SURVIVAL OF DESICCATED ROOT-NODULE BACTERIA

by

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person except where due reference is made in the text of the thesis.

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Summary

It has been demonstrated that the fast-growing root-nodule bacteria are more susceptible to desiccation than the slow-growing species. This reaction to drying has been related to the amount of water retained by the two bacterial groups at any vapour pressure. Invariably, the fast-growing bacteria retained more water than the slow-growing species. This may be a reflection of the higher surface energies involved in wetting the fast-growing species at any vapour pressure, and the larger surface area available for adsorption of water by these bacteria relative to the slow-growing rhizobia.
The different responses of the two bacterial groups to desiccation is probably related to the different amounts of water retained rather than to differences in the rate of movement of water between the bacteria and the environment on desiccation.

Very little effect of desiccation on lysozyme sensitivity could be detected for either fast-or slow-growing rhizobia. Consequently, it is unlikely that sub-lethal damage involves alterations of the integrity of the lipopolysaccharide layer of the bacteria surviving desiccation. However, desiccation did cause drastic changes

to the surface features of rhizobia as determined by fluorescence of bacteria in the presence of 1-anilino-8-naphthalene sulphonate. This could reflect damage to the cytoplasmic membrane and leakage of intracellular material from killed bacteria.

Montmorillonite was found to protect the fast-growing but not the slow-growing rhizobia from desiccation. Protection of the former group of bacteria is dependent upon the precise clay/bacterium association as not all combinations resulted in increased ability of these bacteria to survive drying. It has been postulated that the mechanism of action of montmorillonite is to decrease the intracellular water content of desiccated fast-growing rhizobia below an undefined critical level. As the water content of desiccated slow-growing rhizobia is normally below this value, further removal of intracellular water by clay has no effect upon survival.

Of all the other additives tested, only maltose, sucrose, glucose and polyvinylpyrolidone (PVP) protected both the fast-and slow-growing rhizobia from drying. The effect of polyethylene glycol (MW 6000) was similar to the effect of montmorillonite as the polymer protected the fast-growing bacteria but not the slow-growing species. All other additives tested were either detrimental or had no effect upon survival of desiccated bacteria. The mechanism of protection of sugars and PVP is unknown but it has been suggested that the site of action may involve the cytoplasmic membrane.

1. INTRODUCTION

With the advent of legume seed-inoculation and lime pelleting, many problems associated with legume nodulation during establishment of legume-based pastures have been overcome. Some annual legumes grown in certain sandy soils in Western Australia, have proved difficult to nodulate in the second and subsequent seasons despite adequate nodulation and legume establishment in the first season. The problem was very severe in many areas for clovers, but often nodulation difficulties were not observed in the blue lupin plant (Lupinus digitatus Forsk.). Marshall, Mulcahy and Chowdhury (1963) have termed the condition "second-year clover mortality" and determined that the problem was one of microbial survival. Prompted by the above results, Marshall (1964) investigated the survival of the fast-and slow-growing root-nodule bacteria in sandy soils when desiccated and subjected to high temperatures. Invariably, the ability of the slow-growing rhizobia to survive the stresses was greater than the fast-growing species. Another important fact arising from the investigation was the dramatic increase in survival of Rhizobium trifolii when either dehydrated or heated in sandy soil in the presence of certain fine-particle materials. The protective effect of both montmorillonite and illite was in contrast with the lack of such effect with kaolinite.

Why do the slow-growing species have the ability to survive extremes of desiccation and heat, when the fast-growing root-nodule bacteria die rapidly? Also, what is the

mechanism by which impenetrable additives such as montmorillonite are able to afford protection to *Rhizobium trifolii* from the detrimental effects of dehydration? In this investigation, an attempt has been made to answer some of these questions.

2. REVIEW OF THE LITERATURE

Studies on the maintenance of bacterial life in the dehydrated state or, conversely, the causes of death due to removal of intracellular water have involved several major techniques. Recently, much information has come from investigations using aerosols of bacteria from either the wet state (dehydration) or from dried bacterial powders into atmospheres of high relative humidities (rehydration). Conclusions derived from the use of aerosols have often been substantiated by experiments with lyophilized or air-dried bacteria, although these must differ because of the different techniques employed to achieve dehydration. The advantage of aerosolization, and probably freeze-drying, is that a direct measurement of the response of a bacterium to desiccation or rehydration can be achieved. The result is not complicated by interactions with particulate material as is the case when microorganisms are dried in soils or on glass microbead systems. Ultimately of course, all systems of study are valid but results need to be interpreted with care when extrapolation to a field situation is made.

The literature has been reviewed under four broad sections:-

A. Mechanisms of death of bacteria when dehydrated.

B. The structure of water in cells.

C. A review of the relevant literature dealing with death of desiccated rhizobia.

D. Microorganism - soil-particle interactions.

A. Mechanisms of death of bacteria when dehydrated.

Scott (1958), Webb (1960b) and Bateman et al.(1962) have shown that the water content of bacteria is dictated by environmental relative humidity (R.H.). The nature of the relationship is typical of water sorption isotherms of other biological materials (Bull, 1944). When the amount of water adsorbed is plotted against the percentage R.H. an S-shaped curve is obtained with the slope greatest between 70% and 100% R.H. A small change in R.H. produced a comparatively large change in cellular water content. Survival of aerosolized Escherichia coli was very R.H. dependent, especially in the 70-100% R.H. range and Webb (1960b) and Cox (1968a) suggested that this could be due to the large amount of water lost in that R.H. range. Consequently, these authors postulated that the modus operandi of death due to R.H. changes was via control of bacterial water content. Later, Cox (1970) considered that if bacterial water content was the vital factor involved in death due to dehydration, then phenomena shown by wet disseminated aerosols should be repeated by cells aerosolized from the dry powdered state. For instance, the very critical nature of bacterial survival at high R.H. (Cox, 1966a; 1966b; 1967; 1968a; 1968b; 1969) and the toxic nature of air at low R.H. (Benbough, 1969; Cox, 1966a; 1968b; Cox and Baldwin, 1967; Hess, 1965 and Webb, 1967b) should be identical for the same bacteria whether they were aerosolized from the wet or dry state.

Cox (1970) used Escherichia coli to test this relationship, and concluded that the two aerosil types were not equivalent. This study was extended to Pasturella tularensis by Cox (1971, 1972) and similar results were obtained. Consequently, for these two gram-negative rods survival in the aerosolized state may not be related to the cellular water content. Cox (1972) suggested that R.H. operated through its control on the direction of water flow across the bacterial membrane. The difference between bacteria aerosolized from the wet and dry states resulted from the interaction between the bacterial water content at the time of aerosol generation and the final environmental R.H. It was this factor which determined whether cells adsorbed or lost water. Webb (1969) suggested that the zones of instability reported by Cox (1968a;b) could have been due to toxic substances in the gases used. If this is true, then comparisons between wet and dry aerosols would be difficult. Hess (1965) reported results with Serratia marcescens which tentatively agreed with those of Cox in that aerosolization at 40% R.H. into nitrogen did not result in the death of the microorganisms, i.e. the cellular water content per se was not a vital factor in the destruction of desiccated S.marcescens. This conclusion is similar to that reached by Cox (1972).

The conclusion that the bacterial water content was not the prime cause of death upon aerosolization was in disagreement with a review by Webb (1965), who concluded that the death rate of S.marcescens was dependent upon the level of

bound water remaining in the cell at equilibrium. As water was progressively removed from cells, a corresponding collapse of proteins was assumed to occur first by interactions of -N, = N-H or -OH, groups, when some cells dried while others survived; then, below 50% R.H., further removal of water from =C=O or =P=O groups resulted in irreversible protein denaturation and a higher death rate of S.marcescens.

Cox (1966a;b;1967;1968a;b;1969;1970;1971), Cox and Baldwin (1966) and Leach and Scott (1959) have emphasised the importance of the rate and degree of rehydration as well as the composition of the rehydration medium. Monk and McCaffrey (1957) studied the effect of partial rehydration on the survival of S.marcescens. The bacteria were particularly sensitive to rehydration at a water content of 33%. It was postulated that the detrimental effects of partial rehydration were due to increased surface forces and surface-to-volume ratios with consequent large areas of denaturation within the cells. Incomplete rehydration could differentially reactivate some biochemical pathways within the cell and result in metabolic disruptions and death of the organism (Monk and McCaffrey, 1957).

Rehydration of freeze-dried Salmonella anatum in solutions containing glycerol, lactose, sucrose or milk solids resulted in recoveries higher than when the cells were rehydrated with water (Ray et al. 1971a). Higher

recoveries were obtained by increasing the solute concentration. Leach and Scott (1959) also reported higher recoveries by rehydrating freeze-dried bacteria in glycerol or sucrose solutions. They observed optimum recoveries in solutions of about 0.96 to 0.98 water activity. An optimum concentration for glycerol was not detected, but a relatively greater viable count was obtained in a 29% glycerol solution at a water activity of 0.92. Ray et al. (1971a) reported higher recoveries of freeze dried Salmonella anatum when rehydrated in 20% sucrose and 20% lactose than when a 20% glycerol solution was used. Glycerol failed to increase recovery. Choate and Alexander (1967 - cited by Ray et al. 1971a) obtained optimum recoveries of freeze-dried Spirillum atlanticum by rehydration in a 24% sucrose solution. They speculated that sucrose acted as an osmotic buffer and regulated the rehydration of these freeze-dried bacteria and reduced the dehydration damage. Therefore, in restoring water to dry bacteria, there should be some finite rate of rehydration at which damage to the microorganisms could be minimized (Leach and Scott, 1959). Rehydration in a solution of high osmotic pressure probably controls the rehydration rate.

In their reviews of the literature on microbial survival, Anderson and Cox (1967) and Poon (1966) suggested that the bactericidal action of deacreased R.H. could be dependent

upon the rate at which water evaporated from the aerosol droplet. However, the results presented by Cox (1968a) suggested that the viability of Escherichia coli was not dependent upon the rate of evaporation of water from the aerosolized bacteria.

Benbough (1967), Webb (1969), Cox and Baldwin (1966), Anderson (1966) and Davis and Bateman (1960a) concluded that there were at least two major mechanisms causing death of desiccated bacteria. First a high R.H., death occurred and was dependent upon, the removal of water from bacteria. Secondly, at low R.H. death was largely due to the toxic nature of oxygen in the air. In the first case, bacterial death was quite independent of the presence of oxygen. For ease of presentation and understanding, subsequent literature has been reviewed under two subheadings:

(i) Effects on bacteria of partial dehydration at high R.H.

(ii) Effects on bacteria of dehydration at low R.H. where oxygen becomes toxic.

A(i) Effect of bacteria of partial dehydration at high R.H.

The physiological action of semi-dehydration has been studied in detail and summarized in a review by Webb (1965). (For the purpose of this review, semi-dehydration is defined as desiccation of bacteria at 60% R.H. and above). Webb (1965) concluded that many enzymes were unaffected by aerosolization, because the same enzymatic changes occurred in bacteria protected by inositol as occurred in unprotected organisms. Escherichia coli induced to synthesise B-galactosidase prior to dehydration continued to do so after the stress was removed even though the bacteria were incapable of colony formation (Webb, 1965). Anderson (1966), Anderson and Dark (1967) and Anderson, Dark and Peto (1968) obtained similar results for a range of aerosolized bacterial genera because the activity of B-galactosidase was unaffected, however, impairment of synthesis of the enzyme was reported. In a more detailed study, Benbough et al. (1972) have reported the effect of aerosolization at 75% R.H. upon the transport of various subtrates across the membrane of E.coli. One substrate, methyl-(-D-gluco) pyranoside, was used as a non-metabolizable analogue of glucose. Aerosolization decreased the intracellular accumulation of the analogue of glucose by causing detachment of components involved in its transport across the membrane. No damage to the permease enzymes as such was found. Based on results from further experiments, Benbough et

al.(1972) concluded that the loss of active transport systems was correlated with the lethal effects of aerosolization, but that for short periods of stress, damage was repairable.

Several of the above authors (Webb, 1965; Anderson, 1966; Anderson and Dark, 1967; Anderson et al, 1968; Benbough et al, 1972) emphasised that many enzymes were unaffected by aerosolization or freeze-drying. Recently, Darbyshire and Steer (1973) published data describing semi-dehydration of certain enzymes. The activities of indoleacetic acid (IAA) oxidase and ribonuclease were unaffected by changes in water potential but ribulose diphosphate carboxylase and ketose - 1 - phosphate aldolase were both differentially sensitive to such changes. This sensitivity was attributed to fragmentation of these enzymes which did not take place with either IAA oxidase or ribonuclease. The differential response to desiccation between IAA oxidase and ribonuclease on the one hand and ribulose-diphosphate carboxylase and ketose - 1 - phosphate aldolase on the other, was reflected in their molecular complexity. The first two enzymes were relatively small (molecular weights of 40,000 and 13,700 respectively) whereas the carboxylase and aldolase were very large (molecular weights of 550,000 and 130,000, respectively). The authors concluded that water played an important structural role in the biological integrity of ribulosediphosphate carboxylase and ketose - 1 - phosphate aldolase but not in IAAase or ribonuclease. Darbyshire (1973)

demonstrated that the glycoprotein, glucose oxidase was unaffected by dehydration. Removal of various amounts of the carbohydrate moiety rendered the enzyme highly sensitive to water loss. It was postulated, therefore, that the carbohydrate units were functional in protecting the enzyme from desiccation; the mechanism was via water associated with the enzyme due to the presence of carbohydrate units. It is worth mentioning here, that Mallet (1957, cited by Davis and Bateman, 1960b) reported that several membrane bound or "particulate" enzymes in Serratia marcescens were sensitive to dehydration at 93% R.H., whereas others which were "soluble" or not bound to any particulate fraction were stable to the stress.

Benbough (1967) studied the effect of aerosolization of Escherichia coli into an atmosphere of high R.H. on its ability to synthesise and degrade nucleic acids. Increased survival was associated with decreased s-RNA synthesis and increased release of RNA into the suspending fluids. High ribonuclease activity was associated with aerosol stability of E.coli. The author suggested that the effect of dehydration on s-RNA may result in the synthesis of lethal proteins, therefore, if damaged RNA was not degraded by ribonuclease the proteins manufactured could cause death of the bacterium. No effect on DNA synthesis or breakdown was detected. In contrast, Brewley (1972; 1973) demonstrated that the polyribomes in the moss Tortula ruralis were not degraded during desiccation but were rapidly involved

in protein synthesis after rehydration. Therefore, this organism maintained a stable protein synthesizing apparatus during long periods of desiccation.

Webb and Walker (1968b) demonstrated that RNA phages were produced after desiccation even though uninfected hosts were unable to form colonies, i.e., RNA phage formation within the bacterium was not affected by dehydration. In marked contrast, Webb, Dumasia and Bhorjee (1965) reported that bacterial dehydration caused destruction of intracellular phage DNA. These results, together with the findings of Webb and Dumasia (1967) that dehydration was mutagenic and resulted in the induction of lysogenic phage, agreed with the conclusion of Webb (1965) that the mechanism of death due to dehydration was disruption of the DNA molecule. Webb and Walker (1968a) substantiated this by demonstrating that the incorporation of nucleic acid base analogues into DNA altered the sensitivity of Escherichia coli to partial dehydration. It is worth noting that Falk, Hartman and Lord (1963), showed that the structure of DNA was seriously affected when the molecule was dehydrated. Thus, Webb (1965) claimed that the basis of bacterial death due to desiccation was the removal and reorientation of water molecules bound to DNA and this resulted in the destruction of its biological integrity. It was suggested by Webb (1969) that a mechanism existed in E.coli for combating the detrimental effects of DNA which had been

damaged by semi-dehydration. This involved a repair mechanism which required energy and resulted in the discharge of damaged cell components which absorbed at a wavelength of 260 nm.

Recently, several authors have reported damage to the outer lipopolysaccharide layer (or cell envelope) of gram-negative bacteria caused by aerosolization and freeze-drying. Hambleton (1970) reported induced sensitivity of Escherichia coli B to hydrolytic enzymes such as lysozyme, ribonuclease, deoxyribonuclease and trypsin when the bacterium was aerosolized at 75% R.H. The damage sustained by the cell envelope (which allowed penetration of the above enzymes to the cell interior) was repairable and this repair did not require the synthesis of protein, RNA or peptidoglycan. An intact cytochrome system, however, was essential (Hambleton, 1971). Unlike E.coli B, bacteria such as E.coli Jepp, E.coli commune and Serratia marcescens, which were resistant to the effects of aerosolization, remained resistant to the enzymes mentioned above. Ray et al.(1971a;b; 1972) and Ray and Speck (1972) studied injury and repair in frozen and freeze-dried samples of Escherichia coli and Salmonella anatum. They found that these sublethal stresses resulted in cellular injury. The injury was reversible because the injured bacteria were able to repair the damage when exposed to a suitable environment. Ray et al.(1971b) demonstrated that repair of Salmonella anatum injured by freeze-drying, did not require the synthesis of protein, RNA or cell wall peptidoglycan, but did require energy in the form of ATP.

Freezing and storage of Aerobacter aerogens and E.coli resulted in membrane damage and consequent susceptibility to toxic metal ions (MacLeod et al. 1966). It was suggested that the protective action of amino acids was by binding or chelation of the toxic chemicals. Maltman and Webb (1971) agreed with the above authors in that they demonstrated repairable damage caused by semidehydration of Klebsiella pneumoniae. They could not demonstrate, however, any correlation between viability loss and induced susceptibility to hydrolytic enzymes. In contrast to the conclusions of the above authors, Maltman and Webb (1971) suggested that damage to the lipopolysaccharide layer of the cell wall may only be a prelude to and not a cause of death in semidehydrated bacteria. Recently, Janssen and Busta (1973) studied the effects of various additives on the damage and repair mechanisms of frozen and thawed Salmonella anatum. Their conclusion was that the protective agents may have decreased the extent of death and damage by stablizing the bacterial cell envelope.

A.(ii) Effects of oxygen during dehydration at low R.H.

The point has been made by many authors (Benbough, 1967; 1969; Cox, 1966a, 1968b; 1970; 1971; Cox and Baldwin, 1966; 1967; Cox, Baxter and Maidment, 1973; Cox, Bondurant and Hatch, 1971; Cox and Heckly, 1973; Hess, 1965 and Webb, 1967b; 1969) that the mechanism of death of bacteria dehydrated below a certain R.H. could not be directly attributed to the removal of water molecules, but was due to the toxic nature of oxygen at these low water contents. Authors vary as to the exact R.H. at which oxygen becomes toxic, even when the same bacterium was being studied. Cox and Heckly (1973) acknowledged this and stated that it was probably due to small differences in the manner of storage. No one has reported oxygen toxicity above 70% R.H. but it has usually become evident below this humidity.

Oxygen toxicity is not confined to aerosols, as Lion and Bergmann (1969a;b) and Lion (1963) reported that freeze-dried Escherichia coli was also susceptible to oxygen, as was Serratia marcescens (Benedict et al. 1961; and Dewald 1966a;b). Recently, Schwartz (1970; 1971) reported that Escherichia coli, which had been subjected to stress by freezing, became sensitive to and died when exposed to oxygen gas. Cox and Heckly (1973) however, were unable to repeat this observation.

Cox and Heckly (1973) presented evidence showing that at low oxygen concentrations the kinetics of death of S.marcescens were first order, i.e. log percentage viability was proportional to oxygen concentration. At oxygen tensions of about 5% and above, the kinetics became zero order, i.e. cell survival became unrelated to oxygen concentration. Thus, the site of action of oxygen-induced death became saturated. This was not found to be the case by Dewald (1966a) or Hess (1965), as they did not encounter limiting concentrations of oxygen toxicity. Dewald (1966a;b) derived an empirical mathematical expression which described the kinetics of death of freeze-dried S.marcescens. Cox et al. (1973), however, concluded that the results of Cox and Heckly (1973) closely fitted a different equation and applied Michaelis-Menton theory to describe the kinetics of oxygen-induced death of freeze-dried cells. In general, the equation accurately described the effects of oxygen concentration and time upon oxygen-induced death of S.marcescens.

Most of the postulated mechanisms for oxygen toxicity have centred round the detection of free radicals by electron spin resonance in freeze-dried S.marcescens (Dimmick et al. 1961) and in Escherichia coli (Lion et al. (1961) . Heckly, Dimmick and Windle (1963) and Heckly and Dimmick (1967;1968) reported inverse correlations between the number of viable bacteria surviving freezing and lyophilization, and the

magnitude of free radical production in the presence of oxygen.

