procedure.

3.2.3 Stripping of Unlabelled DNA: the absorbance ratios of labelled DNA were adversely affected by stripping (Method 2.2.5). The stripping procedure was applied to unlabelled DNA of known absorbance spectrum in order to study the phenomenon. No change in absorbance spectrum occurred. The apparent decrease in purity of the DNA when stripped was only evident with labelled DNA.

3.2.4 Ultracentrifugation of Sonicated and Stripped ^{32}P-DNA: the second labelled DNA preparation was placed on an alkaline sucrose gradient and ultracentrifuged. Fractions were collected and the absorbance and radioactivity of each fraction measured (Method 3.2). The results (Figure 12) show that the radioactivity was associated with an absorbance peak near the top of the tube. This indicates that the label was incorporated into DNA. The failure of the separation procedure (Method 4.2) to detect a large proportion of labelled double-stranded DNA

Table IV: Assays of labelled and unlabelled DNA prepared by the Phenol-chloroform method.

Strain number and DNA preparation description	Concentration in $\mu g/cm^3$			
	DNA (3.3)	Protein (3.4.1)	Polysaccharide (3.5.2)	RNA (3.6)
cc275e* 1st preparation	24.7	65.6+	17.1	-
cc275e* 2nd preparation (one purification)	30.5	0.0	44.0	-
cc275e* 2nd preparation (two purifications)	13.0	0.0	22.3	-
cc275e* 1st preparation	324.2	0.0	409.6	45.7
cc275e 2nd preparation	237.5	0.0	1,539.0	-
514/1	571.0	34.0	311.2	55.3

Footnotes: *labelled DNA preparation

+determined by the method of Lowry et al. (1951) (Method 3.4.2)

when homologous DNA was allowed to reassociate with labelled strands, could not be ascribed to the presence of components other than DNA which had been labelled with ^{32}P but were incapable of binding to the HA. Such components would have been eluted by 0.14M PB + 0.4% SLS contributing to the apparent proportion of single-stranded DNA.

It was concluded that the low level of hybridisation was due to the impurity of the labelled DNA for which the polysaccharide was probably responsible. Careful application of the phenol-chloroform method of purification and repurification failed to yield labelled DNA of sufficient purity; another purification technique had to be developed.

3.3 Purification of DNA by the Hydroxyapatite-Urea Procedure

3.3.1 Purification of DNA Prepared by the Phenol-Chloroform Procedure: DNA extracted and purified by the phenol-chloroform procedure (Method 2.1) was repurified by the hydroxyapatite-urea procedure (Method 2.2.3) to see whether an improvement in the absorbance profile could be obtained. Initially DNA was absorbed on HA and impuritiess were removed by repeatedly centrifuging the HA with 0.24M PB, which was 8M w.r.t. urea, and discarding the supernatant liquid. Only 8.6% of the DNA used was recovered when the HA was eluted with 0.4M PB. Material eluted in the first 8M urea + 0.24M PB washes had an absorbance spectrum similar to that of DNA. The residual DNA eluted with 0.4M PB showed improved spectral ratios. It was concluded that the buffer concentration was unsuitable but the method was capable of improving the purity of DNA preparations.

A second purification was carried out, in which the PB concentration was decreased to 0.14M in the 8M urea washes. This resulted in a 3.7-fold increase in recovery and a considerable improvement in the purity ratios of the two DNA samples used (Table V).

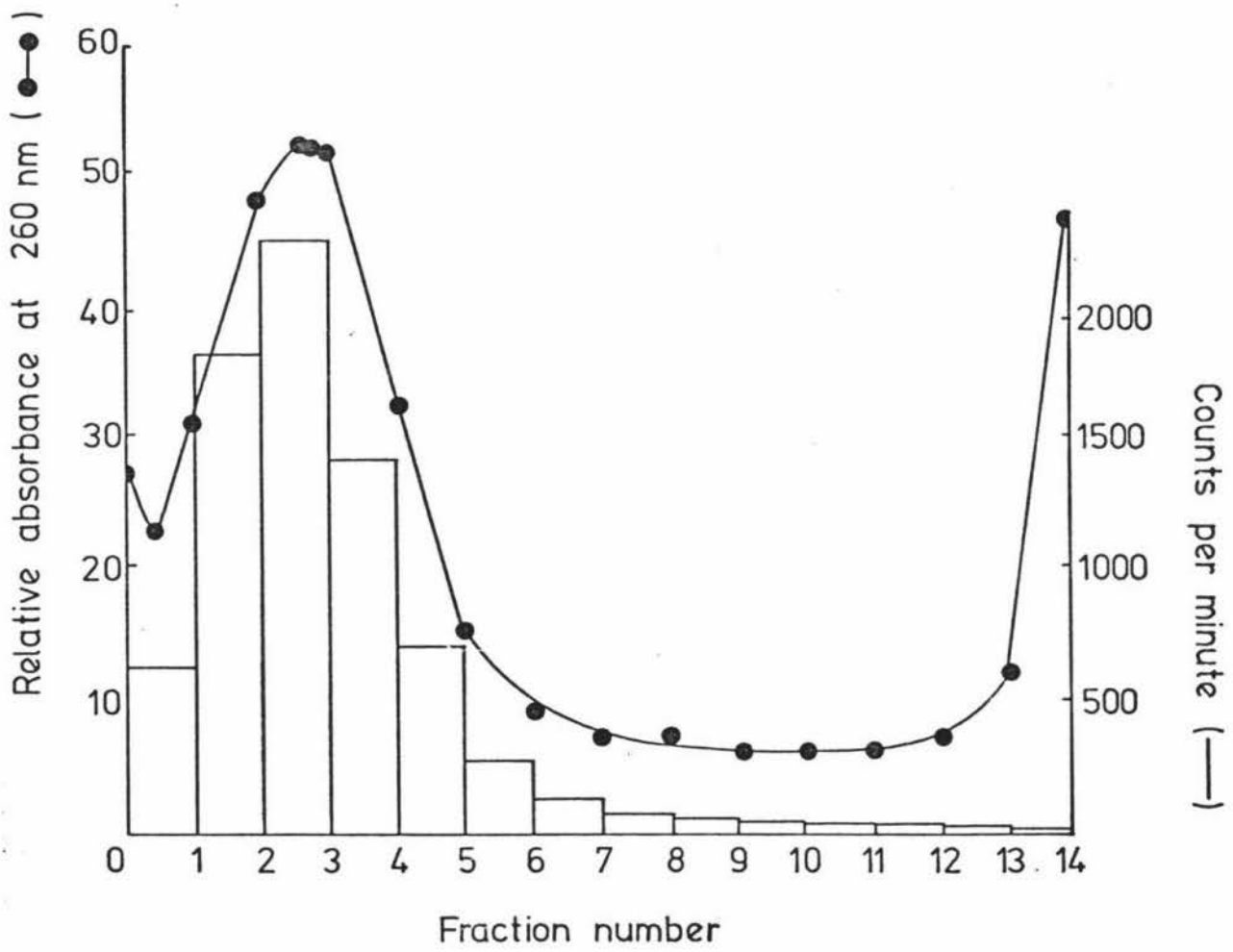


Figure 12. Ultracentrifugation of sonicated and stripped ^{32}P-DNA from Rhizobium trifolii strain cc275e

Table V: Effect of hydroxyapatite-urea purification on the absorbance spectra of two samples of DNA prepared by the phenol-chloroform procedure.

Source of DNA	Effect of hydroxyapatite-urea purification on absorbance			
	before purification		after purification	
	258:230nm	258:280nm	258:230nm	258:280nm
cc275e	1.79	1.74	2.05	1.82
5225	1.91	1.88	2.14	1.90

It was concluded that hydroxyapatite-urea DNA purification can increase the purity of unlabelled DNA samples.

3.3.2 Preparation of DNA by the Hydroxyapatite-Urea Procedure: a preliminary trial of this procedure (Method 2.2) omitted the use of ribonuclease (RNase) in the lysing solution and used only five washes of 8M urea in 0.14M PB. The yield of DNA was lower than that obtained from an equivalent cell mass by the phenol-chloroform technique, but the absorbance spectrum of the DNA was improved. For DNA recovered in the first 0.4M PB wash absorbance ratios at 258:230nm and 258:280nm were 2.5 and 2.1 respectively.

In a second trial RNase was added to the cell lysate and the lysed cell suspension was shaken more vigorously with phenol before the hydroxyapatite-urea treatment. A sixfold increase in DNA yield was observed. DNA recovered in the first 0.4M PB wash had absorbance ratios at 258:230nm and 258:280nm of 2.2 and 1.9, respectively. In subsequent 0.4M PB washes the absorbance ratios remained high but DNA concentrations decreased in each succeeding wash. Since an average of 73% of the DNA was recovered in the first two 0.4M PB washes these were usually pooled and the others discarded.

3.3.3 Assay of Contaminants in DNA Prepared by the Hydroxyapatite-Urea Procedure: Table VI records the results of assays on DNA preparations obtained in the preliminary trials with the hydroxyapatite-urea procedure described above. Fraction 8/1 is the first wash containing 8M urea in 0.14M PB. Since the protein concentration in this fraction was low, it was assumed that protein was removed effectively by the phenol extraction of lysed cell suspensions which preceded hydroxyapatite-urea treatment. The high concentration of polysaccharide in fraction 8/1 is consistent with the failure of the phenol-chloroform procedure to remove polysaccharide, and indicates that DNA can be separated from polysaccharides by this

procedure.

Fraction 4/1 is the first 0.4M PB wash used to elute the DNA from hydroxyapatite. The fraction contained no protein and only a low concentration of polysaccharide compared with DNA prepared by the phenol-chloroform procedure. Fraction 4/1 from the first preparation contained a significant concentration of RNA but the addition of RNase to the lysing cell suspension during the second trial considerably decreased the proportion of RNA to DNA. Since DNA appears to interfere when RNA is measured by the orcinol reaction (Result 3.1.5) it was assumed that the second preparation was essentially free of RNA.

3.3.4 Suitability of DNA for Hybridisation: the experiments described indicated that failure to obtain hybridisation with labelled DNA prepared by the phenol-chloroform procedure was due to impurity of the DNA. The phenol-chloroform procedure was unable to remove polysaccharide and in most samples assayed there was a higher concentration of polysaccharide than DNA. Preparation of DNA by the hydroxyapatite-urea procedure produces DNA which is free from protein and almost free from polysaccharide and material assayed as RNA. To decrease the concentration of these contaminants still further, the number of 8M urea in 0.14M PB washes was increased to ten and the volume of each wash to 100cm^3. Trial hybridisation between labelled DNA prepared by the hydroxyapatite-urea method and homologous DNA, resulted in 69% hybridisation. It was decided that some unlabelled and all labelled DNA preparations for use in hybridisation experiments would be made by this method.

3.4 Hybridisation Conditions

3.4.1 The Effect of Phosphate Buffer Molarity on the Separation of Single and Double-Stranded DNA with Hydroxyapatite: PB of varying molarity was used to elute

Table VI: Composition of fractions obtained in preliminary trials of the hydroxyapatite-urea procedure for DNA preparation.