The site of action of free radicals has been the subject of several papers. The presence of oxygen decreased uptake of pyruvate by aerosolized and freeze-dried Escherichia coli B (Benbough, 1967; 1969). The author attributed this to damage of flavin-linked enzymes connected with pyruvate metabolism. In agreement with this, Lion and Avi-Dor (1963) demonstrated damage to a flavin-linked enzyme in freeze-dried E.coli when exposed to air. As most cellular oxidations are mediated by the pyridine nucleotide-flavoprotein-cytochrome-c cytochrome oxidase system and as the mechanism of flavo-enzyme action involved free radical formation (Fox and Tollin, 1966 cited by Benbough, 1967), Benbough (1967, 1969) speculated that the cytochrome system may be the site of the lethal action of oxygen induced free radicals. Webb (1969) presented results which agreed with this postulate. In contrast, Novick, Israeli and Kohn (1972) speculated that death of freeze-dried E.coli exposed to oxygen, resulted from free radical induced disruption of the points of initiation of cell DNA synthesis, i.e. the mesosomes.

Recently, Cox and Heckly (1973) published results correlating oxygen toxicity with free radical production in freeze-dried and freeze-thawed bacteria. Contrary to the results of Cox and Baldwin (1966), Dimmick et al. (1961), Heckly and Dimmick (1967; 1968), Heckly et al. (1963), Lion

et al. (1961) and Schwartz (1970), they concluded that oxygen-induced free radical production was not positively correlated with viability loss of either the freeze-dried or freeze-thawed Serratia marcescens or Escherichia coli B. They were unable to detect any free radical production by freeze-thawed E.coli B, and maximum free radical production by freeze-dried S.marcescens (stored in 10% oxygen) occurred well after (5 days) all the cells were dead. In fact, after 4 hours storage the viability had decreased to 0.1%, yet no oxygen-induced free radicals were detected. Thus, there was a lag in free-radical production of at least several hours during which most cell death took place. Cox and Heckly (1973) explained that the apparent correlation obtained by others was due to the fact that they did not detect the lag in free radical production at the critical time of bacterial death.

A (iii) Influence of additives on the survival of desiccated bacteria.

There is enormous variation in substances which afford protection to desiccated bacteria. Studies have been made on the effects of chemicals ranging from simple inorganic salts and their mixtures to more complex amino acids, proteins, monosaccharides, polysaccharides and such undefined mixtures as spent culture fluid.

From the above review it is apparent that, as a broad generalization, there are two aspects to be considered in protection, i.e. does the substance protect the cell from damage due to semidehydration or is its influence at the relative humidities at which oxygen becomes toxic?

Webb (1965) suggested that whether an additive was protective or not depended upon its effect on the interaction between cell water content and the atmospheric water vapour pressure. For example, competition for available water between cell macromolecules and added compounds could be important in determining protein structure and, thus, bacterial viability. Molecular configuration was also considered by Webb (1965) to be an important factor in the action of an added compound. It was this feature which determined whether a compound could fit into and combine with cellular macromolecules in such a way as to preserve their functional configuration. Of all the substances tested by Webb (1965), the protective compounds were similar in that they contained groups potentially capable of replacing water molecules and of hydrogen bond formation. All the polyhydroxy, straight-chained hydrocarbons were toxic to airborne cells, however chemicals such as glucose, glucosamine and the six-membered rings of the hydroxycyclohexanes gave good protection. Amino and secondary alcohol groups on 6-carbon ring structures afforded cellular protection, as did increasing the number of hydroxy groups on the cyclohexane nucleus. Unexpectedly, the hydroxy benzenes

were extremely toxic. This was attributed to the basic structure of the benzene ring, the important feature being the lack, of at each carbon atom, of a second hydrogen atom with each of the -OH groups.

Cox (1966a) decided that the protective action of polyhydroxy compounds resulted from the formation of supersaturated, viscous solutions around bacteria which reduced the amount of water loss and the penetration of oxygen. The mechanism of action of protective agents was not via an effect on the rate at which water evaporated from bacteria (Cox, 1968a).

Zimmerman (1962) compared the effects of sugars on viability of Serratia marcescens after aerolization and freeze-drying. The conclusion was that all the monosaccharides tested stabilized cells against freeze-drying, and the oligosaccharides gave protection to aerosol exposure. No one type of sugar protected bacteria against both types of stress. The ability of a particular sugar to protect cells was correlated with their penetrability. Minimally penetrable sugars (oligosaccharides) were those capable of penetrating only to the cell membrane, and were active in protection only during aerolization. Freely penetrable sugars (monosaccharides) capable of crossing the membrane, were protective only during freeze-drying. Zimmerman (1962) explained the effects in terms of intracellular water content, i.e. there was an optimum cell water content for bacterial survival. In the absence of monosaccharides, lyophilization reduced the water content below this critical level. Minimally penetrable sugars served only to

further decrease the cell water content and thus could not protect freeze dried cells. The action of oligosaccharides in protecting aerosols was suggested to be via an increased rate of water loss from the bacteria. Thus the microorganisms were not held in a state of partial dehydration for extended periods of time. This is not in agreement with the results of Cox (1968a) with regard to the mechanism of protection by raffinose of aerosolized Escherichia coli. As mentioned earlier, Cox (1968a) concluded that the sugar did not affect the rate at which water was lost from either the aerosol droplet or the bacterial cell wall.

Hambleton (1970) compared the degree of bacterial cell wall damage due to aerosolization from fluids containing impenetrable raffinose and dextran, slowly penetrable glucose and glutamate, and freely penetrable glycerol. All substances decreased or prevented cell wall damage. In this respect, the results presented by Hambleton (1970) do not agree with the classification of protecting substances put forward by Zimmerman (1962).

As pointed out by Benbough (1969) additives which protected aerosolized bacteria against the toxic effects of oxygen were only required in small amounts. This was in contrast to the large quantities of sugars and polyhydroxy compounds required to give the same effect under similar conditions. Benbough (1967;1969) obtained protection of aerosolized Escherichia coli B when preincubated with electron

transport inhibitors, free radical scavengers (e.g. cysteamine), paramagnetic ions (e.g. Mn^{2+}, NO_2^- and I^-) and compounds with labile hydrogens (e.g. ascorbic acid, anilinothiols and reduced dyes), Hess (1965) also demonstrated that the paramagnetic ions (Co^{2+} and Mn^{2+}) and such substances as glycerol and thiourea could ameliorate the effect of oxygen on aerosols. It was suggested by Benbough (1969) that the sugars and polyhydroxy compounds acted by increased water retention which decreased susceptibility to oxygen. In contrast, the paramagnetic ions free, radical scavengers, and electron transport inhibitors, acted at the site of action of oxygen - induced free radicals. Many chemicals which gave protection at low R.H's were toxic at vapour pressures above 0.70.

B. <u>The structure of water in bacteria</u>.

It has long been recognised, and was often implied in many papers reviewed above, that the ability of a cell to withstand physical stresses such as desiccation, heat, cold and probably irradiation, was related to the hydration state of the protoplasm at the time of application of the stress. A distinction has been made by many workers (note Webb, 1965; Parker, 1972 and Ling, 1972) between cellular water which was bound to macromolecules and that which was free. However, Crafts (1968) considered the existence of

of truly free water in the living cell was unlikely if not impossible. Unavoidably, the definition of bound water is vague, but it is generally considered to be hydration shells of ions and water attracted to polar molecules and hydrophilic colloids. Unlike free water, bound water does not freeze and is not able to act as a solvent (Ling, 1972 and Mazur, 1970).

Adequate reviews of the literature dealing with the state of water in cells have been presented by Parker (1972), Ling (1972) and Walter and Hope (1971). The critical review by Walter and Hope (1971) dealt mainly with the state of water in cells as determined by nuclear magnetic resonance (NMR) spectroscopy. Briefly, the NMR techniques employed by various authors to study cellular water could be divided into steady state methods on the one hand and pulsed techniques on the other. Line broadening and splitting are parameters frequently observed when high resolution steady-state NMR is employed. Because these are subject to error when particulate biological material is introduced, many conclusions based on this technique are open to criticism. The techniques of pulsed NMR are largely free of these errors (Walter and Hope, 1971).

Virtually all the NMR studies of water in cells are compatible with the hypothesis of the existence of a large fraction of free water and one or more fractions of bound water (Walter and Hope, 1971). Molecular exchange occurred

between the various fractions. Although various parameters such as spin-spin (T_1) and spin-lattice (T_2) relaxation times, as well as the self-diffusion coefficient of water (D), in living cells vary somewhat from that of dilute ionic solutions, the existence of water in "ice-like" or "quasi-crystalline" structures within the cell is not required to explain these deviations. For instance, the variation in D can be accounted for by an increase in the diffusion path length around cellular obstructions such as macromolecules (Wang, 1954). In fact, it was reported by Walter and Hope (1971) that between such obstructions, the value of the self-diffusion coefficient and, hence, the structure of cytoplasmic water is very similar to that of dilute ionic solutions. This would seem to be against the tenets held by Ling (1972), who provided evidence which indicated that intracellular water exists in the form of continuous polarized multilayers between fixed charges on such macromolecules as proteins. The water layers nearest the proteins would be most severely restricted and the other layers, less motionally bound.

C. Death mechanisms in desiccated rhizobia.

For the purpose of this review the root-nodule bacteria have been divided into three broad groups:

(a) the fast-growing rhizobia e.g. Rhizobium trifolii, Rhizobium leguminosarum.

(b) the slow-growing rhizobia, e.g. Rhizobium lupini, Rhizobium japonicum and others of the cowpea groups.

(c) Rhizobium meliloti

This tripartite division has been suggested by Graham (1964), t'Mannetje (1967) and Vincent and Humphrey (1970).

The literature describing the response of rhizobia to desiccation stress is not very comprehensive and the results often vary due to differences in experimental procedure used by various authors. As seen from the previous section much of the recent work dealing with bacterial death on desiccation has been carried out using freeze-dried and aerosolized cells of genera other than rhizobia. However, one report by Won and Ross (1969) described survival of R. meliloti in the aerosolized state. Air-borne rhizobia were stable at 90, 70 and 50% R.H. for 5 hours but exposure at 30% R.H. produced an accelerated loss of viability. At the higher relative humidities of 90, 70 and 50%, U.V. irradiation increased the death rate but only after a delay period. This delay was absent at 30% R.H.

Vincent *et al.* (1962) stated that like other gram - negative bacteria, the rhizobia have generally been considered to be relatively susceptible to physical stresses such as desiccation. Using freeze-drying to preserve the bacteria, Vincent (1958) found that survival from a suspension of water was 1%, but increased to 23-44% when the bacteria were suspended in 10% sucrose or peat. Sucrose was found to be superior as an agent for protection of freeze-dried cells than sorbitol, mannitol, lysine, amino acids, milk, and yeast mannitol broth (Vincent, 1958). That freeze-dried rhizobia could survive long periods of storage was recently reported by Antheunisse (1973). Survival of various species of Rhizobium was evident after storage for 6 years. Similarly, Jensen (1961) was able to recover rhizobia from dry sterilized soils 45 years after inoculation. Lyophilization of rhizobia as a successful method of storage has been demonstrated by the fact that cultures gave good survival and nodule formation from commercially produced preparations (McLeod and Roughley, 1961), but this practice was discontinued when it was found that their survival on inoculated seeds was exceptionally short. This was in contrast to the long life of the freeze-dried cells in the laboratory (Vincent, 1972). Amarger *et al.* (1972) studied the effect of culture age on the survival of R. meliloti when freeze-dried and stored. Cells harvested while in the log phase of growth survived the freeze-drying process better than stationary phase cells, however, the latter were able to survive long periods of storage better than the

cells from the log phase of growth.

Reports dealing with survival of rhizobia in soils after prolonged desiccation and/or heat suggest that ecologically significant populations survived these stresses. Thus, Chatel and Parker (1973a) showed that dried R.lupini and R.trifolii WU95 survived heating at 80°C for 6 hours without any decrease in survival as judged by the most probable number technique. The viable count of R.trifolii TA1 dropped markedly after this treatment but significant nodulation was still obtained. Brockwell and Whalley (1962; 1970) Brockwell and Phillips (1970) and Hely and Brockwell (1962) found good desiccation and dry heat resistance of R.meliloti sown into some hot, dry Australian soils.

Marshall, Mulcahy and Chowdhury (1963) reported distinct differences between fast-and slow-growing root-nodule bacteria in their response to desiccation and heat in Western Australian soils. Lupin rhizobia exhibited much greater inherent resistance than clover or medic nodule bacteria. Marshall (1964) substantiated these field observations by similar findings on various soil types in the laboratory. It was suggested by Marshall (1964) that the predominance of legumes nodulated by slow-growing rhizobia in arid and semi-arid regions may stem from enhanced ability of these bacteria to survive desiccation and dry heat.

Chatel and Parker (1973 a and b) extended the studies initiated by Marshall, Mulcahy and Chowdhury (1963) on the problem of establishment of rhizobia in certain soils in Western Australia. They demonstrated that the numbers of R.trifolii strains TA1 and WU95 were critically low prior to the dry, hot summer period. In contrast, the population size of R.lupini WU425 was significantly greater than either strain of clover-nodule bacteria. During the summer months desiccation and heat stresses occurred and aggravated a situation that was already serious. Rhizobium trifolii WU95 was better able to survive desiccation and dry heat than strain TA1, which was the standard commercial inoculant in use at that time. Thus, Chatel and Parker (1973a;b) agreed that the problem was microbological but suggested that lack of soil colonization during the growing season was the cause. That rhizobia have different abilities to colonize soils during the growing season has clearly been demonstrated by Chatel and Parker (1973b). Rhizobium lupini was superior to both R.trifolii strains TA1 and WU95. Not only were there species differences between rhizobia in their ability to adapt and survive field conditions ("saprophytic competence", Chatel, et al. 1968), but Chatel and Greenwood (1973a) showed differences between strains of R.trifolii. Strain TA1 was consistently inferior to several other strains tested in its ability to colonize both the legume host-root system (Chatel

and Greenwood, 1973a) and the rhizopheres of plants other than those of their specific host (Chatel and Greenwood, 1973b). In contrast to the greater inherent ability of the slow-growing rhizobia to survive desiccation and heat compared to the fast-growing rhizobia (Marshall, 1964), Foulds (1971) concluded that three species of rhizobia displayed decreasing resistance to desiccation, in the order R.trifolii, R.meliloti and 'Lotus' Rhizobium. The results are equivocal, however, due to the large differences in the initial viable numbers.

Wilkins (1967) reported that after prolonged desiccation and dry heat, an adequate number of R.meliloti and rhizobia isolated from Acacia, Lotus and Psoralea species, survived to allow nodulation of their hosts. Susceptibility to heat was much greater when soils were moist than when dried. The same result was reported by Bowen and Kennedy (1959). Substantial work with other bacterial genera has resulted in similar conclusions regarding the greater potency of wet heat over dry heat as a cause of bacterial death. Evidence was reported by Wilkins (1967) of an ecological adaptation by native rhizobia to conditions of wet and dry heat which was not demonstrated by the exotic R.meliloti. However, as pointed out by Marshall (1968a), the conclusions are open to criticism because no attempt was made by Wilkins to determine the initial numbers of rhizobia present in the soils. Survival in heated

soil is related to the bacterial strain, but it is also a function of the initial numbers present in the soil.

The mechanism of survival of root-nodule bacteria when desiccated has been the subject of controversy. Bissett (1952) suggested the presence of resistant spores but, in spite of an extensive survey, Graham et al. (1963) were unable to demonstrate endospore production within the genus. Dudman (1968) implicated the capsule as a structure affording desiccation resistance, while Marshall (1964; 1968a;b) demonstrated the envelopment of rhizobia with clay particles and argued that consequent protection may be via altered rates of water exchange between the cell and its environment. Dart et al. (1969) presented electron micrographs of rhizobia in peat cultures and pointed out a fine matrix of fibrous material around R.trifolii. It was suggested that this protected cells from desiccation. Chen and Alexander (1973) reported an inverse correlation between the water activity at which rhizobia and other soil microbes were grown, and their desiccation resistance. The lower the water activity of the growth medium the greater the resistance to desiccation. It was stated that the enhanced survival may have been a result of increased water retention created by a higher internal osmotic pressure. There were, however, anomalies in that some drought susceptible soil microorganisms grew at low water activities and some drought resistant cells would only grow at high water availabilities.

Thompson (1964) studied the effect of nutrition of rhizobia upon death rates during desiccation. Unexpectedly, there was no clear evidence for poorer survival of cells deficient in calcium or magnesium relative to cells grown in media with adequate quantities of these ions. The author concluded, therefore, that lack of cell wall rigidity and the postulated inability of magnesium-deficient cells to multiply (Vincent and Colburn, 1961) were not important factors in the death of desiccated R.trifolii.

Vincent et al. (1962) studied R.trifolii desiccated at various R.H.'s on glass beads, and demonstrated a very rapid loss of cell viability at all R.H. values within the first 24 hours. It was during this time that most water was lost. A suspension of 9% maltose substantially reduced the death rate during both desiccation and periods of storage.

Thompson (1964) described the response of rhizobia to desiccation at various relative humidities and also studied the ameliorating effect of additives such as sucrose and maltose. Very rapid death of bacteria occurred at 0,20 and 60% R.H. in the absence of additives. Contary to the findings of Vincent et al.(1962) sucrose was found to be little better than distilled water as a protective agent. However, bacteria dried from suspensions in maltose solutions resulted in greater cell survival at all R.H. values. The death rates during both desiccation and storage were similar at 0% and 20% R.H. and during dehydration at 60% R.H. Storage at 60% R.H. resulted

in an increased death rate of cells relative to storage at 0 and 20% R.H. The author claimed that this could be due to the slower desiccation rate or a higher level of residual moisture during storage, both of which may be detrimental to cell survival.

Neither Vincent *et al.* (1962) nor Thompson (1964) could explain the superiority of maltose as a protective agent over other additives. There was no correlation between reduced death rate and additive assimilability, osmotic effect, the presence or absence of carbonyl groups, molecular size or solubility. Other less protective substances shared some or all of these properties. Unexpectedly, the B-isomer of maltose (cellobiose), was inferior to the α-glucoside (maltose). This prompted Vincent *et al.* (1962) to postulate the importance of molecular configuration of the additive as a vital factor involved in protection by minimally penetrable solutes. Webb (1965) also emphasised this point when discussing the mechanism of additive protection of aerosolized bacteria.

In peat cultures, Roughley and Vincent (1967) found a differential response of rhizobia to drying conditions, *R.meliloti* surving better than *R.trifolii*. Factors apart from species differences which were found to be important were the type of peat, its pH and the substances used to alter pH to neutrality. Drying the peat at high temperatures

produced inhibitory substances which prevented multiplication of added inocula, and sterilization of peat always resulted in improved rhizobial growth and survival. This was important, especially for the slow-growing species. In non-sterile peat, storage at 25°C led to rapid loss of viability whereas at 5°C negligible loss occurred over a 12-15 week period. Sterilization removed this temperature dependence during storage. The moisture content of adjusted (30%) and high moisture content (60%) were detrimental to both R.trifolii and R.meliloti, but the slow-growing Rhizobium sp. CB756 was able to tolerate the high level. For clover and lucerne strains the optimum water content for storage was 40-50% (w/w). Peat sterilization removed the dependence for survival of the inoculated rhizobia upon a critical moisture content.

Date (1968) studied the relation between survival of R.trifolii on inoculated seed and the effect of subsequent treatment and storage conditions. By peat-slurrying and lime pelleting of seeds inoculated with fast-growing rhizobia, sufficient numbers of bacteria were able to survive to produce prompt nodulation, provided seeds were not stored for periods longer than 2-3 weeks. The results reported by Date (1968) of the effect on bacterial survival of various adhesives used to stick the inoculum to the seed, substantiated the results of others, e.g. Brockwell (1962) and Date et al.(1965). Gum arabic provided the best survival of rhizobia inoculated on seeds, consequently, it is one of the most commonly used

adhesives in temperate regions. A wide range of materials used to coat inoculated seeds have been tested and these range from blood-dolomite and lime-blood mixtures (Brockwell, 1962) to various sources of lime, lime phosphate and phosphate mixtures (Date, Batthyany and Jaureche, 1965; Radcliffe, McGuire and Dawson, 1967).