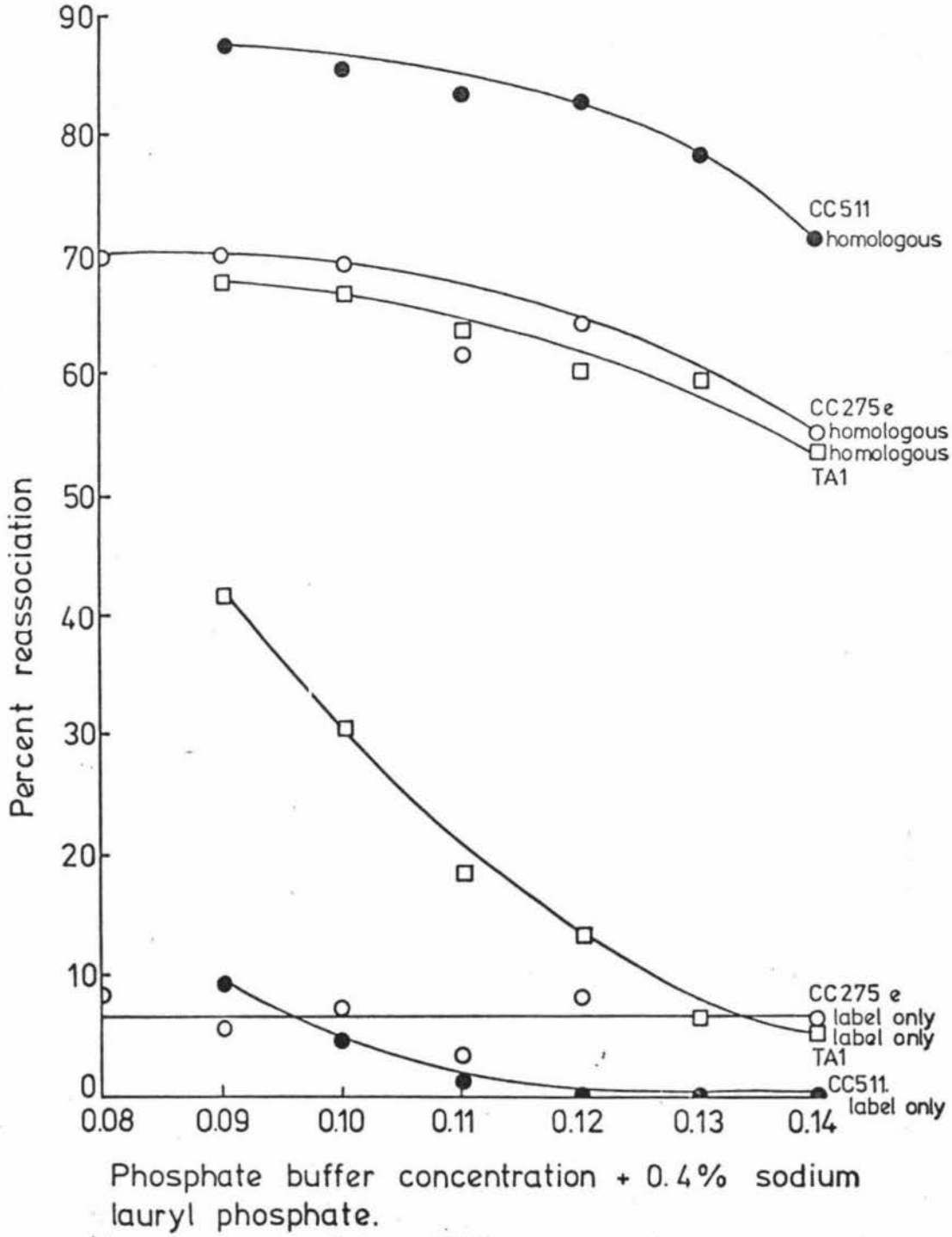
Preparation	Fraction 8/1			Fraction 4/1			
	RNA[2]	Protein[3]	Polysacc.[4]	DNA[1]	RNA[2]	Protein[3]	Polysacc.[4]
1.	67.8	0.0	393.7	49.1	12.8	0.0	0.4
2.	71.0	56.7	223.0	279.5	15.2	0.0	10.8

Footnotes:
1. Absorbance at 258nm (Method 2.5)
2. Orcinol procedure (Method 3.6)
3. Dye-binding procedure (Method 3.4.1)
4. Anthrone procedure (Method 3.5.2)
5. All values in $\mu g/cm^3$.

single-stranded DNA from hydroxyapatite. The concentration of buffer used to elute single-stranded DNA is critical. If it is too high some double-stranded DNA will be eluted with the single-stranded DNA. If it is too low some single-stranded DNA may remain to elute with the double-stranded DNA. To select the optimum PB molarity, separations were carried out with PB of varying molarity in the presence of 0.4% SLS. Each buffer was used to separate (Method 4.2) labelled DNA which had been allowed to reassociate with homologous unlabelled DNA (Method 4.1), and DNA reduced to a single-stranded form by boiling. Figure 13 records the results for three different batches of HA. The optimum PB molarity for hybridisation experiments was considered to be one which gave the largest difference between the apparent % hybridisation of the labelled DNA with itself, and that of labelled DNA with homologous unlabelled DNA. A separate HA suspension was made up for each labelled DNA preparation. The buffers chosen for elution of single-stranded DNA were 0.10M PB + 0.4% SLS for R. trifolii strain cc275e, 0.13M PB + 0.4% SLS for R. trifolii strain TA1, and 0.11M PB + 0.4% SLS for R. phaseoli strain cc511.

3.4.2 The Effect of Cot on DNA Reassociation: Cot is defined as the product of nucleic acid concentration (Co) and the time (t) of incubation. The Cot value is calculated as the product of nucleic acid concentration in optical density units at 260nm, divided by 2 and multiplied by the time of incubation expressed in hours (Brenner and Falkow, 1971). Cot values normally refer to incubations in 0.12M PB. Reassociations in this study occurred in 0.28M PB and a conversion table (Britten et al., 1970) was used to determine the equivalent Cot (E. Cot) in 0.12M PB. Different Cot values were usually obtained by varying the incubation period from 8 to 40 hours and holding the concentration of DNA constant, but the largest value used was obtained by increasing the concentration of unlabelled DNA from 150 to 300$\mu g/cm^3$ and incubating for 40 hours. Reassociation (Method 4.1) and separation (Method 4.2) were

Figure 13. Effect of molar concentration of sodium phosphate buffer (pH 6.8) containing 0.4% sodium lauryl sulphate on the separation of homologous reassociated and labelled unreassociated DNA as determined by the hydroxyapatite batch centrifugation method.


90
80
70
60
50
40
30
20
10
0
Percent reassociation
0.08
0.09
0.10
0.11
0.12
0.13
0.14
CC511
homologous
CC275e
homologous
homologous
TA1
CC275 e
label only
label only
TA1
CC511.
label only
Phosphate buffer concentration + 0.4% sodium lauryl phosphate.

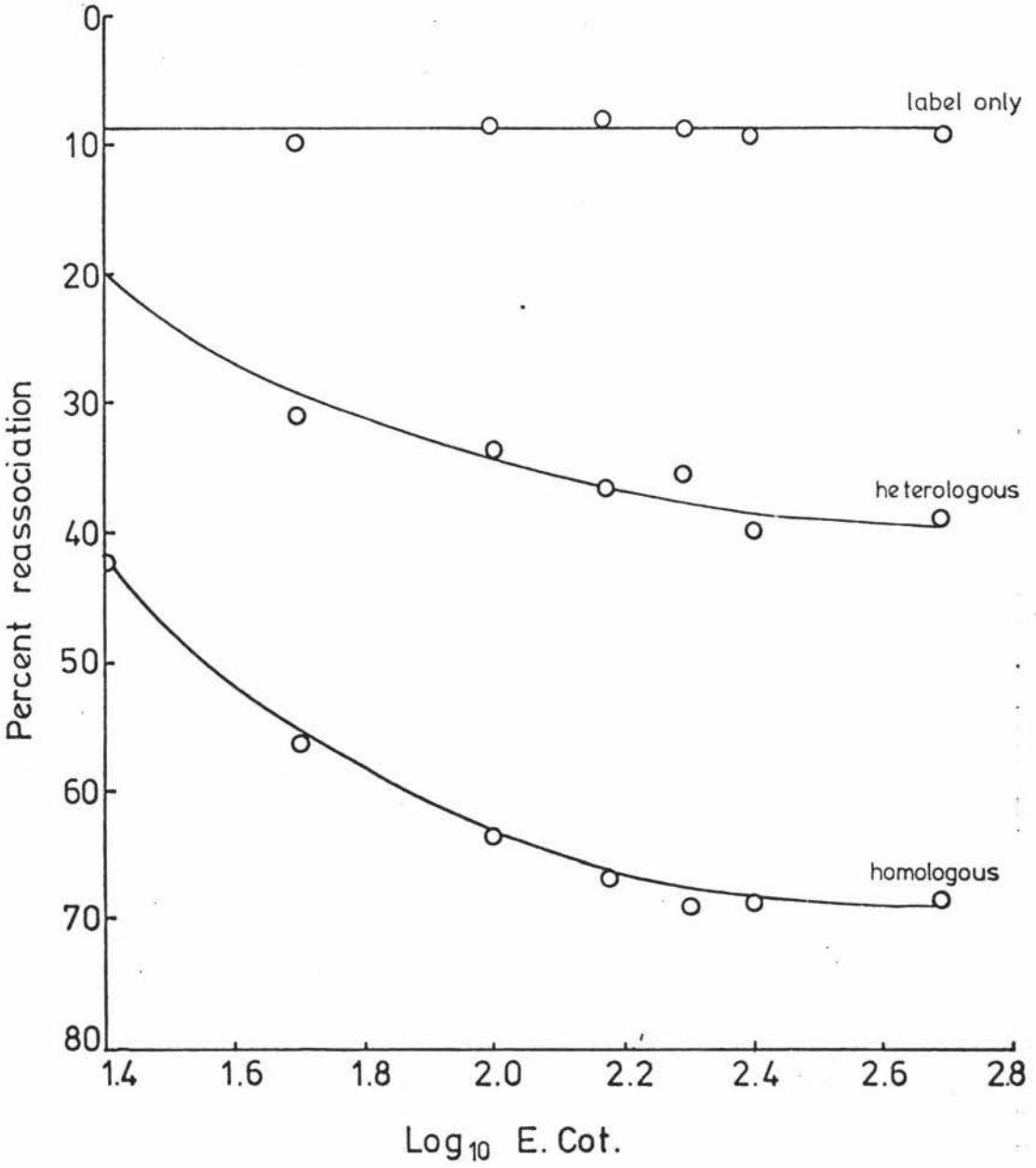


Figure 14. Effect of equivalent Cot value on the reassociation of homologous and heterologous DNA with refernce DNA extracted from Rhizobium trifolii strain TA1

carried out as above.

Homologous DNA (150$\mu g/cm^3$) required an E. Cot of 200 to allow maximum reassociation (Figure 14). Heterologous reassociation between labelled DNA from R. trifolii strain TA1 and DNA from R. trifolii strain 503a required an E. Cot of 250 to allow maximum reassociation. An E. Cot of 250 was obtained by incubating 150μg of unlabelled DNA for 40 hours. Labelled DNA (0.1μg) incubated for 40 hours had an E. Cot Of 0.17 and no increase in self-reassociation of labelled DNA was detected up to this value. In Figure 14 the label only reassociation values are expressed at the E. Cots of the labelled:unlabelled reaction for the same incubation time. It was decided to routinely use an E. Cot of 250.

3.4.3 Experiments to Decrease the Apparent Self-Association of Labelled DNA: reaction mixtures which contain only labelled DNA may show a small percentage of double-stranded DNA when separated by the hydroxyapatite procedure. Low self-association values obtained in this way show that hybridisation is occurring efficiently, and minimise the correction to be applied to the apparent hybridisation of a reference DNA with other homologous or heterologous DNAs.

The % reassociation at 65°C in reaction mixtures containing only reference DNA from R. trifolii cc275e, R. trifolii TA1, and R. phaseoli cc511 averaged 3, 4, and 8% respectively. Self-assocaation of strain cc511 when incubated at 80°C and separated, averaged 5%.

Labelled DNA (0.1$\mu g/cm^2$) was boiled for four minutes in 0.28M PB to reduce it to a single-stranded state and cooled rapidly. The % hybridisation was determined immediately by the hydroxyapatite procedure and again after incubation at 65°C for 40 hours. There was no change in the values. A fivefold increase in Cot value had no effect on the percentage hybridisation of label only,

observed. It was concluded that the observed hybridisation was not due to reassociated DNA.

The apparent self-association of labelled DNA may have resulted from failure to completely dissociate double-stranded labelled DNA on boling. Variation of the PB concentration in which DNA was boiled, from 0.028 to 0.28M PB, and increasing the period of boiling from 4 to 10 minutes had no effect on the values obtained.

The conditions used may have permitted limited non-specific binding of DNA to HA. We were unable to influence this by the addition of up to 10mM Ethylenediaminetetra-acetic acid (EDTA) to the reassociation mixture, or by increasing the amount of labelled DNA from 0.1 to 2.0μg. But when boiled labelled DNA which eluted as double-stranded DNA with 0.4M PB was separated again, 0% reassociation was observed. Material in the labelled DNA preparation which behaved like double-stranded DNA on separation should have been removed by the stripping procedure, but clearly some remained. It may be possible to strip the label more effectively by using a larger quantity of HA or a batch procedure.

4. DNA Homology Among Strains of R. trifolii and Related Species

4.1 Labelled DNA Preparations

4.1.1 Specific Activities: the specific activity of labelled DNA prepared from R. trifolii strain cc275e was only 2,700 cpm/μg. The eluants from these hybridisations were counted for 20 minutes or longer to collect a total of 4,000 counts per comparison. The specific activities of labelled DNA prepared from R. trifolii strain TA1 and R. phaseoli strain cc511 were 134,700 and 72,900 cpm/μg, respectively, They were counted long enough to collect a total of 10,00 counts per comparison.

4.1.2 Hybridisation - self-association of labelled DNA, homologous reaction, and preparation of unlabelled DNA: the self-association of labelled cc275e reference DNA averaged 5%, TA1 averaged 4%, cc511 averaged 8% at 65°C and 5% at 80°C. The homologous reaction for cc275e averaged 69%, TA1 averaged 63%, cc511 averaged 83% at 65°C and 67% at 80°C. Hybridisation of reference DNA with homologous or heterologous DNA gave the same % hybridisation regardless of whether it was prepared by the phenol-chloroform method or the hydroxyapatite-urea method.

This proved that as long as the labelled DNA was of sufficient purity to hybridise, the method of preparation of unlabelled DNA did not affect the hybridisation.