Improved clover nodulation was obtained by Bergersen, Brockwell and Thompson (1958) by the use of seeds pelleted with bentonite and organic material (blood and powdered milk). Brockwell and Whalley (1962, 1970) have shown that lime pelleting gave survival of R.meliloti during storage and, provided that peat was included within the lime pellet, sowing of Medicago and lucerne seeds into dry soils resulted in adequate survival of rhizobia.

Extrapolation of the above results obtained with Medicago and R.meliloti to other cross-inoculation groups should be treated with caution (Brockwell and Whalley, 1970). Norris (1972) and Date (1973) emphasised the need for caution when applying the established principles of legume seed inoculation and pelleting used in temperate regions of Australia, to the tropical scene. Lime pelleting many tropical seeds was usually without effect and in some cases was detrimental to survival of the root-nodule bacteria. Norris (1972) stated that when pelleting was required in the tropical environment, rock phosphate was usually sufficient, but for most purposes inoculation using dilute forms of the adhesive (e.g. 1-2% methyl cellulose or 15% gum arabic) was adequate.

D. Microorganism soil-particle interactions.

The extensive topic of microorganism soil-particle interaction has been the subject of many reviews e.g. Esterman and McLaren (1959). McLaren and Skujins (1968) and Marshall (1971) and books by Hattori (1973) and Griffin (1972). Only a brief review will be given here on the physical interaction of soil particles and microorganisms and the effect of this on microbial ecology. No attempt has been made to review the effect of solid surfaces on the physiology of sorbed cells.

Zvyagintsev (1962) concluded that there were three distinct processes involved in the interaction between soil particles and microorganisms. These were:-

(1) bacterial adsorption to soil particles, i.e. the latter were larger than the living cells.

(2) mutual adsorption, i.e. soil particles and bacteria were of the same size and this resulted in the formation of aggregates.

(3) very small colloidal material adsorbed onto bacteria, i.e. the latter was larger than the soil particles.

Peele (1936), Lahav (1962) and Marshall (1967) recognised the importance of pH ionic strength and type of electrolyte in solution, as these factors influenced the nature of the surface of both bacteria and soil particles. Lahav (1962) demonstrated the reversible nature of sorption of sodium saturated bentonite to Bacillus subtilis and reported that

the bacterial population consisted of two types of cells; those which sorbed clay and those which did not. The two types of cells were indistinguishable by any other features and could only be detected after addition of clay. The results of Marshall (1968a;b; 1969a;b) with various species of Rhizobium and Bacillus subtilis (as reported by Lahav, 1962) were not in agreement with this finding presented by Lahav (1962). Because of the plate-like structure of finely divided montmorillonite, Lahav (1962) postulated that the clay may be oriented in three possible ways at the surface of a bacterium. (1) face-to-face sorption, with the broad platelet surface facing the bacterial surface; (2) edge-to-face sorption, with the edge of the platelets facing the cell surface; (3) mixed sorption, with platelets oriented at the surfaces of bacteria in both ways.

Marshall (1968a;b;1969a;b) found no consistent relationship between the amount of sodium-montmorillonite or sodium-illite adsorbed per cell, and the relative bacterial growth rate, cell ionogenic surface or the surface charge density of the bacteria. There was, however, a definite correlation between the amount of clay adsorbed per unit area of cell surface and the ionogenic properties of the bacterial surface. By electrophonetic mobility studies, Marshall (1967) concluded that Rhizobium japonicum and R.lupini possessed only acidic (carboxyl) surface ionogenic groups, while R.trifolii, R.leguminosarum and R.meliloti possessed predominately carboxyl

groups but also some basic (amino) groups. Cells with simple acidic surfaces adsorbed twice as much Na^+ - montmorillonite or Na^+-illite than bacteria with the carboxyl-amino groups. An explanation for the different ability of species of Rhizobium to sorb colloidal clay was given by Marshall (1968a;b; 1969a;b). The author suggested predominantly an edge-to-face association between the clay platelets and cells with carboxyl surfaces, because the positively charged edges of the clay would be attracted to the negative charges on the bacterial surface. The surfaces with basic groups of the carboxyl-amino type would tend to attract clay particles in face-to-face orientation. Thus, the differences between clay orientation at the surface of the bacterium would account for the differential quantities adsorbed by the various bacterial species.

Marshall (1971) has adequately reviewed the literature dealing with the effects of the solid phase of soils on microbial metabolism, growth and ecology. The literature is full of apparently conflicting results on the effect of solid surfaces on microbial activity and, as stated by Marshall (1971), "it is impossible to reconcile all of those results with any one mechanism that has been proposed." However, from the reviews by McLaren and Skujins (1968) and Marshall (1971) factors such as soil texture must have a profound effect upon the ecology of soil microorganisms. For instance, Marshall and

Roberts (1963) successfully overcame a problem involving the survival of rhizobia in the field by the addition of montmorillonite to a sandy soil. It has been suggested by Marshall (1968a), that the limited distribution of fast-growing rhizobia in sandy soils subjected to desiccation and heat may be related to the lack of a coating of certain soil colloids around the bacteria which would afford protection. Other authors (Giltner and Langworthy, 1916; Vandecaveye, 1927 and Vyas and Prasad, 1960; all cited by Marshall, 1971) have also correlated increased survival of rhizobia subject to desiccation and heat with soils of heavy texture. Another example of the effect of soil texture on microbial ecology is that summarized by Stotzky (1968). The author correlated the spread of Fusarium oxysporum and Histoplasma capsulatum with the lack of 2:1 montmorillonitic clays in soils. It was proposed by Stotzky (1968) that the 2:1 clays regulated or buffered the soil pH to such an extent that acid-sensitive soil bacteria were able to proliferate and competitively exclude the species of Fusarium. In the absence of montmorillonite, soil pH decreased and resulted in a reduced bacterial population. The acid tolerant Fusarium sp. was able to continue growth and proliferate under these conditions.

E. Aims

The aims of the investigations reported in this thesis were:-

1. To confirm the results presented by Marshall (1964) for the comparative desiccation resistance of Rhizobium lupini and R.japonicum, relative to the susceptibility of R.trifolii. The desiccation resistance of other fast-and slow-growing rhizobia has also been tested.

2. To elucidate possible reasons why related bacterial genera should react so differently to dehydration. This has involved investigations of water permeabilities, water adsorption isotherms, and internal osmotic pressures of rhizobia. Desiccation-induced repairable damage has also been investigated in an attempt to determine why the slow-growing rhizobia survived desiccation better than the fast-growing species.

3. To determine the mechanism by which such non-penetrating additives as montmorillonite were able to afford protection from desiccation to living bacteria. The hypothesis has been that the mechanism of protection of bacteria by montmorillonite was via an effect upon the bacterial water content after dehydration. Investigations of this topic has involved experimentation with other additives as well as the use of water adsorption isotherms.

3. METHODS AND MATERIALS

A. Bacteria used at various times during this investigation were:-

Group 1:- Fast-growing rhizobia

Rhizobium trifolii strains TA1, UT6, SU297/31A, SU297/32B, SU298/533A, SU298/531B, SU298/534C, SU298/536D, UT48.

R.leguminosarum TA101

Rhizobium sp. SU343 (ex. *Lotus corniculatus* L.)

Group 2:- Medic rhizobia

Rhizobium meliloti strains SU45, SU47A, SU47B, CC131, CB112, CL-A

(Strains SU47A and SU47B came from different laboratories)

Group 3:- Slow-growing rhizobia

Rhizobium lupini strains UT2, UT12

R.japonicum QA372

Rhizobium sp. CB736 (ex. *Lotononis bainseii* Baker)

Rhizobium sp. CB421

Rhizobium sp. 394b10

Rhizobium sp. 3I1b117

Rhizobium sp. 3I1b125

Rhizobium sp. 3C1f1

Rhizobium sp. UT53

Group 4:- Miscellaneous microorganisms

Escherichia coli M13

Staphylococcus aureus M3

Bacillus sp. M70

Saccharomyces cerevisiae M6

Agrobacterium species 10,24,150,152

B. Culture maintenance

Stock culture of each bacterium consisted of freeze-dried cells stored at 10°C. Initially each bacterium was reconstituted from these ampoules by rehydration, streaked onto the appropriate growth medium, then isolated colonies from these streaked plates were chosen. For storage, these bacteria were then streaked on 5 ml. small screw capped bottles containing a slope of the appropriate growth medium.

C. Tests for nodulation of legumes

The method of Vincent (1970) was used to test the ability of various species of *Rhizobium* to nodulate their specific host plants on Jensen's nitrogen-free-agar.

Bacterium	Host legume
R. trifolii	*Trifolium subterraneum* L.
R. meliloti	*Medicago tribuloides* Desr.
	Medicago sativa L.
R. lupini	*Ornithopus sativus* Brot.

The presence or absence of nodules was noted, but as this test was only used rarely in order to confirm that a particular root-nodule bacterium was not a contaminant, the results have not been included. It was not possible to test nodulation ability of the slow-growing rhizobia, other than strains of R.lupini, because of the size of the host legume.

D. Culture media

For growth of rhizobia, the medium most commonly used had the following composition:

(1) Yeast mannitol broth (YMB)

Mannitol	10 gm
K_2HPO_4	0.5gm
$MgSO_4.7H_2O$	0.2gm
NaCl	0.1gm
Yeast extract	0.5gm
Distilled water	1000 ml

The solid medium (yeast mannitol agar, YMA) contained 15.0 gm agar/l. This medium was suggested by Vincent (1970), but used as modified by Sherwood (1972) by the inclusion of $CaCl_2.6H_2O$ (0.022 gm/l). The yeast extract used was Oxcoid, code number L21. Any requirement for iron was probably met by contaminants, as the addition of $FeCl_3.6H_2O$ (0.01gm/l) did not alter cell growth. The pH of the medium was adjusted to 6.8 prior to the addition of agar and autoclaving for 20 minutes at 15 psi. All chemicals used were analytical reagent grade where possible.

Occasionally, rhizobia were grown on a medium suggested by Bergersen (1961). The medium contained:-

(11) Glutamate agar

Mannitol	10 gm
$MgSO.7H_2O$	0.1gm
$Na_2HPO_4.12H_2O$	0.45gm
Sodium glutamate	1.1gm
$FeCl_3$	0.02gm
$CaCl_2.6H_2O$	0.04gm
Biotin	250µgm
Thiamine	100µgm
Agar	15.0gm
Water	1000ml

The pH of the medium was adjusted to 6.8 before sterilization by autoclaving.

(111) Nutrient agar

Escherichia coli M13, Bacillus sp. M70 and Staphylococcus aureus M3 were all grown using nutrient broth with the addition of agar if required.

The composition of the medium was:

Bonox (Kraft)	3.0gm
Peptone	5.0 gm
Agar	15.0 gm
Distilled Water	1000 ml

Sterilization was achieved by autoclaving at 15 psi. for 20 minutes. Oxoid peptone was used, code number L37.

(4) Malt extract broth

The yeast, Saccharomyces cerevisiae M6, was grown in malt extract broth, with the addition of agar if required.

The composition of the medium was:

Malt extract (Saunders)	25 gm
Agar	15 gm
Water	1000 ml

Sterilization was achieved by autoclaving at 15 psi. for 20 minutes.

E. Soil and soil reaction

The only soil used was that obtained from Dodges Ferry (40 miles east of Hobart, Tasmania) and described as a podzol on cover sands (Loveday, 1957). The soil is a deep sand without any structure with a dark grey surface due to coarse organic matter. The only treatment after collection was to sieve it through a 2.0mm sieve. The reaction of a 1:5 soil-water suspension was pH 6.7.

The particle size distribution of the soil was carried out according to the modified Buoyoucos method (Brewer, 1962). About 50 gm air dried soil was transferred to a 1000 ml beaker to which 150 ml 6% hydrogen peroxide was added to oxidize the organic matter. After standing overnight, the contents were brought to boil and maintained at this temperature until active frothing ceased. After cooling, 10 ml normal NaOH was added, followed by 20 ml of 10% hexametaphosphate. The contents were then washed into a milk bottle and placed on an end-over-end shaking machine overnight. After dispersion, the sediment was

transferred to a 1000 ml cylinder and made up to the mark with distilled water. A blank was prepared by adding 10 ml normal NaOH and 20 ml 10% hexametaphosphate to a cylinder and making up to the mark with distilled water.

The suspensions in the cylinders were mixed for one minute. As soon as they were allowed to stand, a stopwatch was started and hydrometer readings taken at selected times. The hydrometer is calibrated to read in grams per litre. For each sample, the control reading was subtracted from the sample reading to give a corrected value. The corrected hydrometer reading gives the number of particles in suspension and solution at the end of each time interval. Coarse particles settle quickly, whereas fine particles took longer periods of time. From the hydrometer readings and a knowledge of the settling times for various particle sizes, the amount of each constituent in the sediment could be determined. The temperature effects the rate of settling, but this was constant at 20°C, hence no correction was necessary. The hydrometer reading of the blank was 3.0 gm/l. and remained constant throughout the experiment.

The air-dried soil was found to contain 0.006% moisture. The results of this experiment are presented in table 1, and demonstrate that the clay fraction is small while the bulk of the soil consists of sand particles, therefore, the soil used in this investigation is probably similar to the yellow and grey sands described by Marshall (1964).

Table 1

Time since shaking (minutes)	Hydrometer reading (gm/l)	Corrected hydrometer reading (gm/l)	Percentage (w/w) constituents in oven-dried soil.
0.13	12	9	
0.23	8	5	
0.40	7	4	Sand and other constituents = 96.4%
1.08	6	3	
1.56	6	3	
3.10	6	3	
5.00	6	3	
8.20	6	3	
10.47	6	3	Silt and clay = 3.28%
19.49	6	3	amount of silt = (3.28 - 2.20) = 1.08%
29.38	6	3	
43.20	6	3	
60.00	5	2	
120.00	5	2	Clay = 2.20%

Oven-dry weight of soil used was 91.02 gm.

F. Sterilization and treatment of soil samples prior to use.

Both amended and unamended soil samples were moistened with 4-6 ml water then sterilized by autoclaving at 15 psi. for 20 minutes.

Prior to use, the sterilized soil samples were air-dried at 28°C in a forced-draught oven for at least 12 hrs. unless otherwise stated. See attached sheet

G. Estimation of the viable count of bacteria in soil samples.

Each soil sample consisted of 10 gm of soil or soil plus a known quantity (% w/w) of amendment (final weight of soil plus amendment was 10 gm), in a 90 mm diameter glass petri dish.

To estimate the viable bacterial population in each soil sample following the appropriate treatment, the soil from each petri dish was transferred to a 250 ml Erlenmeyer flasks containing 95 ml of 1% peptone. The flasks were shaken for 30 seconds on a Vortex-Genie stirrer (Scientific Instruments, Inc.) and allowed to stand for 30 minutes. This was sufficient time to allow desorption of the bacteria from the soil particles. The flasks were then shaken for a further 15 seconds before serial decimal dilutions were made using 1% peptone as diluent. Each dilution was stirred with the Vortex stirrer prior to plating in either duplicate or triplicate on YMA using the Miles-Misra (1938) technique. To place the drops on the agar surface, a 50 ul Oxford sampler was used. The tips were plugged with a small wad of cotton wool for air filtration, then they were sterilized by autoclaving at 15 psi. for 20 minutes.

No problems were encountered with contaminants in experiments using soils sterilized by this method.

Colonies were counted after 4 days of growth for the fast-growing rhizobia, and 8-9 days for the slow-growing bacteria.

H. Growth of large quantities of microorganisms and preparation for use.

When large quantities of rhizobia were required, the growth on slopes of YMA in 100 ml McCartney bottles was harvested in sterile water. This was used to inoculate a sufficient number of 100 ml McCartney bottles (usually 20-50) to produce enough bacteria to carry out an experiment. After growth (4 days for the fast-growing rhizobia, and 8 days for the slow-growing species), the bacteria were harvested using sterile water and glass beads to remove the growth from the agar surface. The suspension was homogenized to ensure even mixing by the use of a sterile Ten-Broeck glass homogenizer.

Large quantities of Escherichia coli, Bacillus sp. M70 Saccharomyces cerevisiae and Staphylococcus aureus were obtained by growth at 28°C for 24 hrs. in 1 litre quantities of the appropriate broth medium. The containers used were low form culture flasks (Corning Glass Works, No.4422), and 3 were used for each culture. A 24 hr. old culture of the appropriate microorganism was used to inoculate these vessels, which were then placed on a gyratory shaker at 28°C to ensure adequate aeration and growth. After growth, the microorganisms were harvested by continuous flow centrifugation at 12,000-16,000 g. using a Sorval SS4 centrifuge. As experiments using these microorganisms did not require sterile conditions, this apparatus

was not sterilized.

Smaller quantities of Escherichia coli were often required and were obtained by the method outlined above for growth of quantities of rhizobia using nutrient agar as the growth medium.

I. Determination of the response of various species of rhizobia to desiccation.

The root-nodule bacteria were grown on YMA and suspended in sterile distilled water. An initial viable count of the inoculum was carried out, and 4.0 ml of inoculum was added to each of the dried, sterile soil samples. In most experiments these inoculated soils were placed in a forced-draught oven at 28°C and dried overnight. The effect of desiccation on the viable count of rhizobia in the inoculated soils was then estimated. A variation on this procedure was carried out with R.japonicum QA372 and R.trifolii SU297/32B in which soil samples inoculated with these bacteria were dried overnight, sampled, rehydrated and dried overnight followed by further sampling. This was repeated seven times and indicated the rate of decrease of the viable numbers of fast-versus slow-growing rhizobia with each desiccation-rehydration cycle.

J. Determination of the effect of various additives on survival of rhizobia when desiccated.

Various substances were tested for their effect on the viable count of rhizobia subjected to desiccation. The additives used and their concentrations are shown in table 2.

Additives 1 to 7 (table 2) were added to soil samples, in this way an additive concentration of 5% was obtained by spreading 9.5 gm soil plus 0.5 gm of a particular amendment over the base of a petri dish. The amended soil samples were mixed thoroughly and moistened with water. Sterilization was achieved by autoclaving at 15 psi. for 20 minutes.

The usual concentration of montmorillonite used was 5%, however concentrations ranging from 1 to 30% were employed. In all experiments the natural montmorillonite was used, no studies were made with montmorillonite made homoionic to a certain cation. The exchangeable metallic bases of the natural montmorillonite were:-

	meq/100 gm[x]
Sodium	60 to 65
Potassium	1 to 5
Calcium	15 to 20
Magnesium	5 to 10

x Values obtained from American Colloid Co.

The sterilized amended soils were usually air dried at 28°C in a forced-draught oven before the addition of root-nodule bacteria, Occasionally, this step was omitted and the bacteria were added to the sterile, moist, amended soil samples.

An investigation of the effect of montmorillonite added as a suspension rather than as a dried powder was carried out.

Table 2

The additives and their concentrations used in studies on the effect of substances on the survival of desiccated rhizobia.

Additive	Concentration
1. Montmorillonite	varied x
2. Hydrophobic aerosil +	5%
3. Hydrophilic aerosil +	5%
4. Haematite	5%
5. Gaethite	5%
6. Kaolinite	5%
7. Illite	5%
8. Polyvinylpyrolidone 40,000	varied x
9. Polyethylene glycol 400	10%
10. " " 1500	10%
11. " " 6000	10%
12. Maltose	10%
13. Sucrose	10%
14. Glucose	10%
15. Glycerol	5%
16. Tetrabutylammonium bromide	3%
17. Ammonium acetate	varied x
18. Inositol	5.4%

x where the concentrations were varied, see appropriate section of the Results.

+ the hydrophilic and hydrophobic aerosils are colloidal-size silica particles, Degussa Type Nos. 200 and R972, respectively.

Source of additives 1 to 7, see Page 52.

To prepare the suspension, montmorillonite was slowly added to water that was violently stirred with a Sunbeam kitchen homogenizer. This agitation was continued for 30 minutes after the last addition of montmorillonite. The final montmorillonite concentration was 0.035 gm/ml., which was a very viscous suspension that did not precipate or flocculate with time.

To test the effect of this suspended clay on the ability of rhizobia to survive desiccation, the procedure was as follows: The stock suspension of montmorillonite and dilutions of it, were mixed in the ratio 50: 50 (v/v) with a concentrated bacterial suspension in test tubes. The tubes were shaken thoroughly to ensure proper mixing of the two suspensions, then 4.0 ml of each montmorillonite-bacterium mixture were added to sterilized air dried, unamended soil samples. These inoculated soil suspensions were then air dried in the usual way.

Source of additives 1 to 7 shown in table 2 were:-

Montmorillonite - Wyoming montmorillonite,
American Colloid Co. Chicago.