4.2 Thermal Stability of DNA Reassociated at 65°C

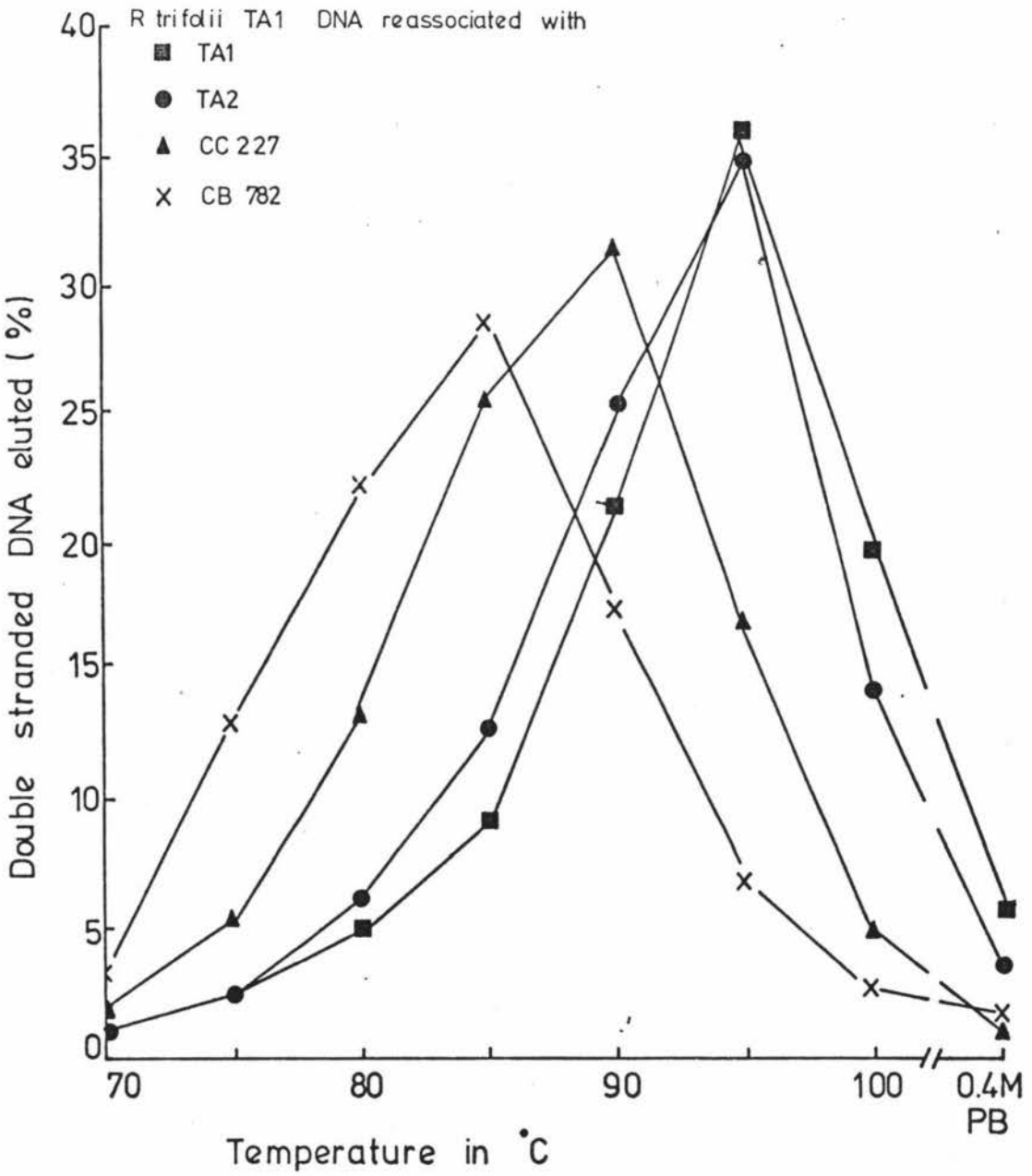
4.2.1 Thermal Elution Profiles: Figures 15 and 16 show thermal elution profiles of reference DNAs from strains TA1 and cc511 with homologous and heterologous DNA. The profiles with heterologous DNA are selected to show the range of relationships observed. Closely related reassociated DNA, TA2 with TA1 reference DNA, elutes at a higher temperature and has a less disperse elution profile than does less closely related DNA (Figure 15).

Thermal elution profiles may be characterised by the temperature at which half the reassociated DNA duplexes have dissociated, Tm(e) (Method 4.3). The difference in thermal elution midpoints of duplexes formed from DNA of homologous and heterologous origins, is ΔTm(e). When ΔTm(e) is plotted against the % relative hybridisation at 65°C a straight-line relationship is obtained (Figure 17). The stability of the hybrids formed at 65°C decreased concomitantly with the degree of DNA relatedness at 65°C.

Table VII: Relative hybridisation of TA1 reference DNA with unlabelled DNA prepared by the phenol-chloroform and the hydroxyapatite-urea procedures.

DNA preparation procedure.	Source of unlabelled DNA	
	TA1	514
phenol-chloroform	100.0%	76.9%
HA-urea	102.6%	77.5%

Figure 15. Thermal elution profiles of reassociated DNA duplexes formed at 65°C between DNA from *Rhizobium trifolii* strain TA1 and DNA from other strains of *Rhizobium trifolii*.


R trifolii TA1 DNA reassociated with
TA1
TA2
CC 227
CB 782
Double stranded DNA eluted (%)
40
35
30
25
20
15
10
5
0
70
80
90
100
0.4M PB
Temperature in °C

4.2.2 Thermal Binding Indices: TBI or the thermal binding index is the ratio of the % relative reassociation at 80°C to that at 65°C. At higher more stringent reassociation temperatures only more stable hybrids are formed and the % relative reassociation of heterologous DNA is consequently reduced. TBI therefore is an index to the degree of mismatching of hybrids formed at 65°C, and is used by some workers as an alternative to ΔTm(e). Figure 18 shows the relationship between Δ Tm(e) and TBI. Δ Tm(e) is directly proportional to TBI and since TBIs contribute no new information their use was discontinued.

4.3 Homologies Among Related Strains

4.3.1 Serologically Related Strains: several of the strains compared are serologically related. Strains TA1 and TA2 are considered to be related but not identical. The difference between the relative hybridisation values of DNA from these two strains with cc275e reference DNA is not statistically significant (64.7 and 69.9%) although Δ Tm(e) values differ by 1.5°C. But when reference DNA from TA1 was used relative hybridisation with DNA from TA2 was 93.6% and a statistically significant difference was observed. This was associated with a value for Δ Tm(e) of 1.3°C. Strain WU290 is a non-nodulating variant of the original strain selected during this work, and WU290iii a non-nodulating variant selected in Australia. These two strains are serologically identical. No statistically significant difference was detected between their relative hybridisation values with either European clover rhizobia reference DNA. Strain 540 was isolated from a seedling used to test nodulation by strain 560 *in vitro*. These strains were effective and serologically identical. They were not significantly different in their base sequence homology with either reference strain. It was concluded that strains which were indistinguishable serologically were unlikely to show different relative hybridisation or Δ Tm(e) values with independent reference DNAs. Partial serological

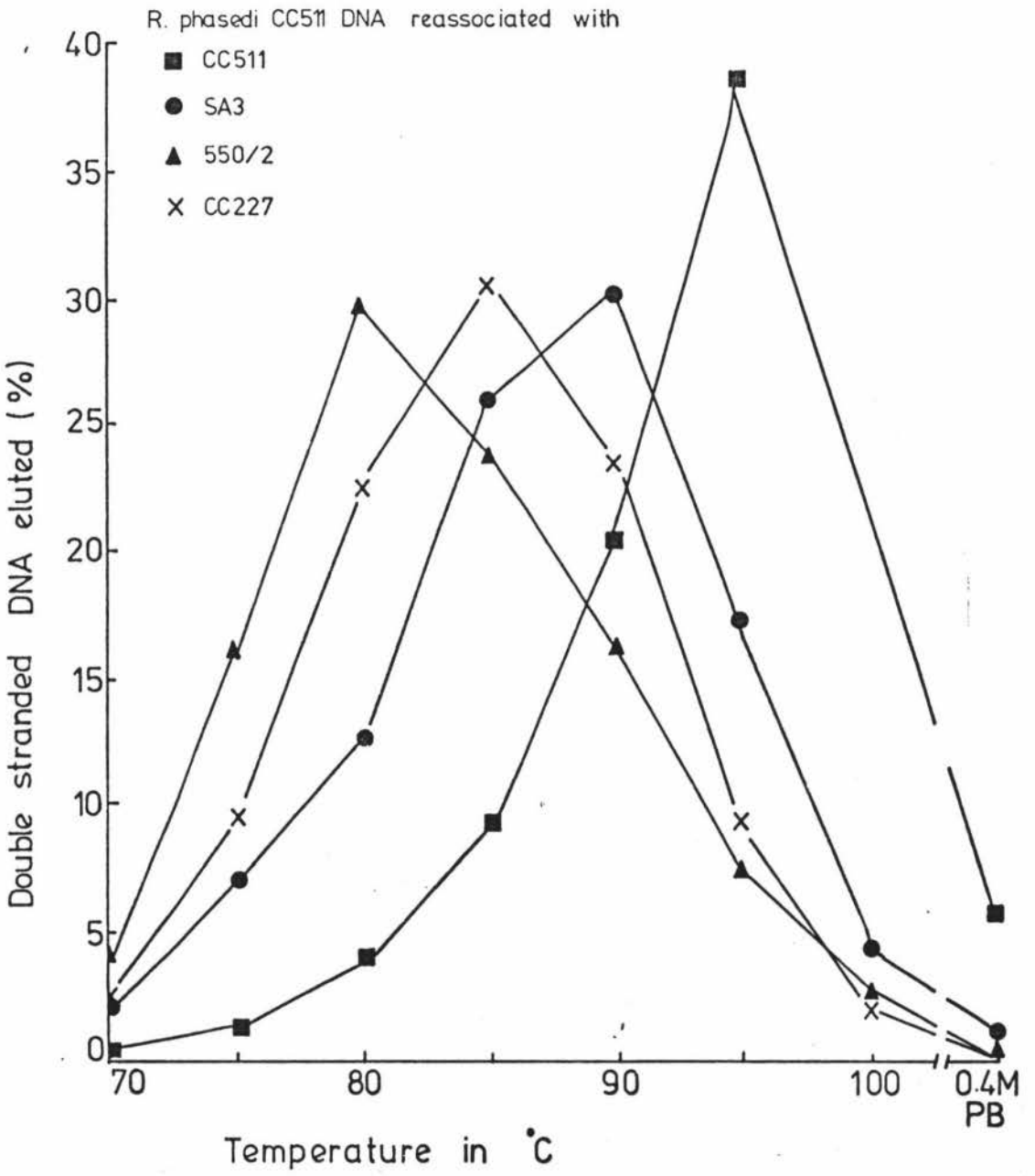


Figure 16. Thermal eltuion profiles of reassociated DNA duplexes, formed at 65°C, between DNA from Rhizobium phaseoli strain cc511 and DNA from strains of R. phaseoli and R. trifolii

Figure 17. The relationship between hybridisation at 65°C and the difference in melting point (ΔTm(e)) of reassociated homologous and heterologous duplexes formed between reference DNAs from *Rhizobium trifolii* strain TA1 and *R. phaseoli* strain cc511 and DNA from *R. phaseoli*, *R. leguminosarum*, and *R. trifolii*.

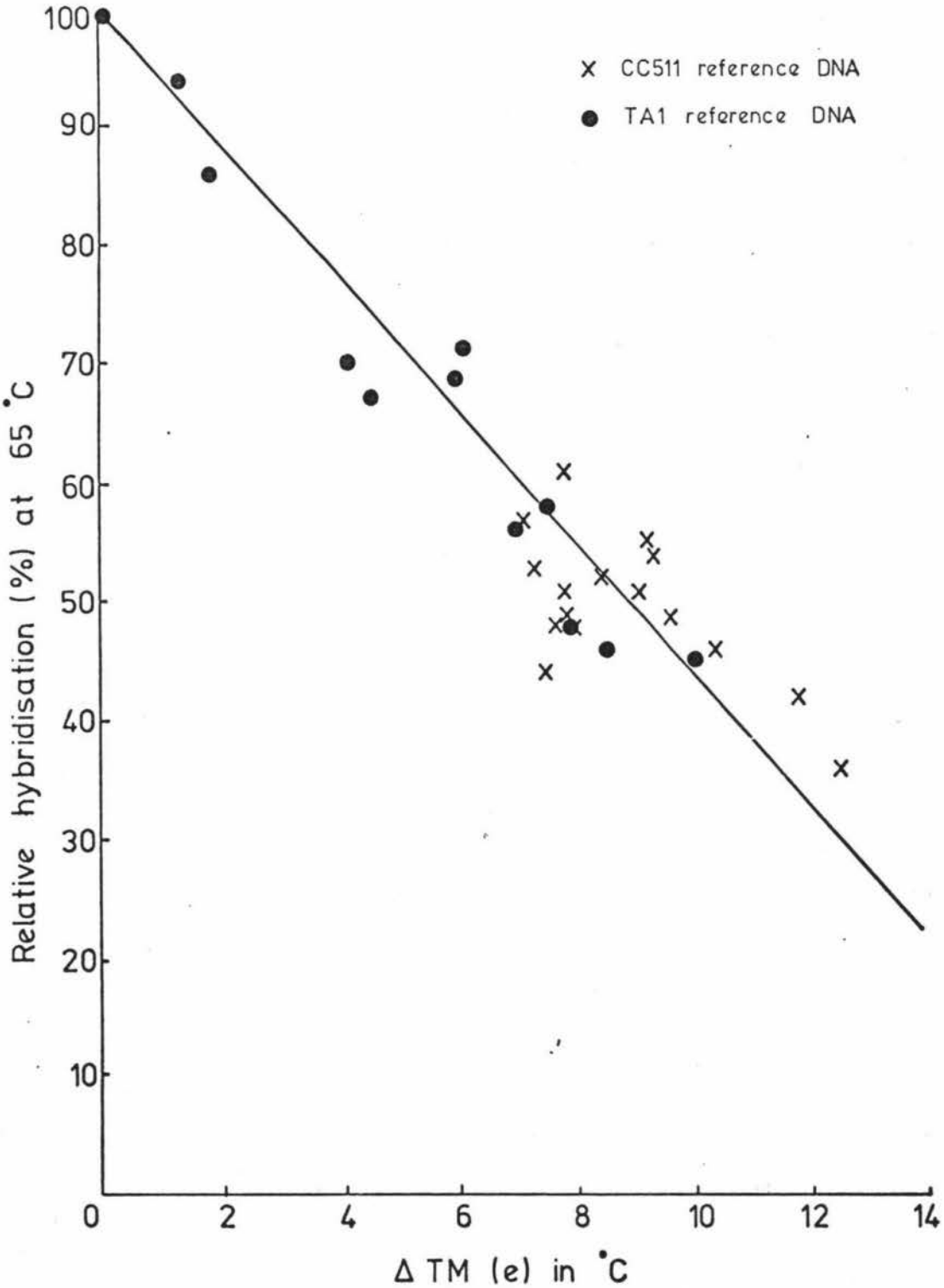
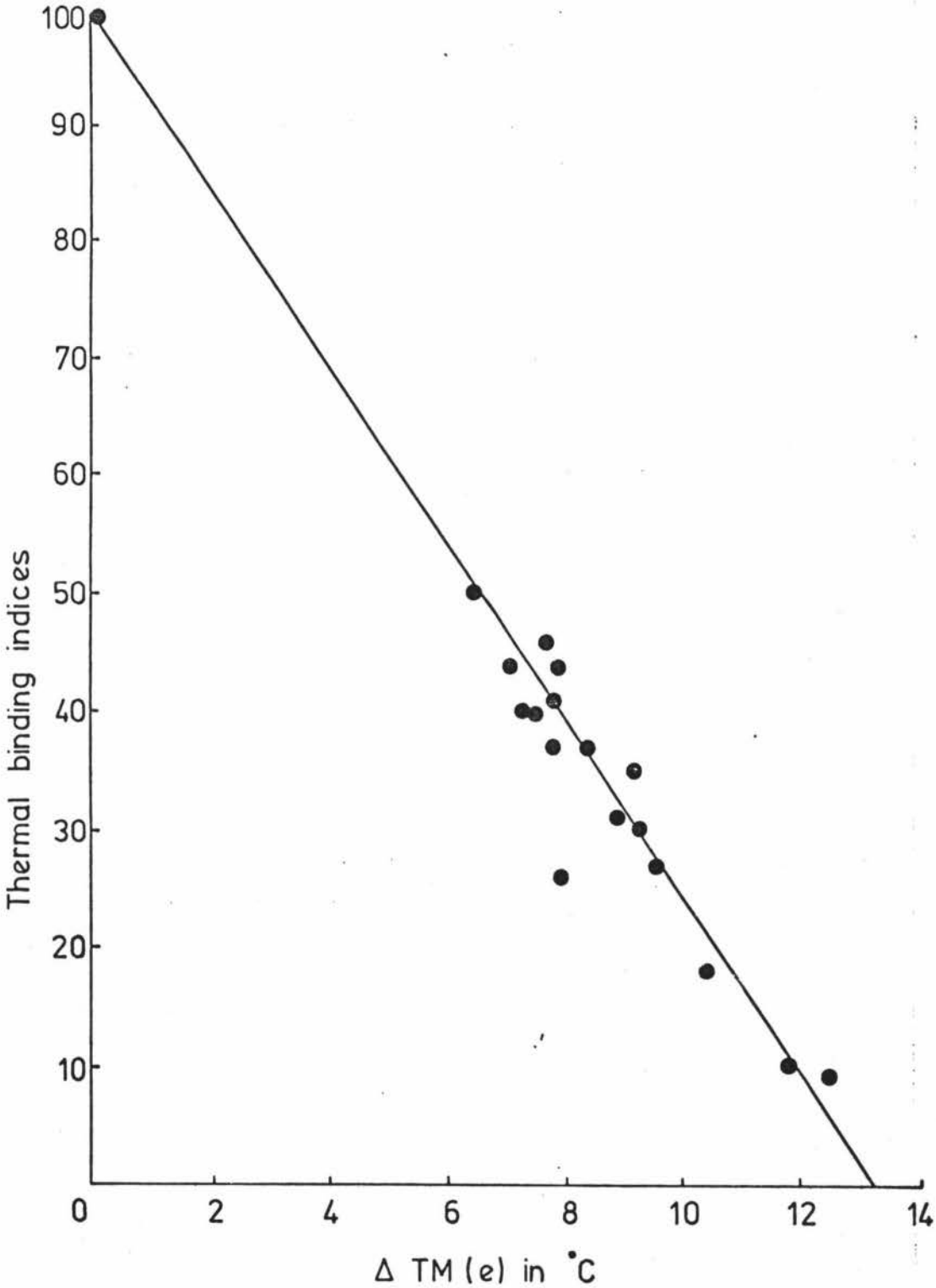

CC511 reference DNA
TA1 reference DNA
Relative hybridisation (%) at 65 °C
Δ TM (e) in °C
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14

Figure 18. Relationship between thermal binding indices (TBI) and the difference in melting point (ΔTm(e)) of reassociated homologous and heterologous duplexes formed between reference DNA from *Rhizobium phaseoli* strain cc511 and DNA from strains of *R. phaseoli*, *R. leguminosarum*, and *R. trifolii*.


Thermal binding indices
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
Δ TM (e) in °C

differences can be reflected by differences in relative hybridisation value and ΔTm(e), especially when reference DNA is prepared from one of the strains under consideration.

4.3.2 Non-nodulating Variants: strain 514 is a non-nodulating variant of an effective strain, 514/1. Relative hybridisation values with reference DNA from TA1 for 514 and 514/1 were 73.7 and 76.9%, respectively, These values are not significantly different from one another. They indicate that the base sequence similarity of DNA from these strains with DNA from TA1 was unaffected by loss of nodulating ability. Similarly, WU290 and WU290iii are non-nodulating variants of WU290 isolated independently, but there is no significant difference in their relationship with the two reference strains. All these results suggest that loss of ability to form nodules on legume roots is associated with a change in the DNA too small to affect overall sequence homology.

4.3.3 Different Histories of Reisolates: strain UNZ29 is considered to be the same strain as 1/6. The culture designated UNZ29 was carried in Uruguay and Australia before being returned to New Zealand. Relative hybridisation sation and ΔTm(e) values with cc275e and TA1 reference DNAs indicate significant differences between the two cultures.

4.3.4 Same Host Origin: strains 5039 and 5117 were isolated from ineffective nodules on a legume indigenous to New Zealand, Clianthus puniceus, both strains form effective nodules on white clover. When DNA from 5039 and 5117 reassociated with reference DNA from R. trifolii TA1 the relative hybridisation values were 67.9 and 65.7 %, respectively. The difference between these values is not statistically significant. Close genetic relationship in this case may be due to their similar host origin and nodulating ability.

TABLE VIII: Relative hybridisation of DNA strains of *Rhizobium trifolii*. *R. phaseoli*, *R. leguminosarum* and *Escherichia coli* with reference DNA from *R. trifolii* cc275e at 65°C and Cot 100.

Source of DNA	Relative Hybridisation (%)	± SE[a]	Relatedness[b]
cc275e	100.0	± 0.00	
540	91.1	± 0.3	
560	87.4	± 1.1	
549	81.2	± 1.3	
SU202	80.8	± 1.5	
5117	80.7	± 0.4	
WU290	80.3	± 3.2	
554	79.1	± 0.6	
WU290iii	74.6	± 2.3	
5225 (c)	73.9	± 3.3	
cc227 (e)	73.8	± 0.9	
1/6	73.8	± 4.2	
WU95	73.6	± 1.0	
514/1	73.4	± 1.0	
K8	72.7	± 0.4	
cc229	72.6	± 2.1	
cc2480a	71.2	± 2.8	
TA101 (c)	70.5	± 2.4	
TA2	69.9	± 1.3	
29	66.4	± 1.3	
cc321a (e)	65.7	± 1.8	
TA1	64.7	± 1.7	
550/2 (h)	62.2	± 0.3	
SA3 (f)	59.6	± 3.1	
5097 (d)	50.3	± 2.7	
CB782 (f)	45.4	± 1.5	
cc511 (d)	43.4	± 2.0	
TLN3 (g)	6.5	± 3.8	
E. coli B	0.0		

Footnotes:

(a) SE = Standard error of the mean

(b) Means that are joined by vertical bars are not significantly different at the 5% level according to Duncan's multiple range test.

(c) *Rhizobium leguminosarum*, effective on *Vicia hirsuta*.

(d) *Rhizobium phaseoli*, effective on *Phaseolus vulgaris* (garden bean). All other strains are classified as *Rhizobium trifolii* but some are members of distinct effectiveness sub-groups within the genus *Trifolium*.

(e) Effective on *T. ambiguum*

(f) Effective on African clover

(g) Effective on *T. lupinaster*

(h) Effective on American clover

Table IX: Relative hybridisation of DNA from strains of *Rhizobium trifolii*, *R. phaseoli*, *R. leguminosarum* and *Escherichia coli* with labelled reference DNA from *R. trifolii* TA1 after reassociation at 65°C and Cot 250

Source of DNA	Relative Hybridisation (%)	± SE[(a)]	Relatedness (b)	$T_{m(e)}$ (°C)	$\Delta T_{m(e)}$ (°C)
TA1	100.0	± 0.0		91.6 ± 0.3	0
TA2	93.6	± 1.2		90.3 ± 0.4	1.3
1/6	87.3	± 1.4			
5225 (c)	85.8	± 0.2		89.8 ± 0.3	1.8
UNZ29	82.8	± 1.0			
514	76.9	± 0.4			
514/1	73.7	± 1.6			
WU95	73.1	± 1.1			
K8	72.6	± 0.7			
SU202	72.0	± 0.9			
cc229 (e)	71.8	± 0.5			
CB596 (c)	71.1	± 0.9			
cc227 (e)	70.7	± 2.1		85.6 ± 0.1	6.0
cc2480a	70.0	± 0.6		87.5 ± 0.2	4.1
cc321a	69.4	± 1.4		85.7 ± 0.1	5.9
5039	67.9	± 0.6			
TA101(c)	67.3	± 1.5		87.1 ± 0.6	4.5
540	67.0	± 1.1			
5117	65.7	± 0.3			
cc277a (h)	64.8	± 0.6			
560	63.2	± 1.3			
cc275e	60.0	± 2.5		84.1 ± 0.8	7.5
SU391 (c)	58.7	± 1.4			
549	58.2	± 0.2			
WU290	58.0	± 1.1			
WU290iii	56.7	± 0.9			
SA3 (f)	56.3	± 0.6		84.6 ± 0.2	7.0

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550/2 (h)	52.5	±	0.8			
554	48.8	±	1.3			
5459 (d)	47.7	±	1.5			
5097 (d)	47.5	±	0.3	83.7	± 0.5	7.9
cc511 (d)	45.9	±	0.8	83.1	± 0.1	8.5
CB782 (f)	45.0	±	1.3	81.6	± 0.2	10.0
CB971 (d)	36.7	±	0.5			
TLN3 (g)	11.9	±	0.4			
E. coli B	0.0					

Footnotes: Table VIII

4.4 DNA Homology and Plant Specificity

4.4.1 Homologies Among the European Clover Rhizobia: in this thesis the term European clover rhizobia will refer to strains of rhizobia which infect Trifolium repens (white clover), T. pratense (red clover) or T. subterraneum (subterraneum clover). Hybridisation values for DNA from 18 European clover rhizobia and R. trifolii cc275e reference DNA, ranged from 59.6 to 91.1%, with a mean of 74.6% (Table VIII). When DNA from 20 European clover rhizobia was compared with reference DNA from R. trifolii TA1 values ranged from 48.8 to 93.6% with a mean of 67.8%. Values of ΔTm(e) of DNA reassociated at 65°C, range from 1.3 to 7.0°C with reference DNA from TA1 (Table IX). These figures indicate considerable genetic variation among the clover rhizobia.