Illite - Fithian illite, Ward's Natural Scientific Establishment, Inc., Rochester, New York.

Kaolinite - British Drug House Ltd.

Goethite - Bayer, needle shaped, of average length 1.0 u.

Haematite - nodular particles of 0.5 to 1.0 u diameter.

The procedure followed for the estimation of the effect of additives 8 to 18, listed in table 2, was similar to that described for the montmorillonite suspended in water, i.e. bacterial suspensions and additive solutions were mixed in the ratio 50: 50 (v/v) and thoroughly mixed. Four ml. of the mixture were added to sterilized air dried soil samples and these inoculated samples air dried in the usual way. The numbers of viable bacteria remaining after desiccation were determined as described previously.

Additives 8 to 18 were made up to double the concentrations shown in table 2, therefore the values in table 2 were the additive concentrations in the bacteria-additive suspension used to inoculate the soil samples. Where the concentration of an additive varied, the final amount is shown in the tables and graphs in the results section. In all cases, 4.0 ml of the bacteria-additive suspension was added to 10 gm soil, therefore, the quantity of the additive per gram of soil can be calculated e.g. 4 ml. of a 10% solution of maltose = 0.4 gm. maltose 10 gm. soil, which gave a soil sample amended with 4.0% maltose.

Where possible, analytical reagent grades were used. The polyvinylpyrrolidone (PVP) used had an average molecular weight of 40,000. Both PVP and tetrabutylammonium bromide were sterilized by membrane filtration, the other additives were sterilized by autoclaving for 20 minutes at 15 psi.

The period of air drying of the amended soil samples was usually 24 hrs., however, one experiment was carried out with R. japonicum QA372 which investigated the effects of various

additives on the numbers of viable bacteria after 120 hrs. desiccation.

Another variation on the above procedure was carried out with R.lupini UT12 in which the viable count was estimated after the removal of a specified percentage (w/w) of water from unamended and montmorillonite-amended soil samples, i.e. 4.0 ml of the bacterial suspension was added to both amended and unamended soil samples. The wet weight was noted and after removal of a known percentage of moisture, the viable count was estimated. The purpose of this experiment was to determine whether rhizobia began to die at the same soil moisture content in both amended or unamended soil samples.

3. The effect of an atmosphere of nitrogen on the ability of microorganisms to survive desiccation.

Soil samples were inoculated with bacteria, and dried for 4-5 days in vacuum desiccators with P_2O_5 as the desiccant. Due to complications, probably introduced by crusting of the surface of the P_2O_5, this desiccant was abandoned in favour of silica get crystals.

Desiccation in an atmosphere of nitrogen required replacement of the air in the desiccators with nitrogen. This was achieved by evacuating the vacuum desiccators to 700 mm. Hg with a water vacuum pump, nitrogen was then introduced until the system was at atmospheric pressure. Pressure changes were monitored by a mercury manometer. This procedure was repeated six times for each desiccator then twice more for every 24 hrs. that the soil samples were in the desiccators. The source of

nitrogen was Commonwealth Industrial Gases (CIG) high purity material, no further purification of this gas being carried out.

After 4-5 days desiccation, the lid of the desiccator was removed and 10 ml 1% peptone were added to each sample as soon as possible. Rapid rehydration was essential to avoid the possible toxic effects of oxygen. The rehydrated sample was added to 85 ml of 1% peptone in an Erlenmeyer flask and the number of viable bacteria estimated as previously described.

K. Measurements of water retention by soil with and without various amendments.

The percent by weight of water retained by the soil with and without various amendments was determined by use of a pressure membrane apparatus. The procedure followed was similar to that used by Richards (1949). Only one pressure was used and that was 218 psi, which is equal to 15 atmospheres.

Water retention was measured in the following samples:-

1. Soil
2. Soil + montmorillonite (powdered)
3. Soil + montmorillonite (as a suspension)
4. Soil + illite
5. Soil + kaolinite
6. Soil + haematite
7. Soil + goethite
8. Soil + montmorillonite (treated as described below)

All amendment concentrations were 5% (w/w). Treatment 8 in the above table consisted of soil amended with montmorillonite that had been heated at 600 °C for 2 hrs. The effect of heating montmorillonite was to suppress interlayer swelling (Mortland and Kemper, 1965). Therefore any water retained by soil amended with this treated montmorillonite which was in excess of that retained by the unamended soil was attributed to water sorbed on the external surfaces of montmorillonite particles. Measurement of this parameter should give an indication of the importance of interlayered water in montmorillonite used as an amendment in this soil.

1. The effects of water activity on rhizobia.

L(i) Growth of rhizobia in yeast mannitol broth adjusted to various water activities.

The water activity (a_w) of a medium is defined as:-

aw = saturation vapour pressure of the solution at a specified temperature divided by the saturation vapour pressure of pure water at the same temperature.

It is a measure of the availability to microorganisms, of water in an aqueous system, and is related to relative humidity (R.H.) as a R.H. of 100% = a_w 1.00.

According to B.J.Marshall (pers. comm.) the a_w of the usual YMB is approximately 0.999 at any temperature between 25-30°C. Yeast mannitol broths with lower water activities were obtained by the addition of various solutes. The table of the amounts of a solute required to give a particular a_w was

supplied by B.J. Marshall and are included in table 3.

Media of specified a_ws were prepared using NaCl as the solute, and the growth monitored by the method of Chowdhury (1965). Ten ml. of each of the adjusted media were placed in L-shaped test tubes plugged by Cap-o-test tops and sterilized by autoclaving. The test tubes had been selected for similar optical properties at 600 nm. A quantity of inoculum was added to each tube except the blank, to which a similar amount of sterile water was added. Immediately after addition of the inoculum, the optical density was measured at wavelength of 600 nm. in a Bausch and Lomb Spectronic 20 colorimeter. The tubes were incubated horizontally on a gyratory shaker at 28°C. The optical density of the tubes was measured either at various time intervals or after four days of growth for the fast-growing rhizobia or 8 days for the slow-growing species. After growth, the contents of each tube were tested for contamination by streaking onto plates of YMA and observation of the isolated colonies following incubation.

L(ii) Effect of the water activity of the growth medium on the ability of rhizobia to survive dehydration.

The root-nodule bacteria were grown at the lowest a_w possible either on agar or in YMB that had been adjusted using NaCl. The agar slopes were inoculated with rhizobia that had been suspended in water adjusted, by the use of NaCl, to the same a_w as the agar. At a_ws less than 0.999, the slow-growing rhizobia were harvested after 14 days growth and the medic rhizobia after 6-7 days growth. Bacteria were washed off the agar surface with water of the same a_w as the growth medium,

Table 3

BASAL MEDIUM 0.999 a_w

	Grams of solutes to be added per 100 gm water to give final a_w									
	0.999	0.997	0.995	0.990	0.980	0.960	0.940	0.920	0.900	0.880	0.860
Na Cl.		0.348	0.700	1.591	3.375	6.857	10.183	13.351	16.385	19.29	22.08
K Cl.		0.447	0.900	2.062	4.421	9.160	13.838	18.446	22.98	27.45	31.84
$Mg\ Cl_2$.		0.414	0.829	1.826	3.683	6.916	9.628	11.989	14.123	16.084	17.913
$Na_2\ SO_4$.		0.661	1.321	3.196	7.396	16.522	25.80	34.21	41.67	48.29	54.27
Sucrose		3.782	7.418	16.43	33.58	64.25	91.80	117.65	142.67	167.01	191.17
Glucose		1.911	3.839	8.729	18.52	37.62	55.87	73.25			
Glycerol		1.022	2.042	4.589	9.679	19.85	30.08	40.37	50.78	61.32	72.05
Urea		0.671	1.349	3.077	6.667	14.371	22.74	31.73	41.10	50.83	60.88
Na Cl. (5)		0.167	0.336	0.756	1.630	3.393	5.080	6.719	8.289	9.718	11.229
K Cl. (3)		0.128	0.257	0.579	1.247	2.597	3.888	5.142	6.344	7.437	8.594
Na_2SO_4 (2)		0.162	0.327	0.734	1.585	3.299	4.938	6.532	8.058	9.447	10.916

The numbers in brackets refer to the proportion of each salt used in a mixture to obtain a particular a_w.

centrifuged and the pellet resuspended in distilled water. The suspension was homogenized, and 4 ml. aliquots used to inoculate soil samples which were then air dried overnight at 28°C in a forced-draught oven. The initial viable count of the bacterial suspension used to inoculate soil samples was determined.

Following desiccation, the number of viable bacteria were determined as described previously.

In an attempt to determine whether the ability to withstand desiccation could be enhanced by acclimatization of bacteria to low a_w, many species of rhizobia were cultured initially in media of the lowest a_w permitting growth. After growth, aliquots were transferred to solutions of slightly lower a_w. In spite of numerous attempts, it was not possible to obtain substantial growth of rhizobia at lower a_w values.

L(iii) The effect of the water activity of the rehydration medium on survival of desiccated rhizobia.

Experiments were carried out in which freeze-dried or air-dried rhizobia were rehydrated in water adjusted to various a_ws. with different solutes.

Soil samples were inoculated with rhizobia grown on YMA (a_w 0.999), dried overnight at 28°C in a forced draught oven, then rehydrated in 95 ml of water adjusted to a_w of 0.995, 0.940 or 0.860 by the use of NaCl, KCl or glycerol (see table 3). The number of viable bacteria in these suspensions were determined as before.

Bacteria were freeze-dried for 12 hrs., using a Dynavac High Vaccuum system, Model No. FD16, by placing 0.5 ml bacterial suspension in sterilized glass ampoules. The initial viable count was determined by conventional techniques. Rehydration of the freeze-dried microorganisms was accomplished by the addition of 1.0ml. of sterile water or by water adjusted to a specified a_w by the use of NaCl, sucrose or glycerol (see table 3). Following rehydration, the suspension was shaken vigorously using a vortex mixer and allowed to stand for 10 minutes before further shaking and estimation of the viable number of bacteria.

M. The effect of desiccation on the cell surface of rhizobia.

M(i) Estimation of the repairable damage caused by desiccation of various microorganisms.

The gram-negative bacterial cell wall comprises an inner rigid layer of peptidoglycan, which can be broken down by the enzyme lysozyme, and a more flexible lipoprotein-lipopolysaccharide (LPS) component (Salton, 1957) which overlays the peptidoglycan. The LPS layer protects the peptidoglycan from the action of lysozyme, possibly by prevention of penetration of the enzyme into the cell wall. Salton (1957) and Noller and Hartsell (1961) showed that gram-negative bacteria can be made sensitive to lysozyme by certain treatments which appear to act by disruption of the outer wall complex (Voss, 1967; Wilkinson, 1968). It seemed possible that desiccation of the root-nodule bacteria might induce changes in the LPS-layer and such changes might be detected by the development of sensitivity to lysozyme,

consequently, the ability of desiccated bacteria to repair such damage has been studied.

Bacteria used in this investigation were Escherichia coli M13 and various species of the rhizobia (see results section). Soil samples were not used in these experiments, instead, 3.0 ml. of bacterial suspension were used to inoculate 10 gm acid washed (Parkinson, Gray and Williams, 1971), sterile glass beads (0.44 mm. diameter) in a petri dish. These samples were air dried at 28°C for 24 hrs. in a forced-draught oven.

The composition of the rehydration medium varied according to the aim of the experiment and the bacteria involved. At no time was 1% peptone used as this caused precipitation of lysozyme.

Each desiccated sample of Escherichia coli M13 was rehydrated in 95 ml. of medium similar to that used by Hambleton (1971), the difference being that ferric citrate was used instead of ferric ammonium citrate. The medium contained:

Glycerol	10 gm
Ammonium citrate	5 gm
$KH_2PO_4 \cdot 3H_2O$	10 gm
$MgSO_4 \cdot 7H_2O$	0.5gm
NaCl	0.5gm
Fe citrate	0.005 gm
Distilled water	1000 ml

This medium was adjusted to pH7, then sterilized by autoclaving at 15 psi. for 20 minutes. In some experiments, E.coli M13 was rehydrated in YMB.

Desiccated glass bead samples inoculated with rhizobia were usually rehydrated in 95 ml of YMB but occasionally were resuspended in Hambleton's medium.

In all cases, subsequent dilution after rehydration was in Sorensen's phosphate buffer (1/15 M, pH 7.0). The sensitivity of desiccated bacteria to lysozyme, actinomycin D and deoxyribonuclease (the selective agents) were tested. The individual selective agents were sterilized by membrane filtration using filters with a pore size of 0.45 um. diameter (Gelman Clemco Pty.Ltd.) and added to the buffer in test tubes at the following final concentrations:-

Lysozyme	100 µgm/ml
Actinomycin D	50 µgm/ml
Deoxyribonclease	50 µgm/ml

The sensitivity of the dehydrated bacteria to these agents was tested at various times after rehydration by mixing duplicate 1.0 ml samples of rehydrated bacteria with either lysozyme, actinomycin D or deoxyribonuclease contained in the phosphate buffer used for dilution for viable count estimates. The change in sensitivity with time after rehydration gave a measure of the repair of the desiccation-induced damage.

To demonstrate the temperature dependence of repair of damaged Escherichia coli M13, rehydration was carried out at 10°C (instead of the usual 28°C.) in Hambleton's (1971) medium. The time at which repair was completed was noted.

Three experiments were carried out (which were not statistically analysable) using Rhizobium trifolii SU297/32B, R.japonicum QA372 and R.meliloti SU45. The purpose was to obtain the range of selective agents that should be used in these experiments on repair. In these trial experiments the selective agents were sterilized by filtration and added to the petri dishes before addition of the agar.

For R.trifolii SU297/32B the agents and their concentration in the agar were:

Glutamate agar

" " + rose bengal 33.3 µgm/1 agar

" " + congo red (3 ml of a 1:400 aqueous solution of congo red to 300 ml of glutamate agar)

" " + polymyxin 2 µgm.ml of agar

" " + penicillin 1.0 µgm/ml of agar

For R.japonicum QA372, the selective agents and their concentrations were:

YMA + chloramphenicol 100 µgm/ml

" + penicillin 1 µgm/ml

" + streptomycin 0.3 µgm.ml

" + polymyscin 2.0 µgm/ml

" + congo red (3 ml of a 1:400 aqueous solution of congo red to 300 ml of YMA.)

For R.meliloti SU45, the effect of desiccation on the tolerance to high NaCl concentrations in the growth medium

was studied. The concentration of NaCl in the medium was 1.59 gm/100 gm YMA, which gave an agar with a water activity of 0.990 (see table 3).

M(ii) Fluorescence of fast-and slow-growing rhizobia with 1-anilino-8-naphthalene sulphonate.

The fluorescence intensity of the probe 1-anilino-8-naphthalene sulphonate (ANS) in water, is negligible compared with its fluorescence when bound at polar/non-polar environments (Radda and Vanderkooi, 1972). In membranes, these sites may include lipid-protein, lipid-water or lipid-protein-water contacts.

In this investigation the relative fluorescence intensity of the fast-and slow-growing bacteria when mixed with ANS has been measured. The effect of bacterial desiccation on the fluorescence intensity has also been measured.

A stock solution of ANS (Nutritional Biochemicals Corp.) at a concentration of 0.05% (w/v) in water was made.

The relative fluorescence intensity was measured with a Beckman Ratio Fluorometer Model 772, with uranium glass reference bar No.6 and the primary and secondary filters used were Schott UG11 and UG2A, respectively. The incident light was at a wavelength of 360 nm.

The fluorescence intensity of bacterial suspensions were measured relative to the fluorescence of a solution of serum albumin. Full scale deflection (100% relative fluorescence) of the fluorometer was given by the following solution:

1.8 ml. serum albumin solution (containing 10 ugm albumin/ml), plus 0.2 ml. ANS stock solution. The zero setting of the fluorometer was adjusted using 1.8 ml. Sorensen's phosphate buffer (1/15 M, pH 7.0) and 0.2 ml ANS stock solution.

To test for linearity over the fluorescence range used, the relative fluorescence at various albumin concentrations in the presence of a constant ANS concentration was obtained:-

Volume of albumin stock solution (ml)	Volume of phosphate buffer (ml)	Volume of ANS stock solution (ml)	Relative Fluorescence (%)
2.0	0.0	0.2	100. 0
1.5	0.5	0.2	75. 0
1.0	1.0	0.2	50. 0
0.5	1.5	0.2	25. 0

Because glutamate broth did not fluoresce when mixed with ANS (in contrast to the fluorescence of YMB caused by the yeast extract), rhizobia were grown on glutamate agar for 4 or 8 days for the fast-and slow-growing bacteria, respectively. The growth was gently washed off the agar slope with distilled water and the total count estimated by use of a Petroff-Hauser counter. The total counts were equalized by dilution and the bacterial fluorescence intensity (as a percentage) was determined immediately after addition of ANS.

Microscopic observation of bacteria in the presence of ANS involved a Leitz microscope with an attached Hg-vapour lamp with primary filters UG1 (1 mm plus 2 mm) and the heat absorbing filter KG1 (2 mm) in place. The barrier filter used was No. K470.

N. Determination of the internal osmotic pressures of various microorganisms.

The internal osmotic pressures of bacteria were determined by two methods.

Method (a). Microorganisms were grown in large quantities and harvested as described previously. After centrifugation, the cell pellet was resuspended in a minimum volume of water and the packed cell volume determined by standard haematocrit techniques. Microorganisms in the suspension were disrupted by sonication and/or autoclaving. The sonicator used was an M.S.E.20 KHz. Ultrasonic disintegrator. During sonication, the bacterial suspension was kept relatively cool by immersion of the container in an ice bath. Cell debris was removed by centrifugation and the osmolality of the supernatant determined using a Fiske Osmometer No. G62 (Fiske Associates Inc.) The results were expressed as milliosmols (mOsM) per unit packed cell volume (PCV).

Due to the large amount of extracellular polysaccharide produced by many species of Rhizobium, highly viscous suspensions of these bacteria were common and these frequently gave erroneous PCV results. Consequently, expression of the results as mOsM /PCV gave incorrect readings. The second method

for determining the internal osmotic pressure, circumvented this problem.

Method (b). Rhizobia were washed off slopes of YMA by a known, minimum volume of distilled water, and the number of viable bacteria/ml determined. This suspension was frozen at -20°C prior to lyophilization. Bacteria dried in this way were rehydrated in a known volume of the non-ionic detergent Triton X100 (BDH) which disrupted any bacteria left intact by the previous treatments. The resulting suspension was halved and one portion was autoclaved for 20 minutes at 15 psi. Cell debris was removed from both autoclaved and un-autoclaved suspensions by centrifugation at 20,000g for 20 minutes and the osmolality of the supernatant measured. Expression of the results was, mOsM per unit $\log_{10}$ of the viable count.

The purpose for autoclaving a portion of each suspension was prompted by a report by DeVries et al. (1970), who described the chemical and physical properties of freezing-point-depressing glycoproteins isolated from the blood of some fish. An indication of whether glycoproteins were involved in the freezing point depression values observed for solutions of disrupted rhizobia would be obtained if proteins were denatured. Autoclaving was used as the denaturing agent. If a substantial decrease of the osmolality was noted after treatment, then aspects of the glycoprotein status of rhizobia would be worthy of further consideration. DeVries et al. (1970) also reported that the osmotically active glycoproteins were not dialysable. Whether the osmotically active ingredients of either the fast-or slow-growing rhizobia could pass through a dialysis membrane was

determined. Rhizobia were grown and disrupted, and a known volume placed in Visking cellulose dialysis tubing. The suspension was dialysed overnight against distilled water at low temperature ($2^{o}C$) in order to stop metabolic activity which caused browning of disrupted bacterial suspensions.

O. Determination of cell dimensions.

The length and width of individual microorganisms were measured using an Olympus OSM filar micrometer mounted on a Leitz microscope. The filar micrometer was calibrated against a known graduated stage micrometer in the usual way.

An average of 20 measurements of each dimension was obtained for each bacterial culture and from these averaged values, the surface areas and volumes were calculated. Rod-shaped bacteria were assumed to be closed cylinders, thus the formulae used were:

$$\text{Volume} = \pi r^2 h$$

$$\text{Surface area} = 2\pi rh + 2\pi r^2$$

where r = the radius of a cylinder

h = length of the bacteria.