4.4.2 Homologies Between R. leguminosarum and the European Clover Rhizobia: two strains of R. leguminosarum DNA (5225 and TA101) were hybridised with R. trifolii cc275e reference DNA (Table VIII). Their relative reassociations to the reference DNA were 73.9 and 70.5%, respectively. Four strains of R. leguminosarum DNA were hybridised with R. trifolii TA1 reference DNA with an average relative reassociation of 70.7%, and ΔTm(e)s for strains 5225 and TA101 of 1.8 and 4.5°C (Table IX), respectively. These values were of the same order as those within the European clover rhizobia group. Thus, base sequence similarity does not necessarily reflect plant specificity.

4.4.3 Homologies Between American Clover Rhizobia and the European Clover Rhizobia: two strains of this study were isolated from American clovers. Strain cc277a was effective on T. parryi and T. dasyphyllum but produced ineffective nodules on T. repens and T. subterraneum and strain 550/2 was effective on T. vesiulosum and produced effective nodules on T. repens and T. subterraneum. Relative reassociation value for strain cc277a with

reference DNA TA1 was 64.8%, and strain 550/2 with reference DNA cc275e and TA1, were 62.2 and 52.5%, repectively. In this case the different geographical origin of the two strains was not associated with a significant decrease in DNA homology and both strains were able to produce nodules on white and subterraneum clover..

4.4.4 Homologies Between Trifolium ambiguum Rhizobia and European Clover Rhizobia: Tables VIII and IX list three strains of rhizobia (551, 552, 553) derived from Caucasian clover (T. ambiguum). These plants require specific root nodule bacteria and the bacteria do not nodulate effectively on T. repens or T. subterraneum (Table III). But relative reassociation and Δ Tm(e) values from DNA from Caucasian clover strains and reference DNA from R. trifolii are generally of the same order as those with DNA from other strains of R. trifolii. Furthermore, the three strains of Caucasian clover rhizobia cluster closely together as seen in Figures 15, 16 and 17. It is concluded that marked differences in plant specificity are not necessarily associated with more general genetic divergence.

4.4.5 Homologies Between African Clover Rhizobia and European Clover Rhizobia: two strains of rhizobia in this study were isolated from African clover. Strain CB782 was effective on T. semipilosim but did not nodulate T. repens or T. subterraneum and strain SA3 was effective on T. africanum but produced ineffective nodules on T. repens and T. subterraneum (Table III). Relative reassociation values for strain CB782 with reference DNA cc275e and TA1 were 45.4% and 45.0% respectively, and ΔTm(e) with TA1, was 10.0°C. Relative reassociation values for strain SA3 with reference DNA cc275e and TA1 were 59.6 and 56.3%, and ΔTm(e) with TA1, was 7.0°C (Tables VIII and IX). Strain CB782 was well outside the range of reassociation values associated with the European clover rhizobia and was significantly different.

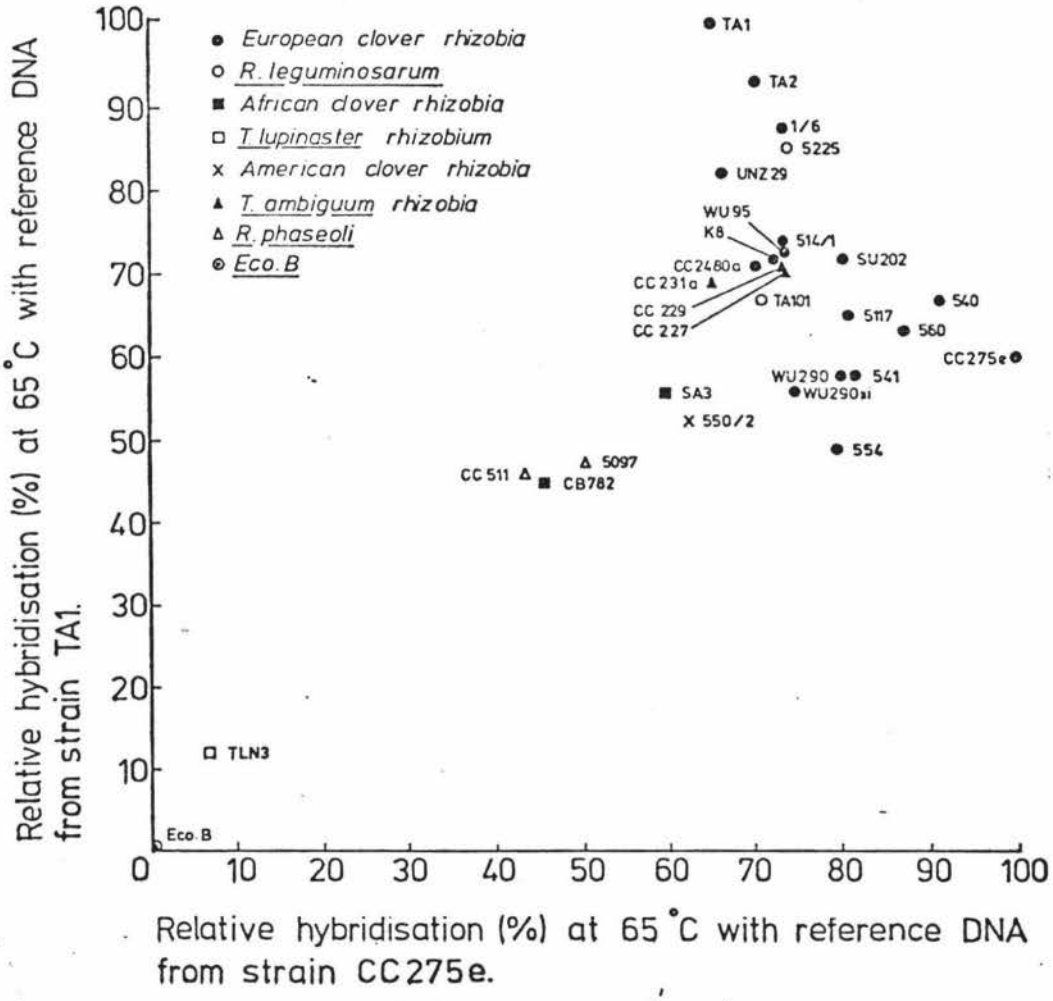

Figure 19. Relationships between the relative hybridisation (%) at 65° C of DNA from strains of rhizobia and reference DNAs from Rhizobium trifolii strains cc275e and TA1

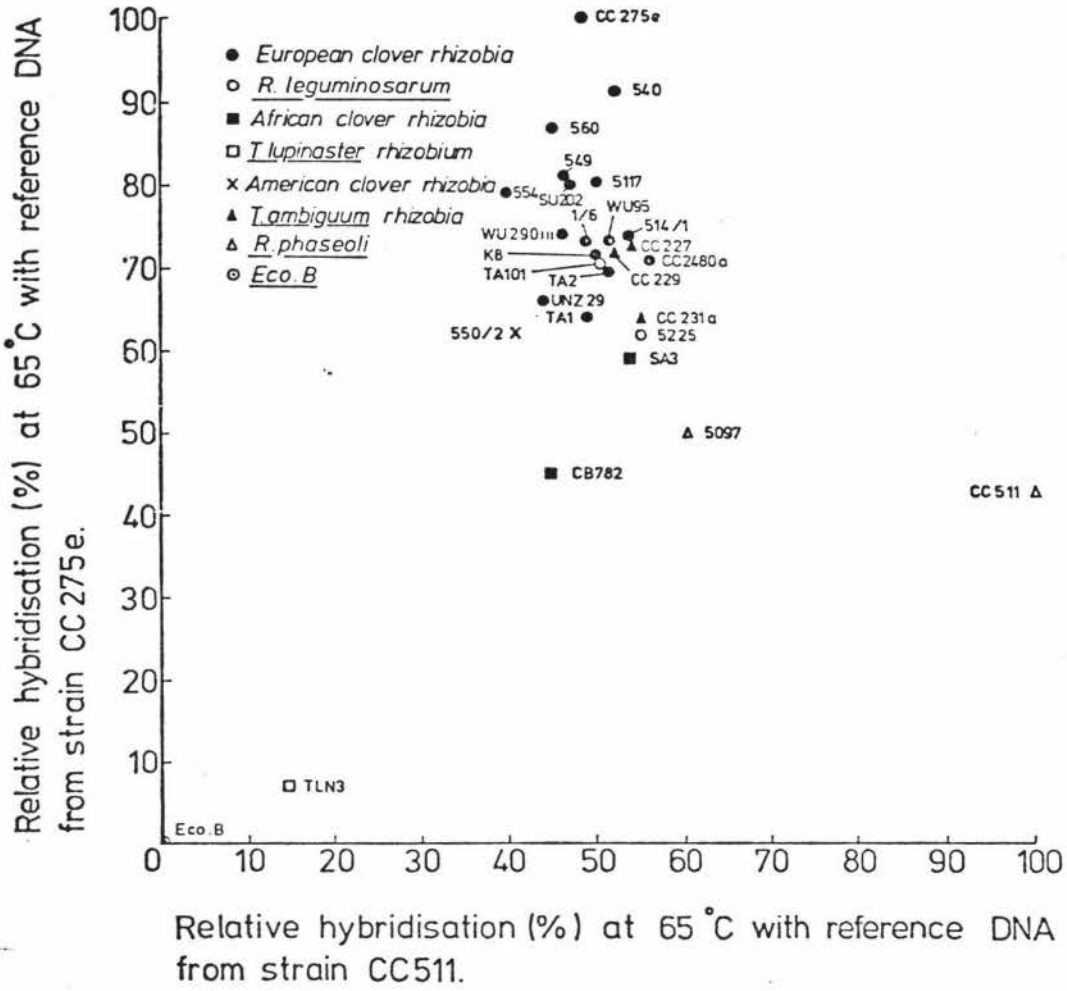

Figure 20. Relationship between the relative hybridisation (%) at 65°C of DNA from strains of rhizobia and reference DNAs from Rhizobium trifolii strain cc275e and Rhizobium phaseoli strain cc511

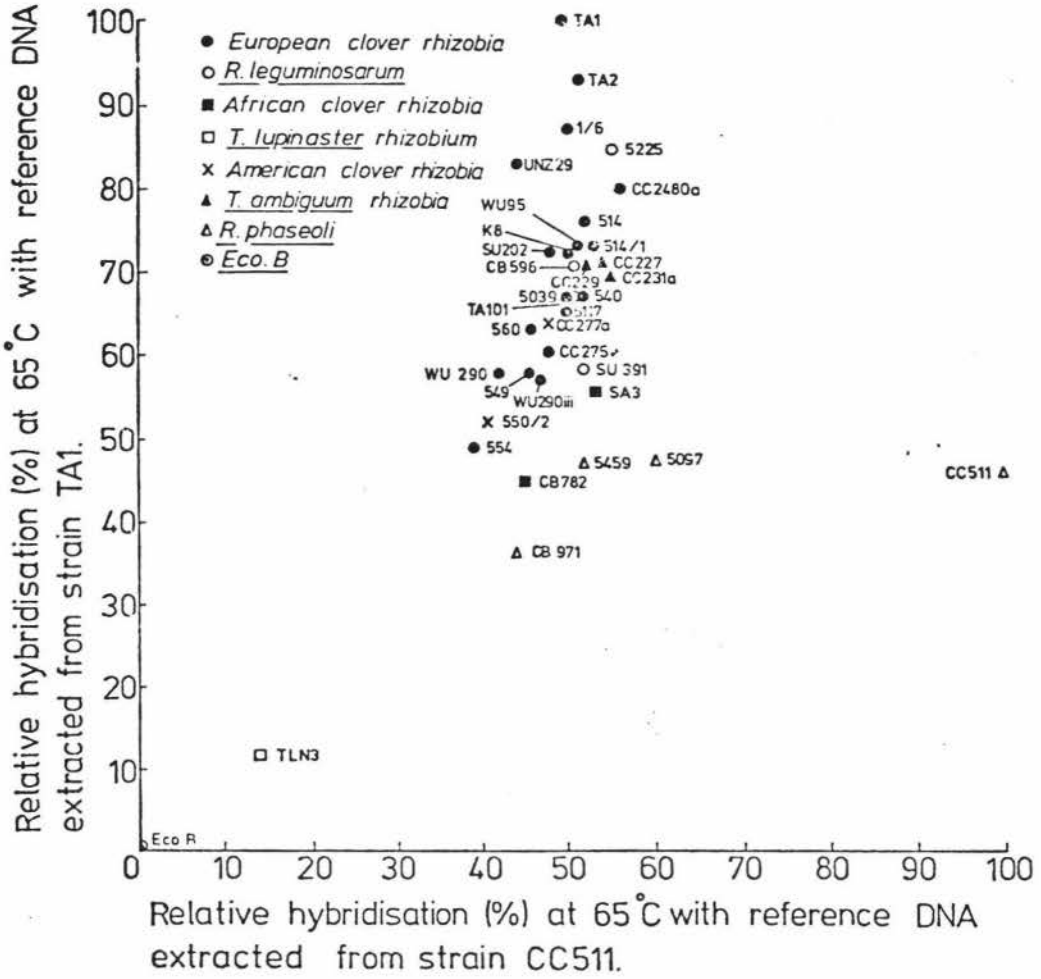

Figure 21. Relationship between the relative hybridisation (%) at 65°C of DNA from strains of rhizobia and reference DNAs from Rhizobium trifolii strain TA1 and Rhizobium phaseoli strain cc511

Strain SA3, which could produce ineffective nodules, was more closely related to the European clover rhizobia but was on the lower limits of the range. With these African clover rhizobia a difference in plant specificity was associated with general genetic divergence from the clover reference strains.