For spherical particles (yeasts), the formulae used were:

$$\text{Volume} = \frac{4}{3}\pi r^3$$

$$\text{Surface area} = 4\pi r^2$$

P. Use of nuclear magnetic resonance spectroscopy to determine the water permeabilities of various microorganisms.

Because nuclear magnetic resonance (NMR) spectroscopy is an unusual technique for the determination of the water

permeabilities of microorganisms an introduction is presented to explain the technique and the reasons for using it in this investigation.

Introduction

In 1967, Fritz and Smith developed a technique using steady-state nuclear magnetic resonance (NMR) spectroscopy which enabled them to differentiate between intra-and extracellular water in frog nerve trunk cells. Conlon and Outhred (1972) modified this technique and used pulsed NMR to determine the rate at which water passed across the membrane of normal red blood cells. The advantages of pulsed NMR over steady-state NMR are explained later. The results were reproducible and were within 10% of the published data obtained by the more complicated isotope tracer technique.
Conlon and Outhred (1972) outline the theory to the technique quite comprehensively. A brief description will be given here.

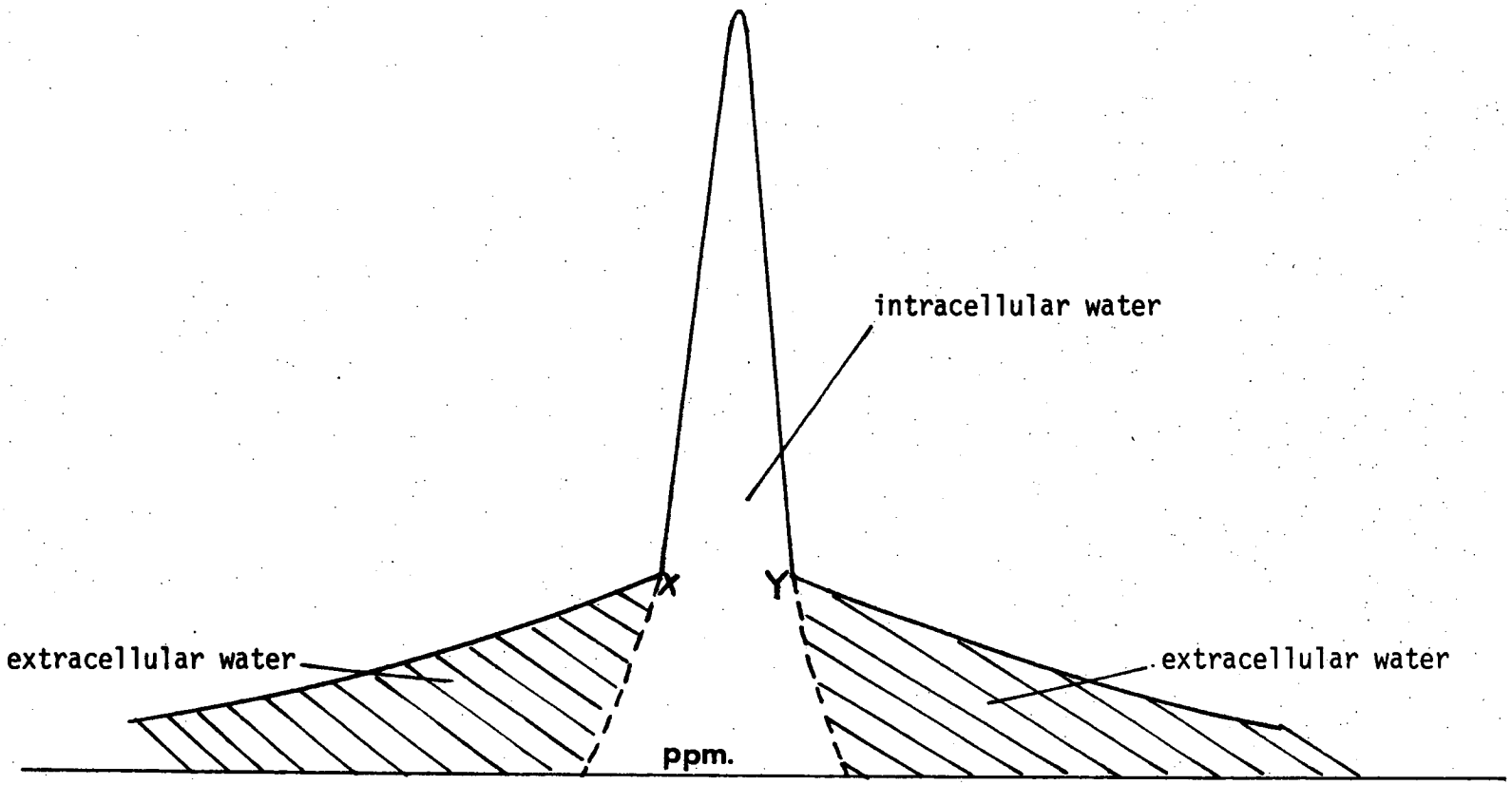
When water proteins are placed in an appropriate static magnetic field they are able to absorb energy from a radio-frequency magnetic field. The absorption and decay of this energy can be monitored by the techniques described by Pople, Schneider and Bernstein (1959) and is described by the spin-spin relaxation time (T_2). If red blood cells are placed in a similar field, the decay time T_2 for water proteins within the cells is about 140 msec. This is much longer than the rate at which water passes from inside the cell into the external solution. This is in the order of 10msec. (Paganelli and

Solomon 1957; Viera, Sha'afi and Solomon, 1970). If T_2 of the extracellular water could be made very much shorter than the cell-environment exchange time (ie. $\ll$10 ms.) the decay of the intracellular water would be dominated by the movement of water molecules out of the cell. The short T_2 of extracellular water protons can be achieved by the addition of a paramagnetic ion such as manganese, to which the cell is impermeable over the time course of the experiment.

In this investigation steady-state (or continuous wave) NMR spectroscopy was employed. A typical spectrum obtained by the use of continuous wave NMR is illustrated in figure 1. The important feature of this spectrum is the abrupt change in the slope of the line at points x and y. The signal in the shaded region to the left of x and to the right of y can be attributed to extracellular water protons. These regions will be called the broad-line areas, as it is obvious that much broadening of the normal peak due to water protons has been induced by the paramagnetic ions. The reasons for this effect of paramagnetic ions are given by Pople et al. (1959). Briefly, paramagnetic ions have unpaired electrons and as the magnetic moment of an unpaired electron is about 10^3 times greater than the moment of a nucleus, the water protons (nuclei) in the vicinity of paramagnetic ions experience very large inhomogeneities in the external magnetic field. Therefore, for all but the lowest concentrations of paramagnetic ions, the proton relaxation time (T_2) is substantially reduced. If the paramagnetic ion concentration is high enough, such broadening can occur that the line attributed to protons is no longer "visible" on high resolution NMR.

FIGURE 1

A representative spectrum obtained from continuous wave (steady-state) NMR of a microorganism suspended in a solution containing paramagnetic ions (Mn^{2+})
The distinction between the intra- and extracellular water can be seen.
ppm = parts per million, which is an arbitrary scale related to the applied field strength.

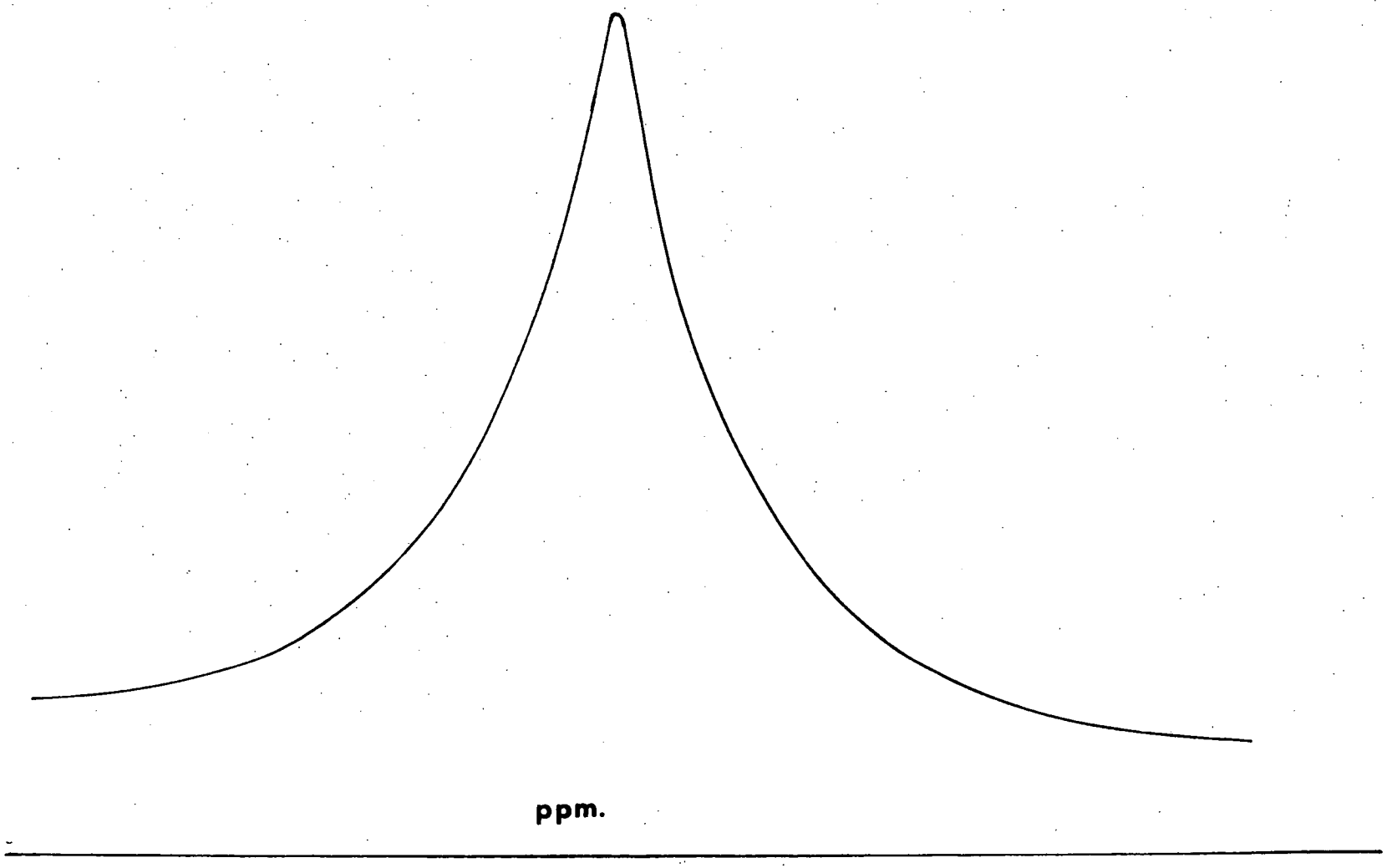

intracellular water
X
Y
extracellular water
extracellular water
ppm.

Between points x and y, (figure 1) the signal due to water protons is quite sharp and can be attributed to intracellular water which is not in contact with the paramagnetic ions. This will be called the narrow line of the spectrum. Theoretically, therefore, it is possible to distinguish between intra-and extracellular water. Now with steady state NMR spectra, the line width at half its maximum height is proportional to $1/\pi T_2$ (Outhred, pers comm.) Thus, estimates of T_2 can be obtained for the narrow line due to intracellular water. Comparisons of narrow line T_2 values may then be made between different microorganisms.

As can be seen from the above equation, there is an inverse relationship between T_2 and peak height. Thus, the broad line (see fig. 1) has a very short T_2, relative to the narrow line. For the complicated reasons outlined by Pople *et al.* (1959), inclusion of particulate matter such as living organisms in the sample leads to line broadening when steady-state NMR is used. Pulsed spectroscopy avoids these errors. Thus, the estimates of T_2 obtained in the present investigation are only relative and not absolute measurements of the water permeability of cells. If the rate of passage of water across the cell membrane approached the T_2 value for the extracellular water, the points of inflection (points x and y, figure 1) become less apparent. Ultimately, the narrow and broad lines merge and the resultant spectrum would appear as in figure 2. When this occurs it is not possible to measure the water permeability as it is beyond the limit of resolution of the technique.

FIGURE 2

The representative spectrum obtained from steady state NMR when the rate of passage of water across membrane of an organism is about the same as T2 for the extracellular water to which paramagnetic ions had been added. As can be seen it becomes impossible to distinguish between intra-and extracellular water under these conditions.
ppm = parts per million, and is an arbitary scale related to the applied field strength.


ppm.

In this study, the method of Conlon and Outhred (1972) was applied to various microorganisms in an attempt to determine the relationship between a variety of cell sizes and their permeability rates. More specifically, it was the aim of these experiments to determine if differences in the rates of water permeability existed between the fast-and slow-growing rhizobia and if these differences could be related to their differential responses to desiccation.

Microorganisms used were:- Saccharomyces cerevisiae M6, Bacillus sp. M70, Escherichia coli M13, Rhizobium japonicum QA372, R.lupini UT12, R.meliloti CC131, R.meliloti SU47B, R.trifolii SU297/31A and R.trifolii SU297/32B.
Large quantities of cells were grown as described previously. After harvesting by centrifugation and resuspension of the cell pellet in distilled water, the packed cell volume was measured using a micro-haematocrit Select-a-fuge No.24 (Biodynamics). The internal osmotic pressure was measured by method 1. From this estimation of the osmotic pressure, it was possible to calculate the concentration of $MnCl_2.6H_2O$ required to yield an isotonic solution. The concentration of the $MnCl_2.6H_2O$ solutions were made double that required for isotonicity so that the final concentration, when mixed with the cell suspension (50% v/v), was the equivalent of an isotonic solution. Unlike the red blood cells used by Conlon and Outhred (1972) where isotonicity was vital otherwise cell lysis would occur the rigidity of the yeast and bacterial cell walls allowed

some deviation from isotonicity. In several instances different concentrations of $MnCl_2.6H_2O$ were used especially with the smaller cells such as Escherichia coli and Rhizobium sp.

Within 2 minutes of mixing the two components, 0.5 ml of the suspension was placed in a standard precision high-resolution NMR tube. The spectometer used was a high-resolution Japan Electron Optics Laboratory Co., Model No. 4H100, and operated at 100 MHz.

It was important to harvest the microorganisms and complete the spectral determination in as short a time as possible when dealing with Bacillus sp. M70, because these bacteria lysed fairly rapidly under the experimental conditions thus allowing Mn^{2+} penetration of the cell. If there was a delay in procedings after cell harvest, it was important to refrigerate the suspension. This did not always prevent cell lysis, which could be observed microscopically and by a decrease of the packed cell volume.

Q. Water sorption isotherms of rhizobia.

The apparatus used to obtain the sorption isotherms was similar to the silica spring balance described by Bateman et al. (1962) and is shown in figure 3. By variation of the temperature of the water in the water chamber, it was possible to control the absolute amount of water in the atmosphere surrounding the bacteria. The ratio of this absolute amount of water (the vapour pressure, p) in the air to the total amount of water that it would be possible to have in the air (the

saturation vapour pressure, P_0) at the temperature of the surrounding water jacket is the relative vapour pressure or relative humidity. The relationship between vapour pressure and relative humidity is such that at a vapour pressure of 1.00, the relative humidity is 100%.

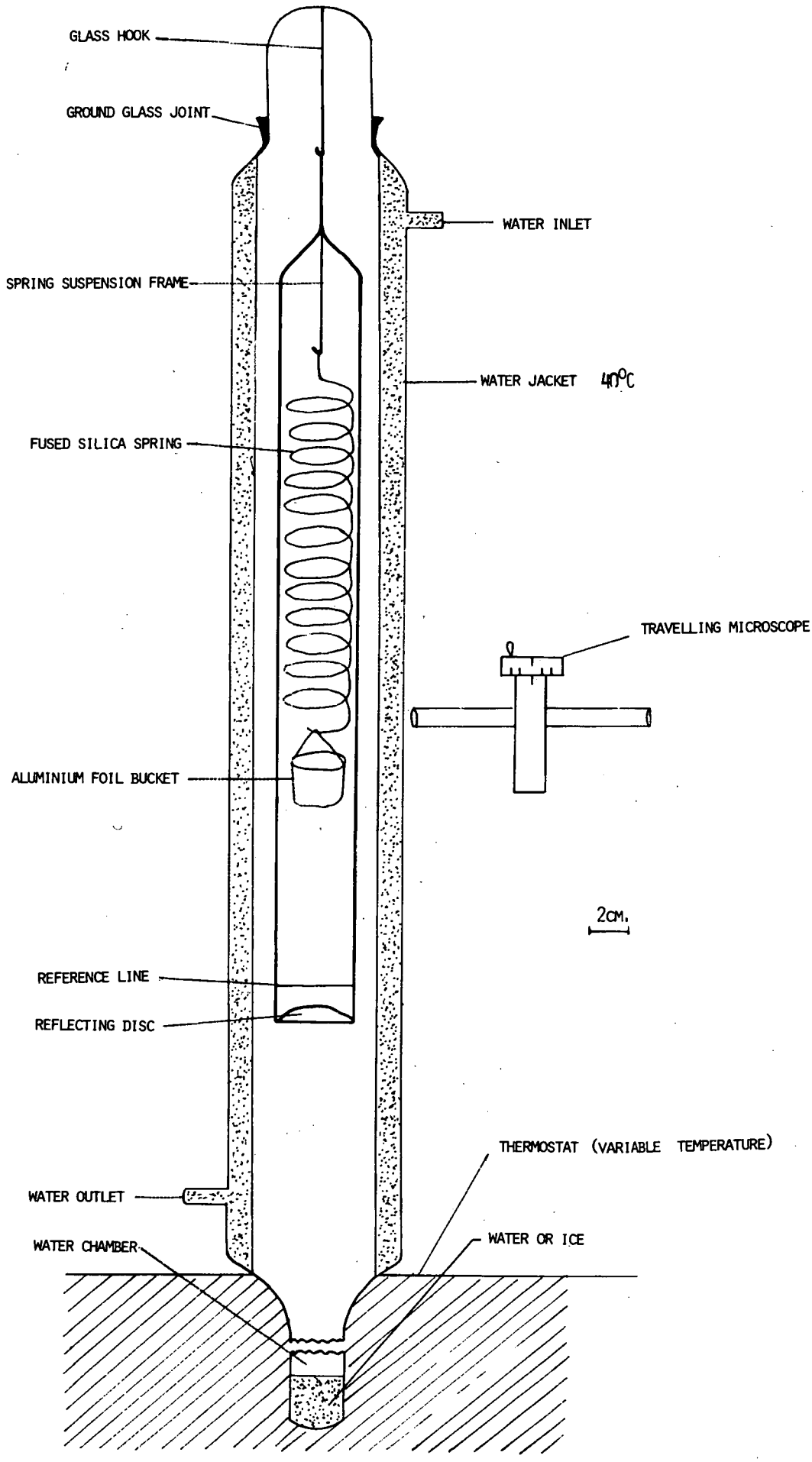
The water temperature in the jacket was kept constant at 40°C. This rather high temperature was chosen because:-

1. The published data of Bull (1944) for serum albumin used to check the proper functioning of the apparatus was obtained at 40°C.
2. This high temperature prevented growth of microorganisms, especially fungi, on the bacterial mass at relative humidities higher than about 80%.
3. It was found to be easier to control the low temperatures of the water chamber to achieve low $P/_{P_0}$ values.

The quartz weighing spring used was Reference No. T/A95/72 No. 11 supplied by Jencons (Scientific) Ltd. It had a maximum load of 0.5 gm and a sensitivity of 35.1 cm/gm. This sensitivity was only approximate, and calibration was required to determine the exact relationship between spring extension and weight added. The extension of the spring was measured by an independently mounted travelling microscope (Swift Vernier Microscope, Thomas Optical Co.) by observing the movement of the end of the spring relative to a

FIGURE 3

Diagram of the silica-spring balance used to measure equilibration of bacteria with water at different vapour pressures. The bacterial sample is located in the aluminium foil bucket.


GLASS HOOK
GROUND GLASS JOINT
WATER INLET
SPRING SUSPENSION FRAME
WATER JACKET 40°C
FUSED SILICA SPRING
TRAVELLING MICROSCOPE
ALUMINIUM FOIL BUCKET
2cm.
REFERENCE LINE
REFLECTING DISC
THERMOSTAT (VARIABLE TEMPERATURE)
WATER OUTLET
WATER CHAMBER
WATER OR ICE

74/5/130

thin reference line mounted on the spring suspension frame (see figure 3.)

Following calibration and confirmation that the relationship between spring extension and weight was linear, an equation for the relationship for that particular spring was obtained. If the spring was broken or another used, this procedure had to be repeated.