4.4.6 Homologies Between Japanese Clover Rhizobium and European Clover Rhizobia: strain TLN3 was isolated from a nodule on T. lupinaster, and it nodulated ineffectively on white and subterraneum clover (Table III). Relative reassociation values with cc275e and TA1 were 6.5 and 11.9%, respectively. Similar results were obtained with a second preparation of DNA from TLN3. These values are well outside the range expected with R. trifolii and it clusters separately from the other strains (Figure 15). This suggests that genetically distinct groups of bacteria may be identified among the rhizobia infecting plants of the genus Trifolium.

4.4.7 Homologies Between Rhizobium phaseoli and European Clover Rhizobia: Tables VIII and IX also list strains effective on Phaseolus vulgaris, the garden bean. The relative reassociation values for DNA from bean rhizobia and the two reference DNAs averaged 45% (range 36.7 - 50.3%) and Δ Tm(e) values averaged 7.8°C (range 6.5-8.5°C). Thus the bean rhizobia were significantly different from the European clover rhizobia.

Reference DNA was prepared from R. phaseoli strain cc511 and reassociated with DNA from 22 strains of European clover rhizobia. Relative reassociation values at 65°C averaged 48.8% (range 39.8-56.6%) and Δ Tm(e) values averaged 8.2°C (range 6.5-11.8°C) (Table X). Relative reassociation at 80°C averaged 17.1% and thermal binding indices, 0.35. This data further emphasises the difference beteeen the bean rhizobia dnd the European clover rhizobia.

4.4.8 Homologies Between *Rhizobium phaseoli* and Other Groups of Rhizobia: hybridisation with strain cc511 produced a relatively small range in % relative reassociation (39.8-60.5%) when compared with clover rhizobia references (36.7-91.1%), excluding strain TLN3. Because of the small cluster of results produced (Figures 16 and 17), its ability to distinguish between the different groups of rhizobia was limited. Table X shows that strain cc511 was not closely related to the three other bean rhizobia, the average % relative reassociation being only 52.3% (range 44.3-60.5%). This suggests that the bean rhizobia are a much more diverse group than the clover rhizobia. Reassociation and ΔTm(e) values were similar for the three strains effective on *T. ambiguum* and four strains of *R. leguminosarum* to those of the European clover rhizobia and within the range found within the bean rhizobia. With the exception of strain SA3, the African and American clovers gave low reassociation and ΔTm(e) with the bean reference, but so too did the bean strain CB771. This bean strain was isolated in the Philippines and was significantly different to the other Australasian bean strains. The reassociation values for DNA from strain TLN3 and cc511 reference DNA indicated that this strain from *T. lupinaster* was no more closely related to *R. phaseoli* than to *R. trifolii*.

Table X: Relative hybridisation of DNA's from strains of *Rhizobium trifolii*, *R. phaseoli*, *R. leguminosarum*, and *Escherichia coli* with reference DNA from *R. phaseoli* cc511 after reassociation at 65°C or 80°C at Cot 250.

Source of DNA	RH (%) ± SE[a]	Relatedness	RH at 80 C (%) ± SE		TBI	Tm(e)
cc511 (d)	100.0±0.5		100.0±	1.0	91.9±0.2	-
5097 (d)	60.5±0.5		24.7±0.3	0.4	84.1±0.4	7.8
cc2480a	56.6±0.6		24.7±1.4	0.4	84.8±0.1	7.1
cc321a (e)	55.4±0.7		19.2±0.8	0.4	82.7±0.1	9.2
5225 (c)	55.3±0.9		21.1±1.9	0.4	84.6±0.3	7.3
cc227 (e)	54.2±0.7		16.0±0.9	0.3	82.6±0.1	9.3
514/1	53.8±0.7		20.0±1.21	0.4		
SA3 (f)	53.8±1.2		27.0±1.3	0.5	85.4±0.1	6.5
514	52.4±0.5		20.2±1.1	0.4		
cc229 (e)	52.2±0.4		17.8±0.9	0.3		
SU391 (c)	52.2±1.3		22.4±1.6	0.4		
5459 (d)	52.1±0.7		18.7±0.4	0.4		
540	52.0±0.8		19.2±0.9	0.4		
WU95	51.9±0.7		20.8±0.7	0.4		
TA2	51.6±0.7		19.3±1.2	0.4	83.5±0.2	8.4
CB596 (c)	51.2±1.9		22.6±1.1	0.4		
5039	50.6±0.5		19.5±0.9	0.4	84.0±0.4	7.9
TA101 (c)	50.6±0.7		15.8±1.2	0.3	83.5±0.2	8.9
K8	50.2±0.8		18.6±0.6	0.4		
5117	50.1±0.6		19.7±1.3	0.4		
1/6	49.7±1.2		21.9±1.4	0.4		
TA1	49.3±0.7		18.0±1.2	0.4		
cc275e	48.4±0.3		22.3±1.8	0.5		
cc277a (h)	48.3±0.5		12.6±0.4	0.3	84.2±0.1	7.7
SU202	47.8±1.1		18.9±0.3	0.4	84.0±0.2	7.9
WU290iii	46.7±1.4		14.6±1.2	0.3		
549	46.5±0.5		17.9±0.7	0.4		

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560	46.0±0.7	12.1±0.8	0.3		
CB782 (f)	45.8±0.9	8.4±1.1	0.2	81.4±0.1	10.4
CB971 (d)	44.3±0.6	17.5±1.0	0.4	81.4±0.1	10.5
UNZ29	43.9±0.6	12.1±0.7	0.3		
WU290	42.4±0.4	8.6±0.2	0.2		
550/2 (h)	41.5±1.3	4.0±0.9	0.1	80.1±0.3	11.8
554	39.8±0.5	3.1±1.0	0.1		
TLN3 (g)	14.5±0.7	0.4±0.4	0.0		
E. coli B	0.0	0.0			

RH = Relative Hybridisation

Footnotes: see Table VIII

DISCUSSION

1. Growth of Rhizobia

1.1 Growth Curves

The growth curves of three representative strains of Rhizobium trifolii in YM medium were determined in order to decide how long the cultures should be incubated before harvest. During active growth phases replicating DNA may contain more than one copy of early replicating genetic markers. Seidler and Mandel (1971) demonstrated that the stage of growth of the cells influenced the renaturation rate. They concluded that to obtain consistent results all the cultures should be harvested at the same stage of growth. The stationary phase was recommended. During the stationary phase of growth strains of Rhizobium continue to produce polysaccharide slime. Phenol-chloroform purification of DNA does not completely remove polysaccharides (Edelman, 1974) and polysaccharides have been found to interfere with reassociation (De Ley, 1970). It was necessary, therefore, to avoid holding cultures for lengthy periods in the stationary phase. Cultures were harvested after 48 hours to ensure that all strains reached the stationary phase but did not produce copious quantities of polysaccharide.

The turbidity of cultures grown in Yeast Mannitol (YM) broth without K_2HPO_4 was less than that of cultures grown in complete YM broth. In the absence of K_2HPO_4 buffering capacity the pH of the medium dropped to an average of 6.0. The lower limit of the pH range which rhizobia tolerate is 5.0 (Jordan and Allen, 1974) and media is normally buffered at pH 6.8-7.0. Although the initial growth of Rhizobium (Figure 6) was the same, acid production by the organisms (Jordan and Allen, 1974) in the absence of K_2HPO_4 buffering capacity resulted in a reduced yield in the stationary phase.

1.2 Improvements in Cell Yield

Initially this work was undertaken to increase the yield of cells containing the labelled DNA. Cellular protein concentration and cell mass are proportional to the DNA content of the cell (Brunschede et al., 1977). Total growth of cells in the stationary phase was measured by two methods: cell dry weight and cellular protein concentration (Method 1.5), both techniques gave similar results (Figure 8). It is concluded that although extracellular polysaccharide production may have contributed to the cell dry weight, the cell dry weight is still proportional to cellular protein concentration. Both methods give a valid indication of the DNA content of the labelled cultures.

Date (1972) and Sherwood (1972) have described the effect of yeast extract (YE) on the growth of rhizobia. Both experimenters found that increasing YE concentration up to a certain point increased growth as measured by turbidity, but further increases resulted in depression of growth. Different responses were encountered by the same strain to varying sources or different batches of the same source of YE. The present study confirmed all of these points. Sherwood (1972) identified the primary cause of YE toxicity to R. trifolii as glycine. The inhibition was increased by the presence of monovalent cations and prevented by calcium. Depression of growth with increasing YE concentration in the presence of K_2HPO_4 in this study can be explained by the interaction between potassium ions (K^+) and glycine from the YE. R. trifolii strain TA1 growth was depressed by concentrations of YE in excess of 3.2g/litre, even in the absence of K_2HPO_4. This toxicity could be explained by the interaction of sodium ions (Na^+ from NaCl or YE in the medium) with increasing amounts of glycine.

Further investigation of increasing YE concentration to improve cell yield became unnecessary when the

hydroxyapatite-urea method of purifying labelled DNA was developed, since a high yield of DNA for ethanol precipitation was no longer important. Instead, emphasis was placed on getting DNA of the highest specific activity. This could be achieved best with a lower concentration of YE asthis supplied less phosphate ions to compete with the ^{32}P. To maximise growth of cells for unlabelled DNA preparation, it would be desirable to increase the YE concentration to 1.6g/litre and also to include calcium ions to eliminate the possiblity of YE toxicity at this concentration. Sherwood (1972) added $CaCl_2.6H_2O$ (0.22g/litre) for this purpose.

2. Molecular Weight Determination of DNA

Sonication of unlabelled DNA for 75 seconds with a 19mm probe reduced the DNA to fragments with an average molecular weight of 230,000. The same effect could be produced in 56 seconds with ^{32}P-labelled DNA. The rate and extent of renaturation is influenced by the molecular weight of the participating fragments of DNA. The extent of renaturation increases with the molecular weight of the fragments (Marmur and Doty, 1961). The fragment size in most investigations has been between 125,000 and 250,000 Daltons (Britten and Kohne, 1966). A fragment size within this range was chosen for this study.

The shorter time required to reduce labelled DNA to 230,000 Daltons is partly due to the purification procedure used. The hydroxyapatite-urea purification method requires vigorous stirring to resuspend the hydroxyapatite in the buffer, this results in shearing of the DNA. Burgi and Hershey (1963) found that ^{32}P-labelled preparations of DNA showed extreme fragility under shear, as if radiochemical action had introduced randomly placed weak spots into the molecules. This is also consistent with the reduced sonication time necessary for ^{32}P-labelled DNA.

3. Difficulties Encountered in Hybridisation

3.1 Preparation of DNA

3.1.1 Phenol-Chloroform Method: this method is satisfactory for the removal of proteins, lipids and RNA but less effective for polysaccharides (Edelman, 1974). Segovia et al.(1965) used this method and found that their DNA preparations contained polysaccharide equal to 54% of the DNA by weight, this was not removed by repeated ethanol precipitations. Table IV records the composition of labelled and unlabelled DNA preparations prepared by the phenol-chloroform method. The first labelled preparation contained considerable protein, another contained protein equivalent to 6% of the DNA by weight but no protein could be detected in the remaining four preparations. Two of the preparations were assayed for RNA and found to contain 12 and 14% respectively. The assay also indicated that RNA was present in Sigma calf thymus DNA suggesting that the assay was not specific for RNA in the presence of DNA. Markov and Ivanov (1974) reported that the orcinol reaction overestimated RNA because of interference by other substances, among them DNA. Segovia et al. (1965) found that 8μg polysaccharide gave an orcinol reaction equivalent to 1μg RNA. The apparent RNA contamination assayed in these preparations was probably overestimated and may not, in fact, have existed. The percentage of polysaccharide to DNA assayed in the six prepartions varied from 55 to 646% (mean 202%). Of the three contaminants assayed for, polysaccharide was the only one consistently found in large quantities.

3.1.2 Hydroxyapatite-Urea Method: this method for purification of DNA was first described by Britten et al. (1970) and has since been used in a modified form in the purification of DNA, for DNA hybridisation, by Hontebeyrie and Gasser (1977). Britten et al. (1970) found that DNA absorbed to the hydroxyapatite while RNA passed through in the presence of 8M urea plus 0.24M PB. Protein

and other cellular constituents bound with very little being eluted under the conditions that eluted the DNA (0.4M PB, 25°C). The advantage of this method was that DNA of high purity was recovered in large amounts. Markov and Ivanov (1974) found that hydroxyapatite-urea purification did not suffice to separate DNA from all protein contaminants. They suggested that a simple and efficient way to achieve such a separation was a treatment of the cells with phenol prior to hydroxyapatite chromatography. The method used here involved phenol treatment of lysed cells to remove protein and ribonuclease (RNase) treatment to remove RNA before hydroxyapatite chromatography. Protein could not be detected by the protein dye-binding method of Bradford (1976) in DNA prepared by this method. Most of the protein was extracted with phenol, some irreversibly bound and in one case some eluted in 8M urea + 0.14M PB. The RNA, expressed as a percentage of the DNA by weight, was 26% in the first preparation but was reduced to 5% by the addition of RNase. This amount could be accounted for by interference to the orcinol reaction by DNA or polysaccharide. The polysaccharide in each preparation, expressed as a percentage of the DNA by weight, was 1 and 4% respectively. This represented a substantial improvement on the average of 202% found in phenol-chloroform DNA. The polysaccharide and RNA passed through the hydroxyapatite and were found in large quantities in the 8M urea + 0.14M PB eluants.

3.1.3 Effect of Polysaccharide in the Labelled DNA Preparation on Hybridisation: after the two initial attempts, labelled DNA was prepared by the hydroxyapatite-urea method. These DNA preparations were not assayed since time and quantity of DNA were both limiting factors. The purity of the labelled DNA was assumed to be of at least equal purity to that of the unlabelled DNA since the method of purification was the same. Hybridisation between homologous strands of DNA occurred at low levels with labelled DNA prepared by the phenol-chloroform method and contaminated by polysaccharide whereas homologous

hybridisation with labelled DNA prepared by the hydroxyapatite-urea method averaged 72%.

Homologous hybridisation between labelled DNA prepared by the hydroxyapatite-urea procedure and unlabelled DNA prepared by the hydoxyapatite-urea or the phenol-chloroform procedure, gave similar values. It was concluded that polysaccharide in the unlabelled DNA preparations did not effect hybridisation. The same observation was true of heterologous hybridisation. De Ley (1970) found that small amounts of protein did not effect hybridisation but polysaccharide caused erratic results. Interactions between denatured DNA and polysaccharides that could account for such results have been described by Graves (1968). It is concluded that polysaccharide was responsible for the inability of the phenol-chloroform prepared labelled DNA to hybridise, and that this could be overcome by the hydroxyapatite-urea purification method.

3.2 Optimisation of Hybridisation

3.2.1 Effect of Phosphate Buffer Molarity on the Separation of Single and Double-Stranded DNA with Hydroxyapatite: many of the important parameters in hydroxyapatite chromatography, including phosphate buffer (PB) concentration, vary from batch to batch and control measurements are required to establish the conditions for each batch in each laboratory (Britten et al., 1974). As previously explained (Results 3.4.1) a low PB concentration must be chosen for the first washes to achieve an efficient elution of single-stranded DNA but not premature elution of double-stranded DNA. The conditions used by different workers have varied (Jarvis et al., 1977; Brenner et al., 1969). In the work reported here a new hydroxyapatite (HA) suspension was made for each labelled DNA preparation and the buffers chosen for elution of single-stranded DNA varied from 0.10M PB + 0.4% SLS to 0.13M PB + 0.4% SLS.

3.2.2 Effect of Cot on the Reassociation of DNA: the initial concentration of DNA and the time of reassociation are particularly critical in free-solution reactions (Brenner and Falkow, 1971). Their product is referred to as Cot when assessed in 0.12M PB, or equivalent Cot (E. Cot) when measured in different buffers but adjusted to 0.12M PB (Britten et al., 1974). With homologous Escherichia coli DNA, reassociation is essentially complete at an E. Cot of 100 (Brenner and Falkow, 1971) and this value has been assumed correct for other species and for heterologous DNA in taxonomic studies. The extent of cross reassociation is important in taxonomic studies and the merits of relative % hybridisation can not easily be evaluated unless the reaction is essentially complete. Bonner et al. (1973) found that base sequence divergence had a significant effect upon the rate of reassociation, so reaction conditions optimised for the homologous DNA may not be appropriate for the heterologous DNA reactions.

The results of Cot curves on Rhizobium DNA (Results 3.4.2) indicated that an E. Cot of 200 was required to achieve maximum reassociation of homologous DNA and 250 for heterologous DNA. The heterologous reaction chosen was labelled R. trifolii strain TA1 DNA with unlabelled R. trifolii strain 503a DNA which had 67.9% hybridisation at 65°C for Cot 250. Less related DNA may require an even greater E. Cot as Bonner et al. (1973) found that where the ΔTm(e) was 24° C, a factor of more than 100 greater Cot (E. Cot 10,000) was required to reach 90% completion of the reassociation, than was necessary for the homologous DNA. An E. Cot of 250 was chosen so that matching sequences in heterologous DNA had time to reassociate. Even at this value the % relative reassociation of less related DNA, for example TLN3, may be underestimated.

3.2.3 Zero-time Binding of Denatured Labelled DNA to Hydroxyapatite: zero-time or non-specific binding is the amount of label bound to HA immediately after the DNA is denatured (Brenner et al., 1978). The stripping process (Method 2.2.5) is designed to reduce the amount of zero-time binding since material in the denatured labelled DNA that binds to HA is discarded. The material responsible is thought to be cross-linked DNA and other contaminating material that retains the ability to bind to HA after boiling and quick cooling. Brenner et al. (1978) found that zero-time binding was usually less than 3% but Britten et al.(1974) found that it varied from batch to batch of HA. Zero-time binding in this work varied from 3-8% depending upon the labelled DNA preparation and the HA suspension used.

Several experiments were carried out in an attempt to reduce the zero-time binding. Britten et al. (1974) suggested that low salt concentrations may be required for the complete dissociation of native DNA with a high guanine plus cytosine content. The % GC of fast-growing rhizobia was found to be in the range 58.6-63.1% (De Ley and Rassel, 1965). Consequently, the PB concentration in which the DNA was dissociated was varied from 0.028 to 0.28M PB with no effect on the zero-time binding.

Dissociation of the DNA strands occurs when the DNA is heated at a temperature in excess of the thermal melting point (Tm). Since fast-growing rhizobia DNA has a high % GC it also has a high Tm (Mandel and Marmur, 1968). More stringent conditions may be required for the dissociation of rhizobia DNA than DNA with a lower GC content. Gibbins and Gregory (1972) dissociated DNA at 105°C for 15 minutes. Initially DNA in this study was dissociated at 100°C for 4 minutes, as did Jarvis et al. (1977). Up to 10 minutes at 100°C resulted in no reduction in zero-time binding.

Metal ions strongly effect the rate of reassociation of DNA but can be complexed by low concentrations of EDTA (Britten et al., 1974). Concentrations in the reassociation mixture of up to 10mM had no effect on the zero-time binding.

Britten et al. (1974) also found that the amount of zero-time binding varied with the amount of DNA added to the HA. Increasing the amount of labelled DNA from 0.1 to 2.0 µg in the reassociation mixture had no effect on the zero-time binding. This indicated that the high zero-time binding was not the result of excess label in the reassociation mixture.

When the material responsible forzero-time binding was separated again 0% reassociation was achieved. The material responsible, which should have been removed on stripping, had thus been removed from the labelled DNA. It appeared that the stripping process was not working efficiently and a modified or second stripping would be desirable. Since the material was removed using a larger proportion of HA and the batch procedure, it is suggested that this technique could be used as a second stripping treatment.

4. DNA Homology Among Strains of Rhizobium trifolii and Related Species

4.1 Thermal Stability of DNA reassociated at 65°C.

Figure 17 shows that the difference in thermal elution midpoint (ΔTm(e)) of heterologous hybrids with homologous DNA is directly proportional to the % relative reassociation. This is in agreement with the results of De Ley et al. (1973) who found that the stability of hybrids decreased concomitantly with the degree of DNA relatedness. They concluded that the less two races of agrobacteria

appeared to be evolutionarily related, the more mutations occurred in the common part. Since the two values are proportional the ΔTm(e) can be predicted from the % relative reassociation at 65°C. A direct relationship was also found between ΔTm(e) and the thermal binding index (TBI). Consequently, the TBI could also be predicted from the % relative reassociation of the rhizobia strains in this study. Laird et al. (1968) and Ullman and McCarthy (1973) found that ΔTm(e) of 1°C was approximately equal to 1% divergence between the base sequences of DNA from different sources. The ΔTm(e), calculated or predicted, can then be used to ascertain the degree of divergence between the two strains.

4.2 Relationships Between Specific Pairs of Strains

4.2.1 Non-nodulating Variants: the classification of legume root nodule bacteria accepted in the eighth edition of Bergey's Manual describes six species and places them in one genus, Rhizobium (Jordan and Allen, 1974). The fundamental characteristic of this genus is ability to nodulate the roots of a leguminous plant but it is recognised that both effectiveness and nodulating capacity are readily lost in culture (Labandera and Vincent, 1975; Roughley, 1976). These results with variants of R. trifolii 514 and WU290 indicate that loss of ability to nodulate had no significant effect on overall genetic homology with an independent Rhizobium reference strain. Furthermore, no marked difference occurred between the DNA homologies of non-nodulating variants of Caucasian clover rhizobia and R. phaseoli, with their respective effectively nodulating forms. It is thought that the loss affected a relatively small portion of the DNA and in other respects the DNA remained similar to rhizobia recognised by plant tests. If this is the case additional, more stable criteria are necessary for the recognition of bacteria belonging to the genus Rhizobium.

4.2.2 Serologically Related Strains: two pairs of *R. trifolii* strains were known to be serologically identical. In both cases no statistically significant difference could be detected in DNA homology with the clover rhizobia reference DNA. One pair of strains was considered to be serologically related but not identical. A statistically significant difference in DNA homology was detected with one of the clover rhizobia reference DNAs. The limited results available suggest a correlation between DNA homology and serological relationships. Serology is already widely used in the identification of *Rhizobium* strains (Vincent, 1970). Because serological results agree with the relationships established its use in the identification of strains of rhizobia becomes more meaningful by DNA hybridisation.

4.2.3 Historical Aspects: two strains isolated on a legume indigenous to New Zealand, *Clianthus puniceus*, were found to be not statistically different. In this case the similar host origin and effectivity of the two strains was reflected in similar DNA homologies. Both strains produced effective nodules on *Trifolium repens* and clustered with the European clover rhizobia. Nodulation and homology data indicate that these two strains are European clover rhizobia that were isolated from a host which they are not normally associated with.

A statistically significant difference was detected between the DNA homologies of reference DNA cc275e with DNA from two strains (1/6 and UNZ29) thought to be the same but with different histories. Assuming that the cultures had remained pure during their different histories the geographical isolation of the two cultures has resulted in two significantly different strains evolving. This fact is important when considering results from different groups of workers using the same strains. It also indicates genetic instability in the genus *Rhizobium*, a fact well documented especially in respect to nodulating

capacity and effectiveness (Labandera and Vincent, 1975; Roughley, 1976).

4.3 Homology and Plant Specificity

4.3.1 Relationships Within Sub-groups of *R. trifolii*

All bacteria which nodulate plants of the genus *Trifolium* are placed in one species, *R. trifolii* (Jordan and Allen, 1974). But Vincent (1974) summarised previous work and recognised seven sub-groups within this species. Strains were allocated to these groups according to their relative host specificity and effectiveness. Group 1 nodulated best on *T. repens* and *T. pratense*; Group 2 on *T. subterraneum*, *T. incarnatum* or *T. glomeratum*; Group 3 on *T. ambiguum*; and Groups 4-7 on individual central African species.

Reference DNA was prepared from *R. trifolii* strain cc275e and TA1 representative of Vincent's Groups 1 and 2, respectively. This DNA was reassociated with DNA from other clover rhizobia whose plant specificity was known. No distinction is obvious between the clover rhizobia when the relative hybridisations of the two reference DNAs are plotted against each other as in Figure 19. The relative hybridisation values and thermal stability of the reassociated DNA do not support the sub-division of strains from European clovers into two sub-groups. These strains seem to belong to one rather diverse genetic population. Relative hybridisation values with reference DNA from *R. trifolii* strain cc275e and TA1 range from 60-91% and 49-94%, respectively. These values are comparable to those found in the related genus *Agrobacterium* (Dé Ley, 1974) as are the thermal stabilities of the hybrid DNAs (De Ley *et al*., 1973), but the information is not sufficient to identify genetic races of *R. trifolii* such as those found in the agrobacteria.