Water adsorption isotherms for the bacterial cultures were obtained as follows: approximately 0.1 gm. dry weight of the bacterial culture was placed in a small, accurately weighed aluminium bucket and freeze dried for at least 24 hrs. As soon as possible after lyophilization, the dry weight of the bacteria plus bucket was accurately determined and the true dry weight of bacteria was obtained. It was found that this was the most crucial part of the operation, as an error in the bacterial dry weight was magnified resulting in apparently meaningless water sorption results.

The ~~temperature of the~~ water in the water chamber (see figure 3) was cooled to between -3 and -7^{o}C prior to positioning the spring frame, spring and freeze dried sample. Temperature control of this water chamber was achieved by a Colora-Kryo-Thermostat type WK5, the water bath in this instrument was an ethylene-glycol/water solution which had a freezing point of -40^{o}C (Handbook of Chemistry and Physics, 28th Edition, page 1739, Chemical Rubber Publishing Co.).

The detailed water adsorption isotherm for the first batch of rhizobia was obtained by warming the water in the water chamber by increments of 2°C. Subsequently, increments of 4°C were used, beginning at 0°C. Sufficient time at all temperatures was allowed for equilibration between the water adsorbed by bacteria and the surrounding relative humidity (R.H.). At lower R.H.s this took only 2-3 hours, however, at higher vapour pressures, longer times were required because of the greater amounts of water transferred. Collapse of the "fluffy" structure of the sample upon rehydration also slowed the rate of equilibration. It was observed that the collapse of the "fluffy" structure with the slow-growing rhizobia was invariably more complete than with the fast-growing root-nodule bacteria.

Because of the time involved, complete adsorption-desorption isotherms for all bacteria have not been obtained and only the adsorption isotherms are presented. That hysteresis is involved in the adsorption-desorption cycle for rhizobia has been shown for Rhizobium japonicum QA372. Roderick and Demriel (1966), working with montmorillonite suggested that the adsorption isotherm was the true equilibrium curve for the hydration of this clay. Similarly Walker et al. (1973) applied the Bradley isotherm equation (Adamson, 1967) to their adsorption data for the proteins myosin A and B.

The adsorption isotherm of calcium saturated montmorillonite was also obtained. Preparation of the sample was as follows:-

About 5 gm montmorillonite was added to 200-300 ml distilled water in a 600 ml beaker. Dispersion of the clay in water was

achieved by using a kitchen homogenizer for about 20 minutes. The suspension was then transferred to centrifuge bottles and centrifuged at 2,000 rpm for 80 minutes. The supernatant was separated from the pellet and the centrifugation repeated on the supernatant. A resin column, 1 inch diameter and 3 feet long, was set up and the resin soaked in electrolyte ($1NCaCl_2$), for 5 minutes. One hundred grams of resin was poured into the column, and about 100 ml distilled water was passed through the resin till the electrolyte concentration was about 10^{-2} - 10^{-3}M. The montmorillonite suspension was passed through the column once, then the column was washed with distilled water, the electrolyte, followed by distilled water (about 100 ml of each was passed through the column). The montmorillonite suspension was passed through the column for a second time. The volume of the suspension was measured, then placed in pressure membrane (Visking sausage membrane, Visking Corp. Chicago), until only 50-100 ml remained. The suspension was then removed and the Ca^{2+}- montmorillonite scraped from the membrane and dried in an oven.

Measurement of the water adsorption isotherm of Ca^{2+}- montmorillonite involved the same procedure as outlined above, for the bacteria.

Adamson (1967) has reviewed the theories dealing with gaseous adsorption to solid surfaces. The theory of Brunauer, Emmett and Teller (B.E.T.) (1938) deals with multilayered

adsorption of gases on solid surfaces. These authors derived the equation:-

$$\frac{P/P_0}{W(1-P/P_0)} = \frac{(C-1)}{WmC} P/P_0 + \frac{1}{WmC}$$

where W=the amount of water vapour sorbed at a pressure, P.

P_0 =the saturation vapour pressure.

Wm=the amount of water required to form a monolayer over the adsorbing surface.

C=a constant from which the average heat of adsorption for the monolayer can be obtained.

If the term $\frac{P/P_0}{W(1-P/P_0)}$ is plotted against P/P_0, a straight line should be obtained (figure,4) from which the values of Wm and C can be calculated. The B.E.T. theory has been applied to the results of water sorption isotherms for both fast-and slow-growing rhizobia and for montmorillonite.

Apart from the B.E.T. theory, there are many other equations put forward to describe the adsorption of gases to solids (Adamson, 1967). One used for biological material by authors such as Ling (1965, 1972), Hoover and Mellon (1950) and Walker et al.(1973), is that derived by Bradley for the adsorption of gases with permanent dipoles onto charged surfaces. Water is a molecule with a permanent dipole and many biological solid surfaces are charged. Ling (1972) has used the closeness of fit of adsorption isotherm data to the Bradley theory as circumstantial evidence for his theory that water

FIGURE 4

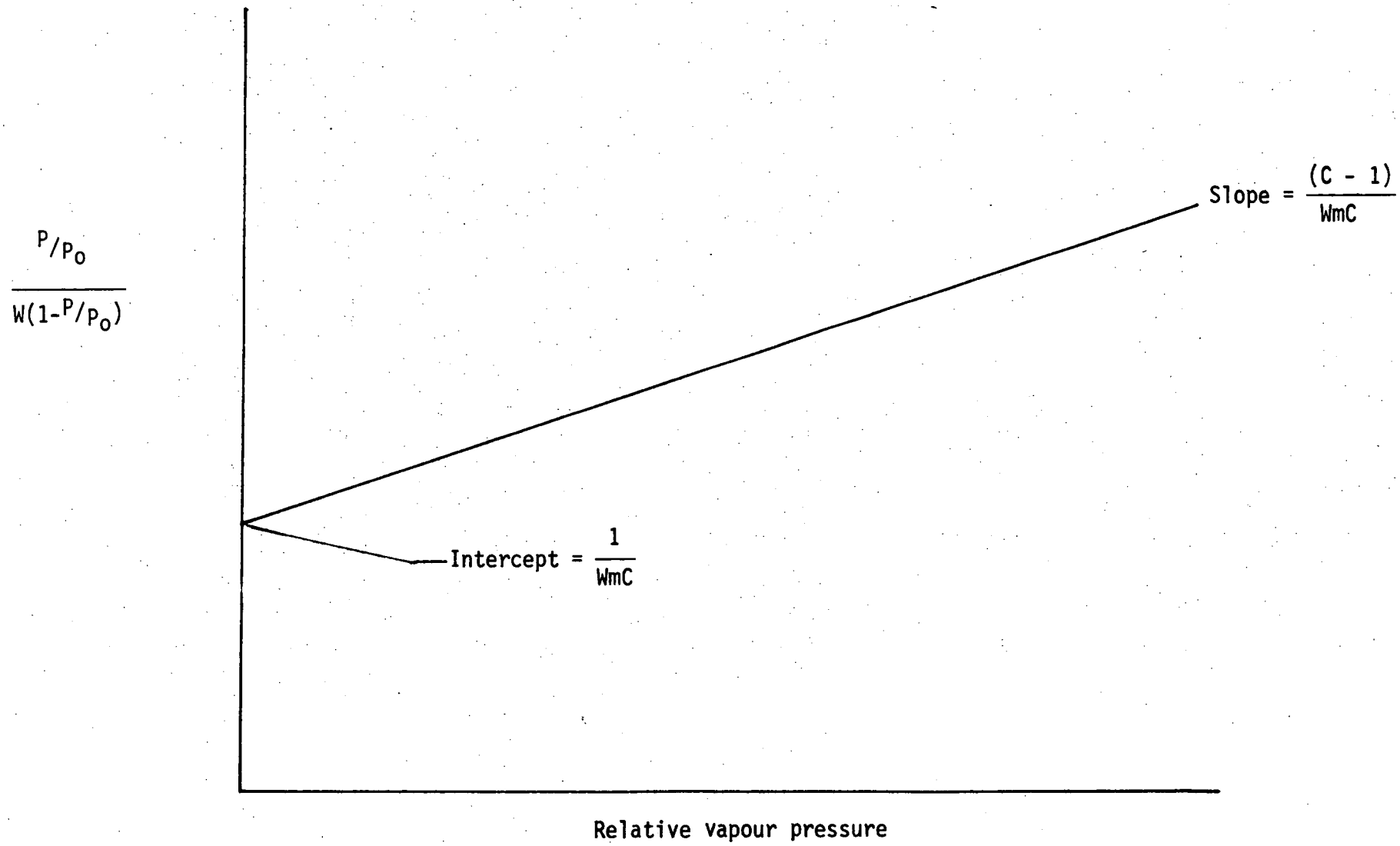
Model diagram of the application of B.E.T. theory to water adsorption isotherms.

W=the amount of water adsorbed at a vapour pressure, P.

P_0 =the saturation vapour pressure.

Wm=the amount of water required to form a monolayer over the adsorbing surface.

C=a constant from which the average heat of adsorption for the monolayer can be obtained.


$\frac{P/P_o}{W(1-P/P_o)}$
Slope = $\frac{(C - 1)}{WmC}$
Intercept = $\frac{1}{WmC}$
Relative vapour pressure

exists in cells as multiple polarized layers. It is suggested that the Bradley theory provides a mechanism whereby dipolar forces are propagated through the dipolar water molecules.

The Bradley isotherm equation is:-

$$\log (P_0/_p) = K_1 \; K_2^{\;W}$$

Thus $\log (\log P_0/_p) = W \log K_1 + \log K_2$

where

W=the amount of water sorbed at pressure, P

P_0 =the saturation vapour pressure

K_1 and K_2 = constants which are functions of the field of the sorptive polar groups, the dipole moment of the sorbed gas, the polarizability of the sorbed gas and of the temperature.

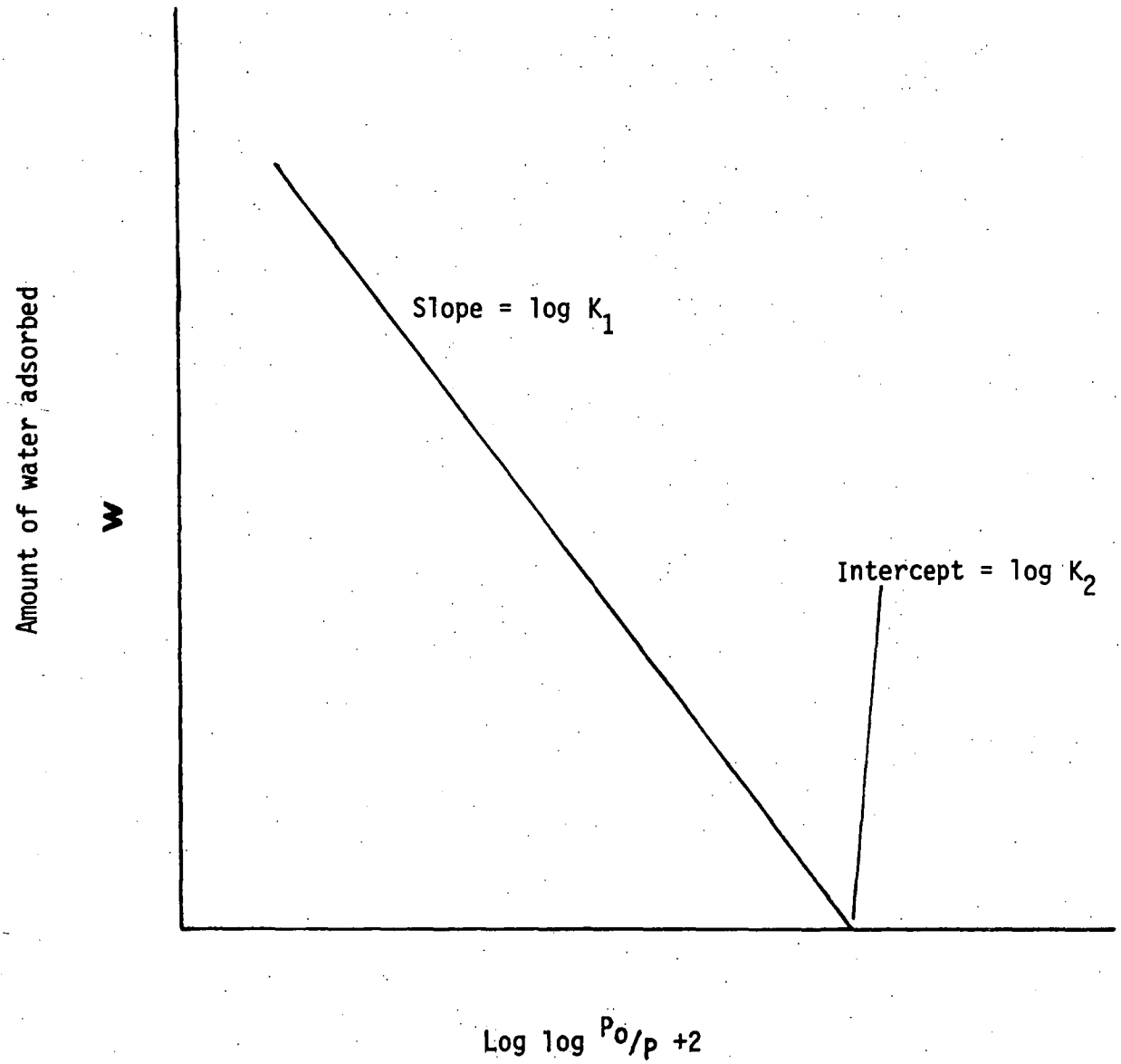
A plot of log log $(P_0/_p)$ verses W should give a straight line as demonstrated by the model diagram in figure 5. The intercept on the pressure axis when W=0 gives log K_2, and the slope of the line gives log K_1. The Bradley theory has been applied to the sorption isotherms for both the fast-and the slow-growing rhizobia to test the closeness of fit of the equation with the experimentally derived data. However, application of both the Bradley theory and the B.E.T. equation to biological systems must be viewed with caution as the theories have been derived for "pure" systems. and need not describe water adsorption to complex biological systems.

FIGURE 5

Model diagram of the application of the Bradley theory to water adsorption isotherms.

W=amount of water sorbed by solid surface at a vapour pressure P.

P_0 =saturation vapour pressure.


Amount of water adsorbed
W
Slope = log K_1
Intercept = log K_2
Log log P_o/P +2

Several authors have determined the change in free energy involved when proteins (Bull, 1944) or montmorillonite (Roderick and Demriel, 1966) adsorbed water vapour by application of Bangham's (1937) free energy equation to the isotherm data. The equation can be expressed as:

$$\Delta F = -\frac{RT}{MA}\int_0^1 W/P/Po \quad d\ (P/Po)$$

R = the gas constant

T = the absolute temperature

M = the molecular weight of water

A = the specific surface area of the adsorbing material

W = the weight of water adsorbed at a pressure, P.

Po = the saturation vapour pressure

ΔF = the change in free energy involved with wetting

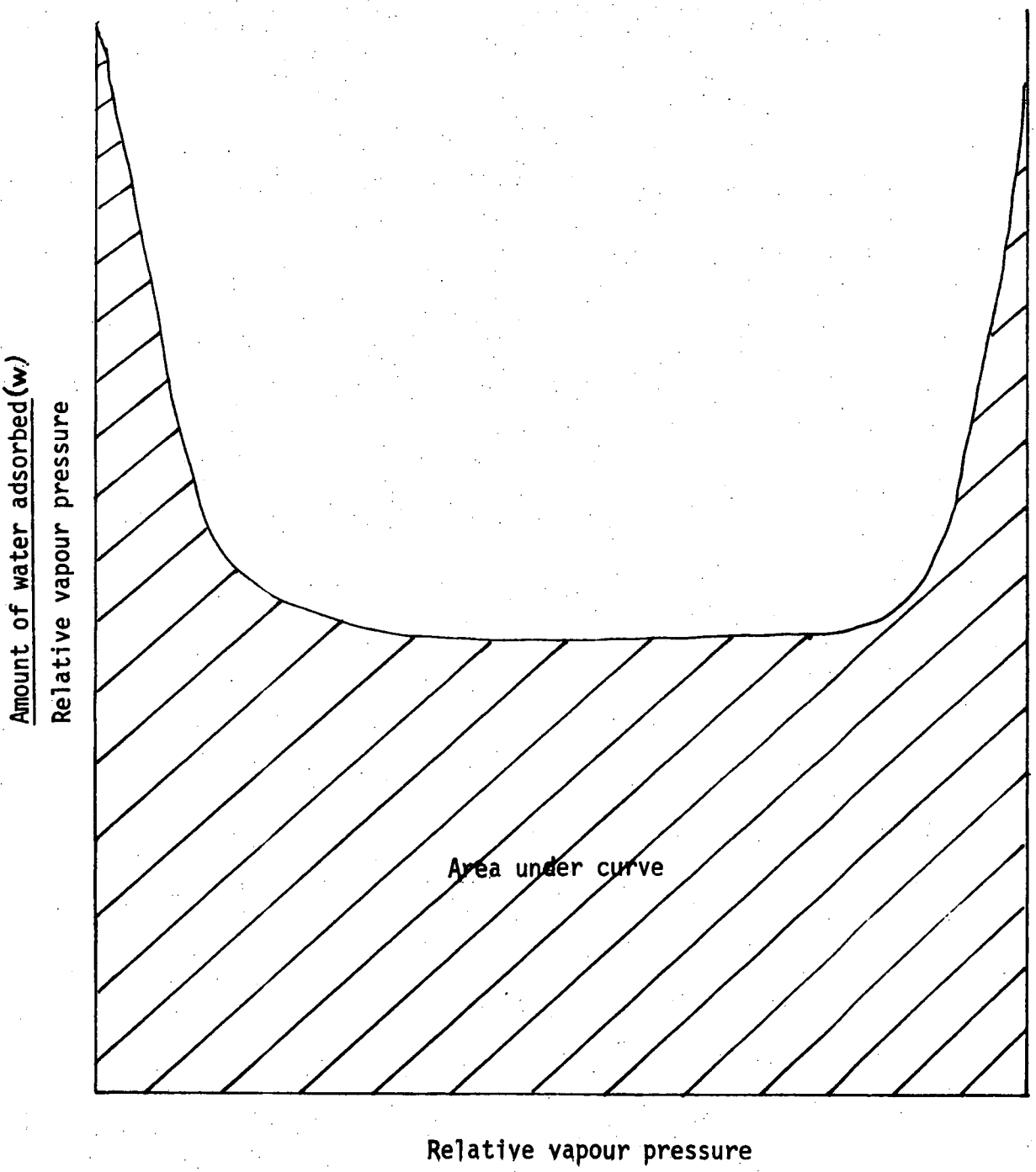
The equation states that ΔF is proportional to the area under the curve obtained by a plot of $\frac{W}{P/Po}$ versus P/Po. Therefore, ΔF can be evaluated by graphical integration of a plot of $\frac{W}{P/Po}$ versus P/Po as illustrated in the model diagram (figure 6). The free energy of wetting of fast-and

FIGURE 6

Model diagram demonstrating the application of the free energy equation of Bangham (1937) to water adsorption isotherm data. Evaluation of $\triangle F$ is obtained by measuring the area under the curve of a plot of $\frac{W}{P/P_0}$ versus P/P_0 (shaded area)

W=amount of water adsorbed at a vapour pressure, P.

P_0 =the saturation vapour pressure.


Amount of water adsorbed (w)
Relative vapour pressure
Area under curve
Relative vapour pressure

slow-growing rhizobia has been determined by application of this equation to the water adsorption isotherm data in an attempt to decide whether the energy state of the adsorbed water molecules in the two groups of rhizobia was similar. Bull (1944) stated that a measure of the free energy of wetting described the affinity of the adsorbing material for the gas. Therefore, application of this equation to the isotherm data for the fast-and slow-growing rhizobia could describe the affinity of these bacteria for water during desiccation.

The reports by Bull and Breese (1970), Walker *et al.* (1973) and Steer (1973) noted that the presence of residual salt markedly affected the water sorption isotherms of various proteins. To determine whether this was an important factor in the water sorption characteristics of fast-and slow-growing rhizobia, the isotherms of salt-free suspensions of rhizobia were obtained. Rhizobia were grown and disrupted as described previously. The suspension was placed in Visking cellulose dialysis tubing and dialysed overnight against tap water. Further dialysis was against a large volume of distilled water for 24 hrs. The disrupted suspensions of the slow-growing rhizobia were dialysed at 2°C to prevent metabolic activity which caused browning of suspensions at room temperature. After dialysis, the suspensions were freeze-dried and the water sorption isotherms determined as described above.

R. Statistical analyses

All statistical analyses were carried out on the logarithms of the viable counts. Viability is expressed as

log viable count per 0.05 gm of soil, i.e. no corrections were made to account for the fact that the number of viable bacteria in 50 ul was determined.

Throughout this thesis the levels of significance have been denoted by the following:

x	$P < 0.05$
xx	$P < 0.01$
xxx	$P < 0.005$

Determination of the significance of differences between means was achieved by Duncan's new multiple-range test (Steele and Torrie, 1960) at $P < 0.05$.

4. RESULTS

A. Survival of various species of Rhizobium in desiccated soil.

Experiments were carried out on a variety of rhizobia to test the generalization that Marshall (1964) predicted, i.e. that the slow-growing rhizobia were inherently more resistant to dehydration than the fast-growing rhizobia. A sandy soil was utilized in these experiments because the report by Marshall (1964) demonstrated substantial protection of bacteria by certain fine particle materials.

Results of experiments determining the relative ability of the three groups of root-nodule bacteria (i.e. the fast- and slow-growing rhizobia, and the medic bacteria) to survive desiccation are presented in figures 7 to 9. The results confirm Marshall's (1964) prediction that rhizobia differ in their ability to survive dehydration. Invariably, the fast-growing species were more susceptible than the slow-growing bacteria and, frequently, the strains of R.meliloti fell somewhere in between these two groups. The fast-growing Rhizobium sp. SU343 (ex. Lotus corniculatus L.) was similar to R.trifolii in its response to drying (figure 8). Results in figure 8 show the similarity of response of seven slow-growing species to the applied stress. Generally under the conditions employed, the log viable count per 0.05 gm soil after desiccation was 6.0 - 7.0 for slow-growing bacteria, 3.0 - 4.0 for fast-growing rhizobia and between 4.0 and 6.0 for R.meliloti. From figure 9 it should be noted that two species of R.meliloti (strains SU45 and SU47A) exhibited poor survival when desiccated. This is in contrast to the result in figure 7

N O T E

In all the figures following where the results are presented as histograms, unless otherwise stated, the broken histogram represents the log of the initial viable count and the solid histogram represent the log of the viable count after desiccation.

FIGURE 7

Response of various rhizobia to desiccation.

A=*R. trifolii* SU297/31A (fast-growing rhizobia)

B=*R. trifolii* SU297/32B (" " ")

C=*R. leguminosarum* TA101(" " ")

D=*R. meliloti* SU47B (medic rhizobia)

E=*R. lupini* UT12 (slow-growing rhizobia)

Detailed results and analysis of variance are given in appendix table I.

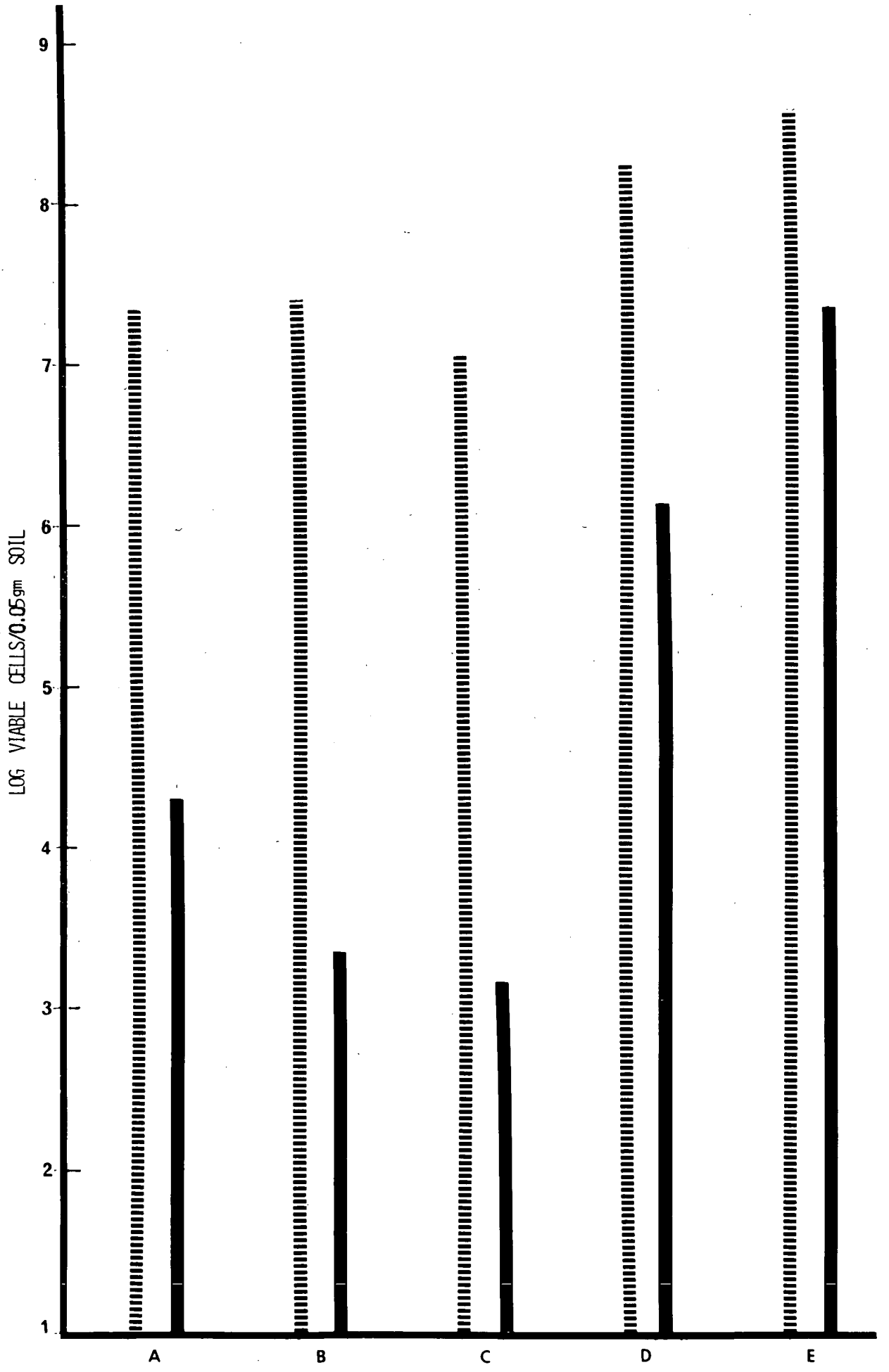

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
A
B
C
D
E

FIGURE 8

Response to various rhizobia to desiccation.

A=*Rhizobium* sp. SU343 (fast-growing rhizobia/m)

B=*R. meliloti* CB112 (medic rhizobia/m)

C=*Rhizobium* sp. CB421 (slow-growing rhizobia/m)

D=*Rhizobium* sp. 3G4b10 (" " ")

E=*Rhizobium* sp. 3I1b117(" " ")

F=*Rhizobium* sp. 3I1b125(" " ")

G=*Rhizobium* sp. 3C1f1 (" " ")

H=*Rhizobium* sp. C.B.756(" " ")

I=*Rhizobium* sp. UT53 (" " ")

Detailed results and analysis of variance are given in Appendix table 11.

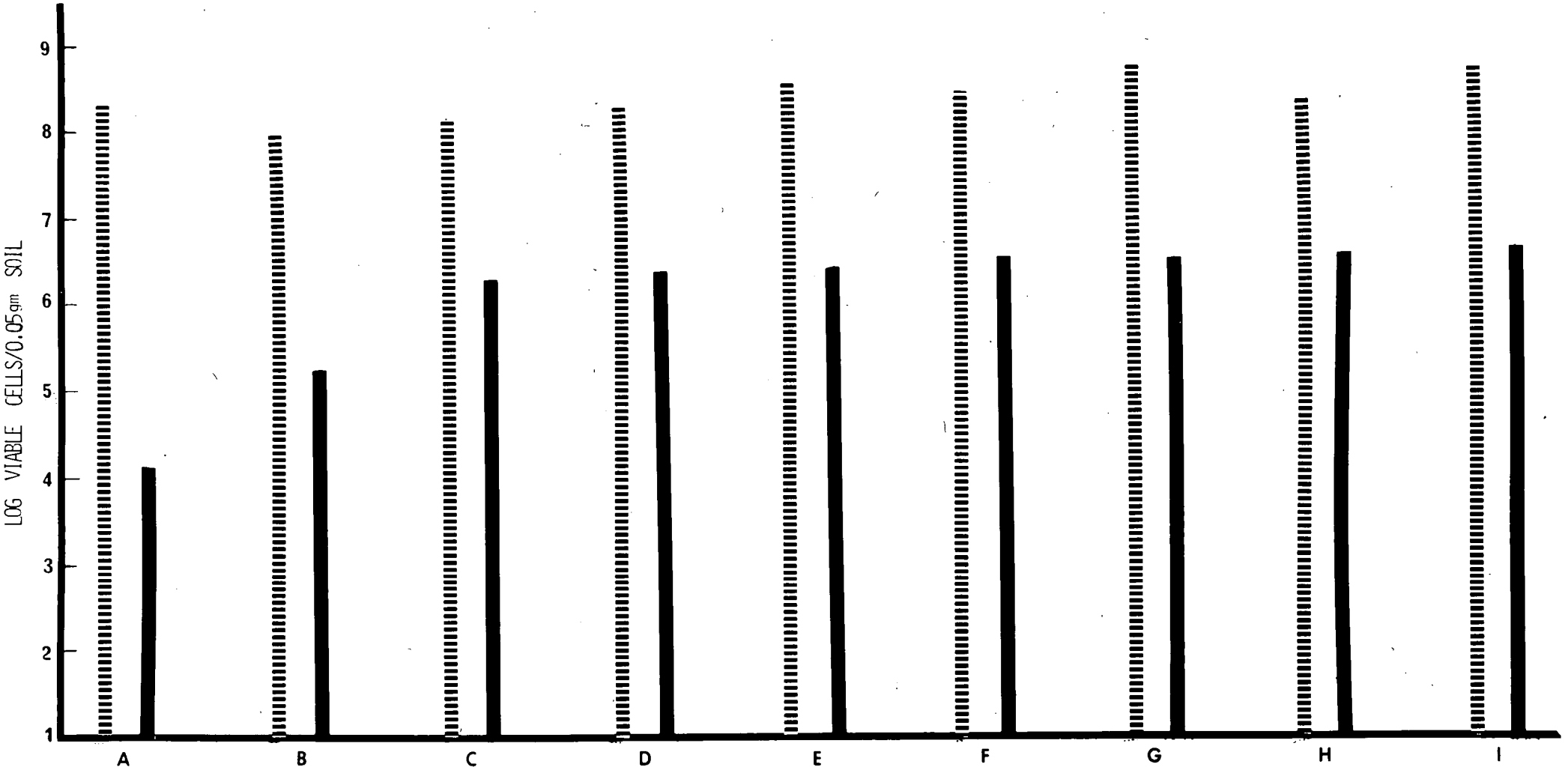

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
A
B
C
D
E
F
G
H
I

FIGURE 9

Response of various species of rhizobia to desiccation.

A=*R. meliloti* SU45 (medic rhizobia)

B=*R. meliloti* SU47A(" ")

C=*R. trifolii* SU297/31A (fast-growing rhizobia)

D=*R. trifolii* SU297/32B (" " ")

E=*R. trifolii* SU298/531B(" " ")

F=*R. trifolii* SU298/534C(" " ")

Detailed results and analysis of variance are given in Appendix table 111.

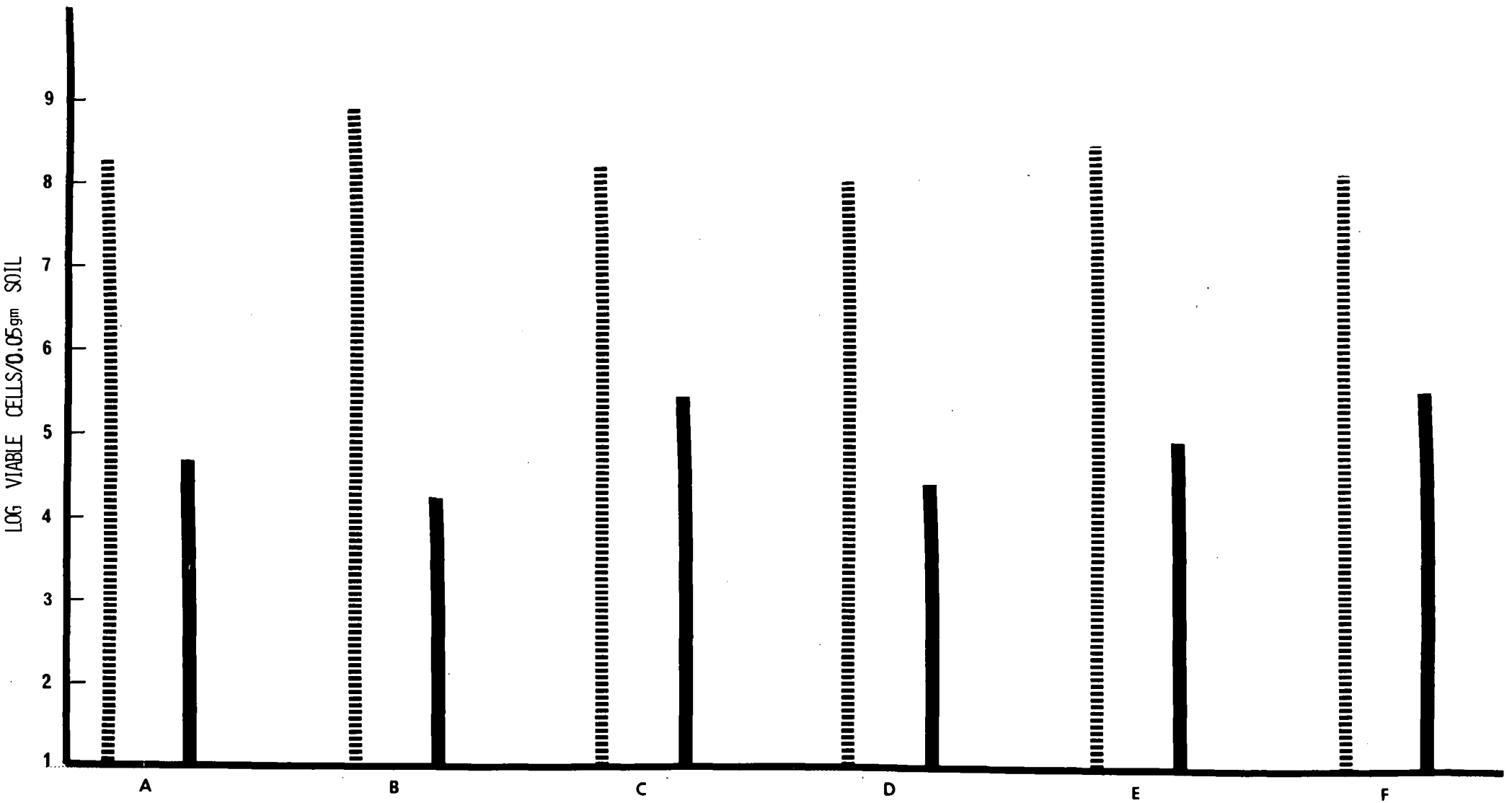

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
A
B
C
D
E
F

FIGURE 10

Response of two species of Rhizobium to alternate dehydration-rehydration cycles.

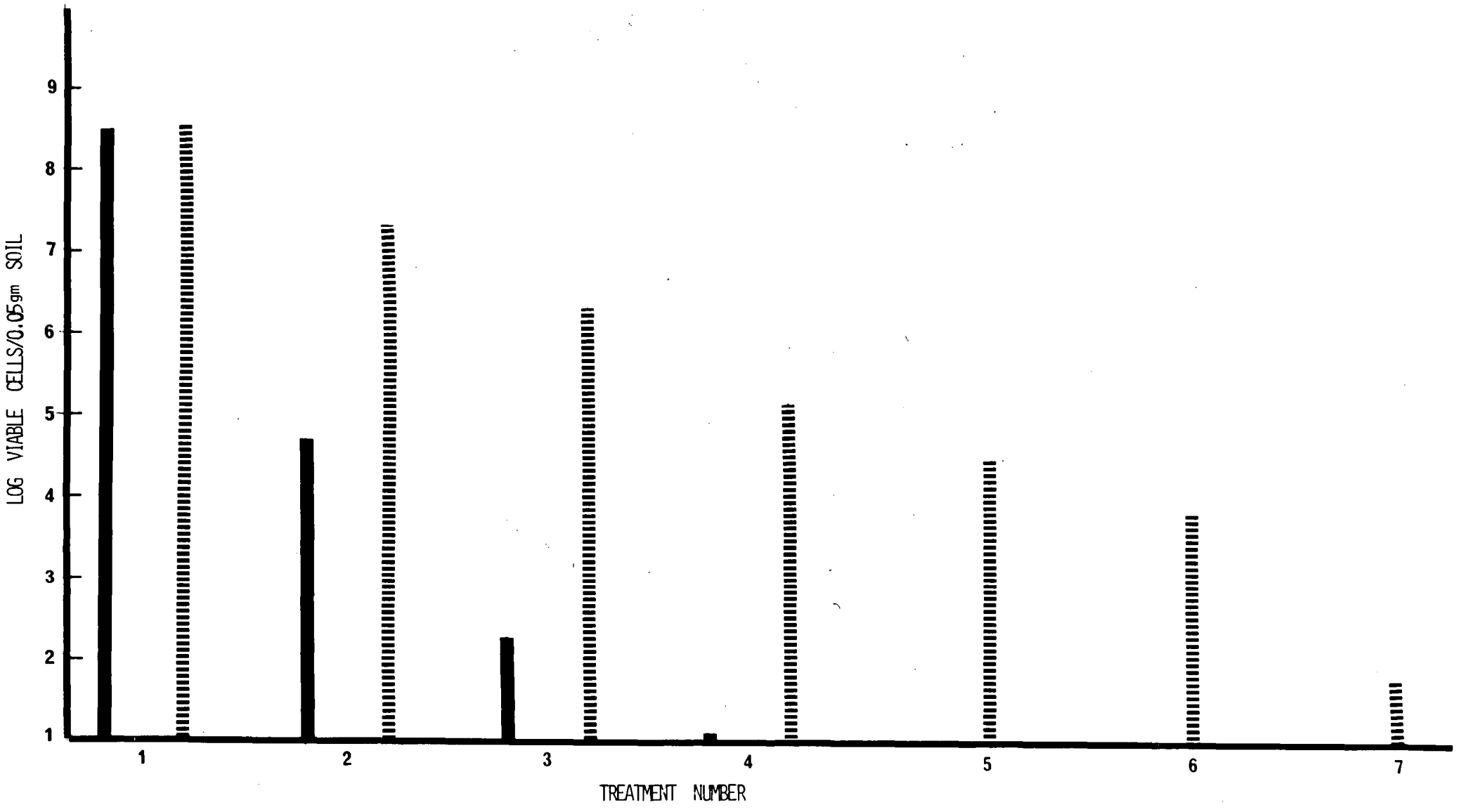
▮▮▮▮▮▮▮▮ = R. japonicum QA372

▬▬▬▬ = R. trifolii SU297/32B

The treatment numbers are:-

1. Initial viable counts.
2. Log viable counts after first dehydration.
3. " " " " soil samples had been rehydrated and dried.
4. |
5. | repeats of number 3.
6. |
7. |

Detailed results and analysis of variance are given in Appendix Table IV.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
TREATMENT NUMBER

which shows relatively good survival of R.meliloti SU47B. That the strains were not contaminants was demonstrated by their ability to nodulate Medicago trunculata Desr. A possible reason for this is that the two strains of R.meliloti came from different laboratories and, as reported by Sanderson (1962) and Luscombe and Gray (1973), laboratory cultivation decreased the ability of bacteria to withstand stresses.

The results presented in figure 10 emphasise the greater resistance of the slow-growing rhizobia relative to the fast-growing species to alternate dehydration-rehydration cycles. Clearly, R.trifolii SU297/32B was more susceptible to the continued application of this stress than was the slow-growing R.japonicum QA372.