DNA from rhizobia effective on *T. ambiguum* reassociated with *R. trifolii* reference DNA to the same extent as DNA

from European clovers and the thermal stabilities relative to TA1 reference DNA were also similar. DNA homology between European clover reference DNA and DNA from the two strains isolated from American clover fell within the normal range for European clover rhizobia. In these cases DNA from the rhizobia of geographically separated hosts did not reveal any significant difference in DNA homology from the other clover rhizobia. Rhizobia effective on T. ambiguum should therefore be included in a combined group composed of Vincent's Groups 1, 2, and 3 rather than constituting a separate group. The American clover rhizobia should also be placed within that combined group.

Vincent (1974) places rhizobia effective on African species of Trifolium in four additional effectiveness subgroups. Two strains of African origin were included in this study, SA3 from T. africanum and CB782 from T. semipilosum; these are representatives from Vincent's Groups 4 and 6, repectively. DNA hybridisation indicates that both are more distantly related to the R. trifolii reference strains than most strains from Europenn clover species, T. ambiguum, American clover or Vicia. Examination of a larger group of rhizobia from central African species of Trifolium may establish that they represent a sufficiently distinct genetic popluation to warrant designation as a separate species.

In this study only one strain of rhizobia isolated from T. lupinaster was examined. Its DNA homologies with the three reference DNAs averaged 11%, and it showed no more homology with the R. trifolii reference DNAs than with the R. phaseoli reference DNA. A thermal binding index of 0.0 with the bean reference DNA showed that the DNA homology at 65°C contained a lot of unmatched base pairs and considerable genetic divergence has occurred even within the section of DNA homologous at 65°C. An examination of Gibbins and Gregory's (1972) and Heberlein et al. (1967) results revealed that homologies with R. leguminosarum

reference DNA of this magnitude were detected amongst such unrelated organisms as Serratia and Bacillus. DNA homolgies of 30-42% were obtained with Chromobacterium DNA hybridised with an R. leguminosarum reference DNA. More strains of T. lupinaster rhizobia would have to be examined, but on the basis of this one strain, it appears that genetically distinct groups of bacteria may be identified among the rhizobia infecting plants in the genus Trifolium. These may represent not only different species but possibly different genera. The relevance of this finding to the evolution of rhizobia is considered later.

4.3.2 Relationship Between R. leguminosarum and European Clover Rhizobia: strains of rhizobia effective on species of Vicia, Pisum, Lathyrus and Lens are designated R. leguminosarum by the Bergey classifcation (Jordan and Allen, 1974). Reassociation between DNA from these strains and reference DNA from R. trifolii indicated that they belonged to the same genetic population as the strains of rhizobia effective on European and American clover, and T. ambiguum. A close relationship between R. trifolii and R. leguminosarum has been reported on several occasions (De Ley and Rassel, 1965; Gibbins and Gregory, 1972; Graham, 1964; Jarvis et al., 1977; 'tMannetje, 1967; White, 1972) and there seems little taxonomic justification for continuing to maintain a distinction at species level.

4.3.3 Relationships Between R. phaseoli and European Clover Rhizobia: Reference DNA from R. phaseoli strain cc511 confirmed that rhizobia from bean species are genetically distinguishable from those from European clovers, T. ambiguum, Vicia or Pisum. It also showed that although DNA from rhizobia of beans and T. semipilosum had similar relative hybridisation values with reference DNA from European clover rhizobia, they probably represented distinct populations since the strain from T. semipilosum was no more closely related to the bean reference strain than to the clover reference strains.

Several taxonomic studies have proposed combining R. phaseoli with R. trifolii and R. leguminosarum to form a single species for 'fast-growing acid-producing' rhizobia (De Ley and Rassel, 1965; Graham, 1964; 'tMannetje, 1967) but DNA homology indicated that R. phaseoli is not as closely related to R. leguminosarum as R. trifolii (Gibbins and Gregory, 1972). The results in this study agree with this conclusion.

4.3.4 Proposed Classification of the Fast-Growing Rhizobia: average relationships between the strains discussed in this study are shown in Figure 22. Strains are identified by plant specificity and linked together according to their average relative hybridisation values at 65°C. Strains from T. ambiguum, T. repens, T. subterraneum, Vicia and Pisum form the main group. Strains from T. semipilosum and T. lupinaster are clearly separate from this cluster. The strain from T. africanum was plotted separately because although its relationship to reference strains from European clovers is within the lower range for that cluster, it has a somewhat higher relationship with the bean reference strain than most European clover strains.

De Ley (1968) commented that the nomenclatural division into genera and species was mainly a matter of personal preference and opted for a genus line at 40% DNA homology for the phytopathogenic bacteria, which include the agrobacteria and rhizobia. More recently, Seki et al. (1978) tentatively formulated a definition of a species: "a species is a group of strains of which the intraspecific DNA homology indices are higher than 50%". Moore et al. (1978) concluded that the different levels of DNA homology chosen to determine a particular taxon would vary from one bacterial group to another. Application of the above taxon limits results in the separation of rhizobia effective on Trifolium into two genera: one solely for the T. lupinaster strain and the other containing

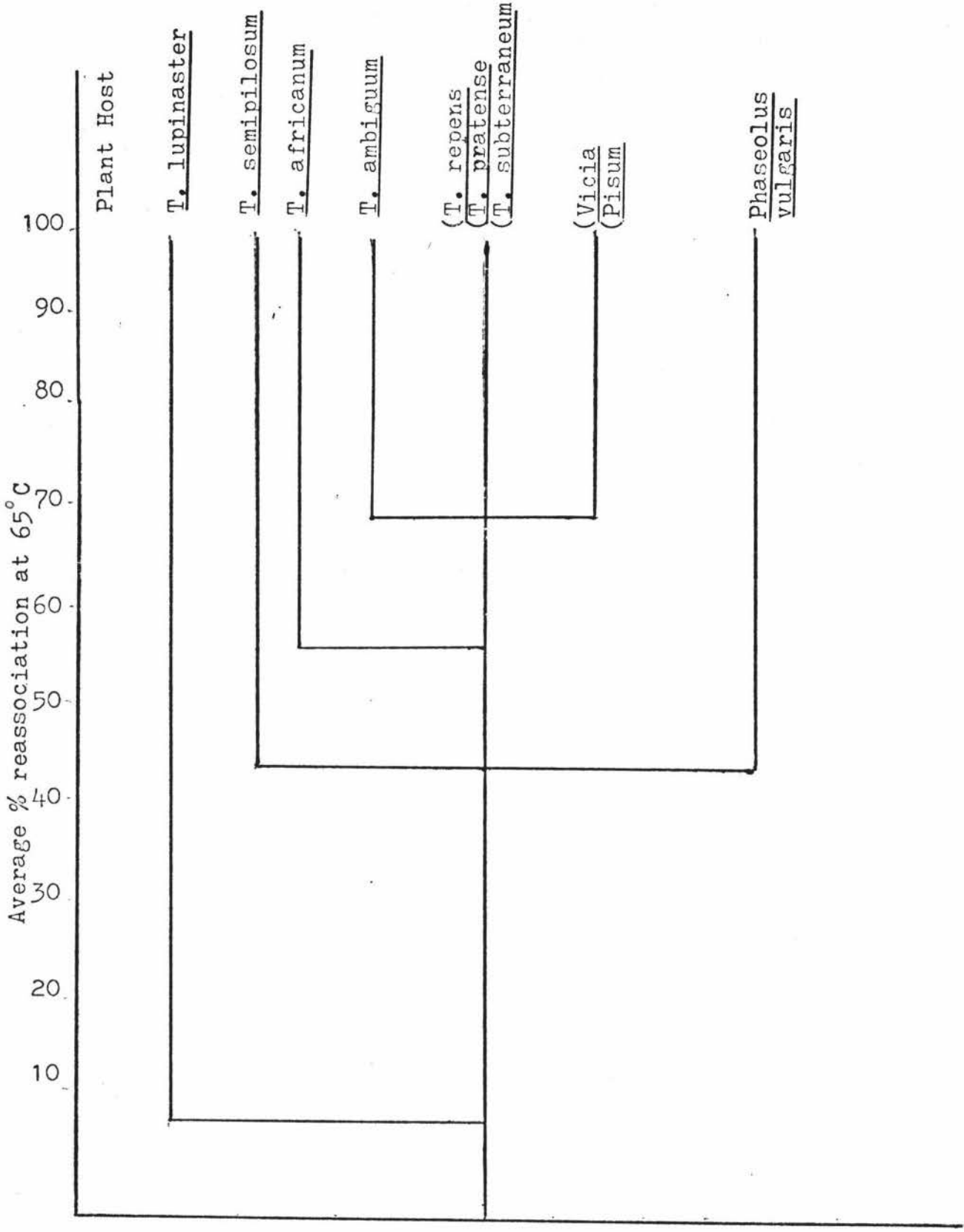

Figure 22. Genetic relationship between strains of Rhizobium from plants in the genera Trifolium, Vicia, Pisum and from Phaseolus vulgaris

the remainder. The species limit at 50% would divide the large group (Rhizobium) into three species: one each for the T. semipilosum strain, Phaseolus vulgaris strains, and one for the group of strains effective on T. ambiguum, T. repens, T. pratense, T. subterraneum, T. dasyphyllum, T. vesiculosum, T. africanum, Vicia and Pisum.

The rules of nomenclature require that this combined group be named Rhizobium leguminosarum (Frank). Within the species, biotypes could be identified according to the species name of the plant on which they nodulate most effectively. R. trifolii would become R. leguminosarum biotype T. repens, T. subterraneum, T. ambiguum, and so on. This proposal reflects the genetic relationship and accepts the common usage among persons working with root nodule bacteria from legumes. Rhizobia effective on Phaseolus vulgaris would remain R. phaseoli (Dangeard). Further investigation using DNA homology on a larger number of strains is recommended before rhizobia effective on T. lupinaster and T. semipilosum are placed in different taxons.

4.3.5 Evolutionary Considerations: Figure 22 indicates bacteria with specialised plant effectiveness may gain this function without loss of genetic similarity with other bacteria whose plant specificityis different. There are also cases where different plant specificity is associated with varying levels of genetic divergence. The first observation is at variance with the hypothesis of "symbio-taxonomy" proposed by Norris. (Norris, 1965; Norris and 'tMannetje, 1964). This suggests that as a legume evolves to fit a specialised ecological niche its root nodule bacteria also evolve and tend to become specific for the new species of legume. Evolutionary advanced legumes would be nodulated by genetically specialised bacteria while legumes which have remained similar to the ancestral form would be nodulated by genetically unspecialised bacteria capable of nodulating a wider variety of legume

species. Trifolium is considered to be an evolutionary advanced genus and should be nodulated by distinct bacterial populations. These populations should show considerable genetic divergence from one another and from other legume root nodule bacteria. The results in this study indicate that rhizobia from T. lupinaster and T. semipilosum show the expected genetic divergence but there is little evidence of it among strains in the R. trifolii-leguminosarum cluster, although some of these strains exhibit comparable symbiotic specialisations.

Parker (1968) strongly criticised Norris' hypothesis and suggested two hypotheses that he considered equally probable. Whereas Norris suggested that the slow-growing rhizobia were the ancestral type, Parker's first hypothesis was that the fast-growing rhizobia could have equally served as the ancestral type. The results of Jarvis et al. (1977) showed that slow-growing rhizobia with specialised plant effectiveness may also gain this function without loss of genetic similarity with other slow-growing bacteria whose plant specificity is different. Parker's first hypothesis therefore seems equally improbable. Parker's second hypothesis was that there was more than one occasion on which a bacterium-legume symbiosis was developed. This would accomodate distinct progenitors of both slow-growing and fast-growing rhizobia. The numerous and considerable differences that exist between the two major groups lend some credence to this proposition (Vincent, 1974). In that case, the possibility of T. lupinaster rhizobia and even other rhizobia evolving from distinctly different progenitors arises, since T. lupinaster rhizobia appear to be no more closely related to the R. trifolii-leguminosarum cluster than do slow-growing rhizobia.

4.3.6 Plasmids in *Rhizobium*: an alternative explanation for these observations may be based on the existence of plasmids in *Rhizobium* (Nuti *et al.*, 1977; Sulton, 1974; Tshitenge *et al.*, 1975; Zurkowski and Lorkiewicz, 1976). Evidence is accumulating which suggests that plant specificity in *Rhizobium* may be a plasmid-borne character (Brill, 1974; Dunican and Cannon, 1971; Higashi, 1967; Johnson and Beringer, 1977). If this is accepted it seems likely that specific plasmids would confer plant specificity on basically similar strains of bacteria and thus provide an alternative mechanism for the acquisition of plant specificity which does not require evolutionary specialisation and consequent genetic divergence.

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