B. Water retention characteristics of the sandy soil with and without amendments.

A pressure membrane apparatus was used to determine the effects of various soil amendments on the water retention characteristics of the sandy soil. The amounts of water retained at 15 bars pressure by the sandy soil with and without amendments are presented in table 4. The addition of powdered montmorillonite to the soil almost doubled the weight of water retained by the unamended samples. If, however, interlayered swelling of this expanding 2:1 lattice clay was prevented by heating at 600°C for 2 hrs. (Mortland and Kemper, 1965) prior to addition to the soil, the change in the water retention characteristic was negligible. This suggested that most of the water held by the expanding montmorillonite was within the expanded clay lattice. This was also suggested by the fact that soils amended with illite did not retain very much more water than the unamended soil. Illite is a non-expanding 2:1 lattice clay ,therefore, water retained by these soil particles would be restricted to the external surfaces.

An important finding was the effect of the physical state of montmorillonite on the amount of water retained by the amended soil. When added as a dried powder, the additive increased the amount of water retained by 96%; however, when the clay was added as an aqueous suspension to the soil (the final montmorillonite concentrations were the same in both cases), the amount of water retained was increased by about

Table 4

The effect of various additives on the amount of water retained by a sandy soil.

Soil sample	Weight of water retained ($gm.H_2O/100$ gm. sample) Individual tests	average
Unamended soil	2.12 1.99 2.70	2.27
Soil + montmorillonite (powder)	4.38 4.15 4.36 4.41 4.30 4.69 4.47	4.45
Soil + montmorillonite (suspension)	5.49 5.39	5.44
Soil + montmorillonite (heated at 600°C for 2 hours)	3.03 2.96 2.60 2.70	2.83
Soil + illite	2.60 2.40 2.38 2.27	2.41
Soil + kaolinite	3.50 3.40 3.14 3.14	3.30
Soil + haematite	1.50 1.50 1.61 1.67	1.57
Soil + goethite	2.21 2.11 2.12 2.15	2.15

140%. In subsequent experiments, this was found to have drastic effects on the survival of rhizobia when desiccated with montmorillonite in these two physical states.

Soils amended with 5% kaolinite retained approximately 1% more water than the natural soil, and goethite had no effect. The effect of 5% haematite was to decrease the quantity of water held by the soil.

C. The effect of various additives on the ability of root-nodule bacteria to survive desiccation.

Of all the additives studied during this investigation, the effect of montmorillonite upon the response of all groups of rhizobia to dehydration, has been of prime interest. Basically, the reason for determining the effects of other additives was to help explain the effects of this clay and, if possible, form generalizations as to which substances afforded protection in the system used. The results of the influence of montmorillonite on survival of desiccated fast-growing rhizobia have been presented first, followed by the response of the slow-growing species. Finally, the results using other additives have been presented, first for the

fast-growing species and subsequently for the slow-growing rhizobia.

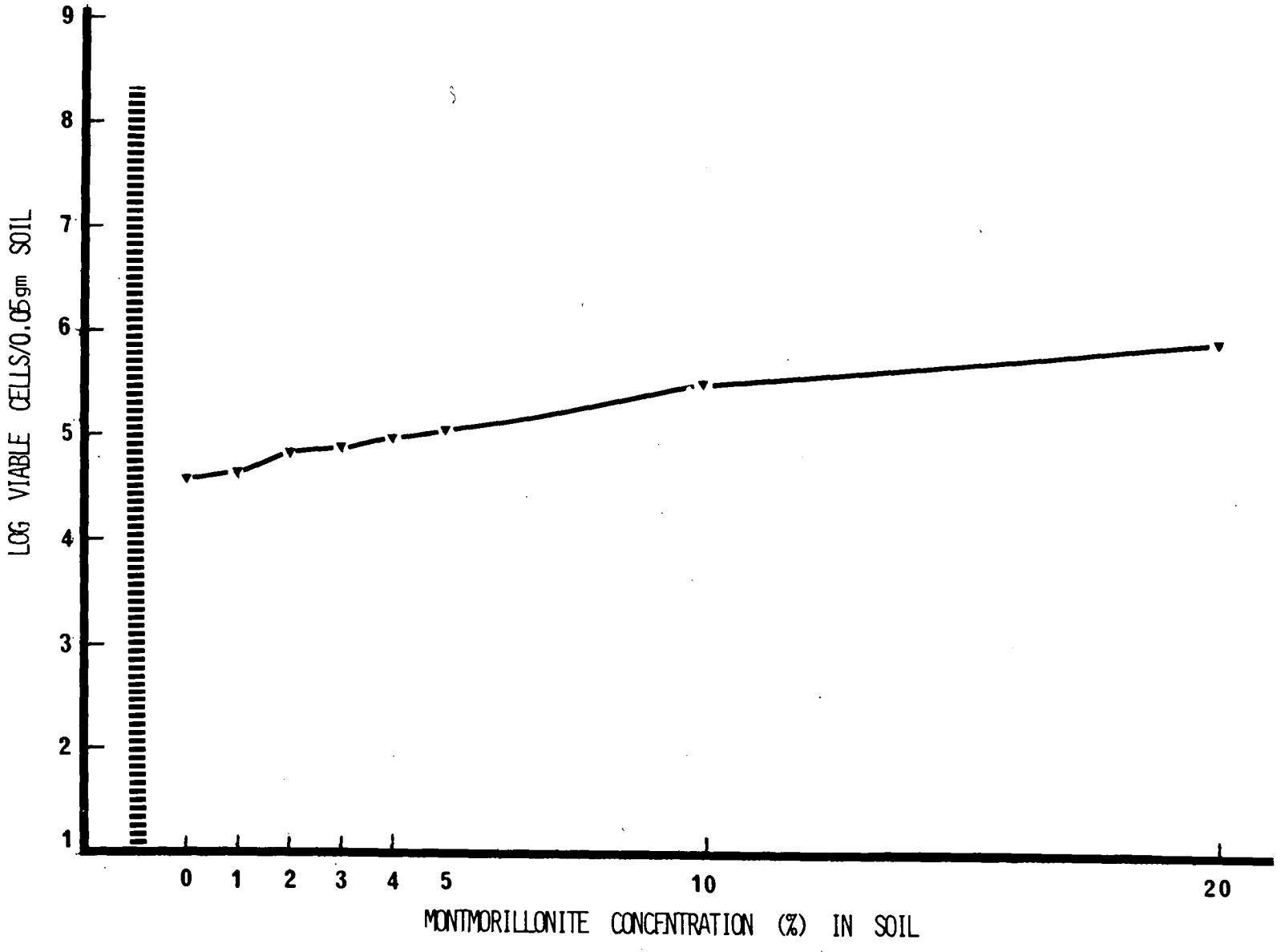
Results of the survival of various species of fast-growing root-nodule bacteria desiccated in soil samples amended with powdered montmorillonite are presented in figures 11 to 16. Included in this group of bacteria were species ranging from R.trifolii (figures 11, 12 and 13), Rhizobium sp. SU343 (figure 14) and R.leguminosarum (figures 15 and 16). The results are in agreement with those presented by Marshall (1964) for R.trifolii, as in all cases a greater proportion of the bacterial populations which were dried in montmorillonite amended soils survived dehydration than of those dried in unamended samples. Montmorillonite had the ability to protect fast-growing root-nodule bacteria from some of the effects of dehydration. Generally, there was a linear increase in survival with increasing montmorillonite concentration. Above a concentration of 10%, however, further increments had little effect, although survival of R.trifolii TA1 was significantly ($P < 0.05$) greater at an additive concentration of 20% (figure 11).

Included in figures 12, 13 and 14 are the effects of maltose and PVP on the ability of two strains of R.trifolii and Rhizobium sp. SU343 to withstand dehydration. Both maltose and PVP increased the survival of the two strains of

FIGURE 11

Effect of various concentrations of montmorillonite on the survival of desiccated *R. trifolii* TA1.

Detailed results and analysis of variance are given in Appendix Table V.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
10
20
MONTMORILLONITE CONCENTRATION (%) IN SOIL

74/3/55

FIGURE 12

Effect of PVP, maltose and various montmorillonite concentrations on the survival of desiccated *R.trifolii* SU297/31A.

For detailed results, see Appendix Table V1.

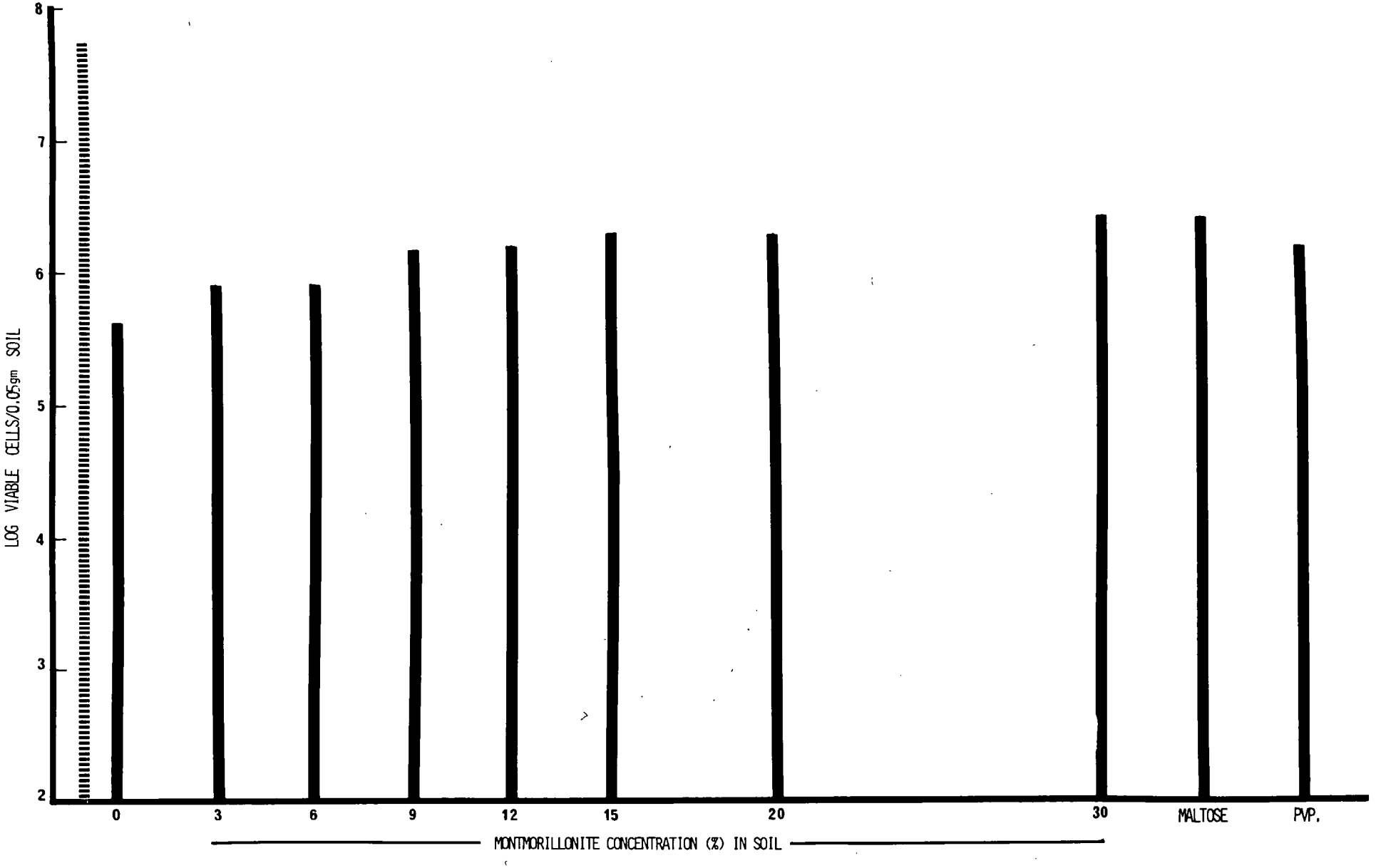

LOG VIABLE CELLS/0.05gm SOIL
8
7
6
5
4
3
2
0
3
6
9
12
15
20
30
MALTOSE
PVP.
MONTMORILLONITE CONCENTRATION (%) IN SOIL

FIGURE 13

Effect of maltose, PVP and various concentrations of montmorillonite on the survival of desiccated R. trifolii SU297/32B.

Detailed results and analysis of variance are presented in Appendix Table V11.

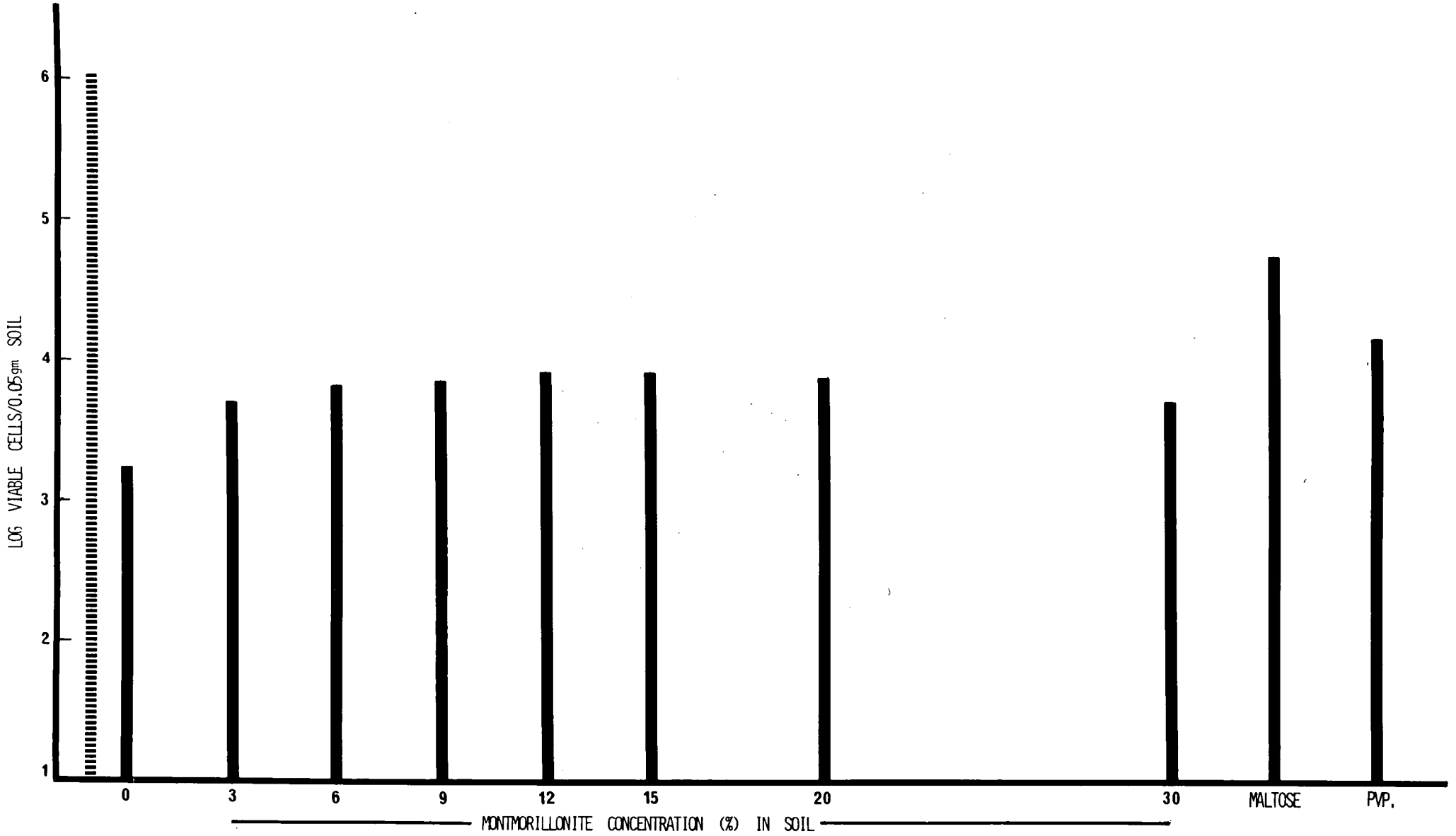

LOG VIABLE CELLS/0.05gm SOIL
6
5
4
3
2
1
0
3
6
9
12
15
20
30
MALTOSE
PVP.
MONTMORILLONITE CONCENTRATION (%) IN SOIL

FIGURE 14

Effect of various additives on the survival of the fast-growing Rhizobium sp.SU343 when desiccated.

Detailed results and analysis of variance are presented in Appendix Table V111.

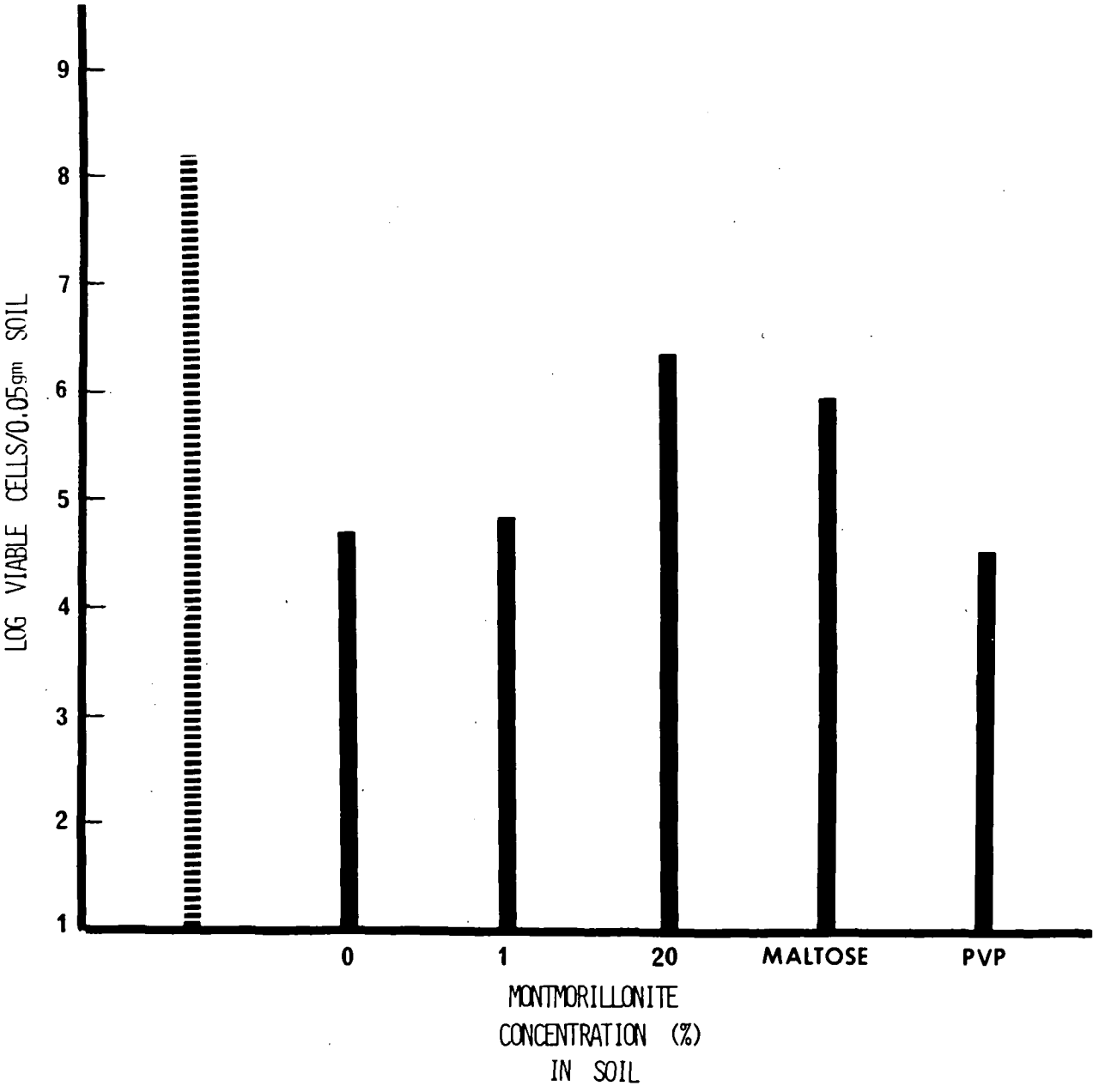

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
1
20
MALTOSE
PVP
MONTMORILLONITE
CONCENTRATION (%)
IN SOIL

FIGURE 15

Effect of various montmorillonite concentrations on the survival of desiccated R.leguminosarum TA101.

Detailed results and analysis of variance are presented in Appendix Table IX.

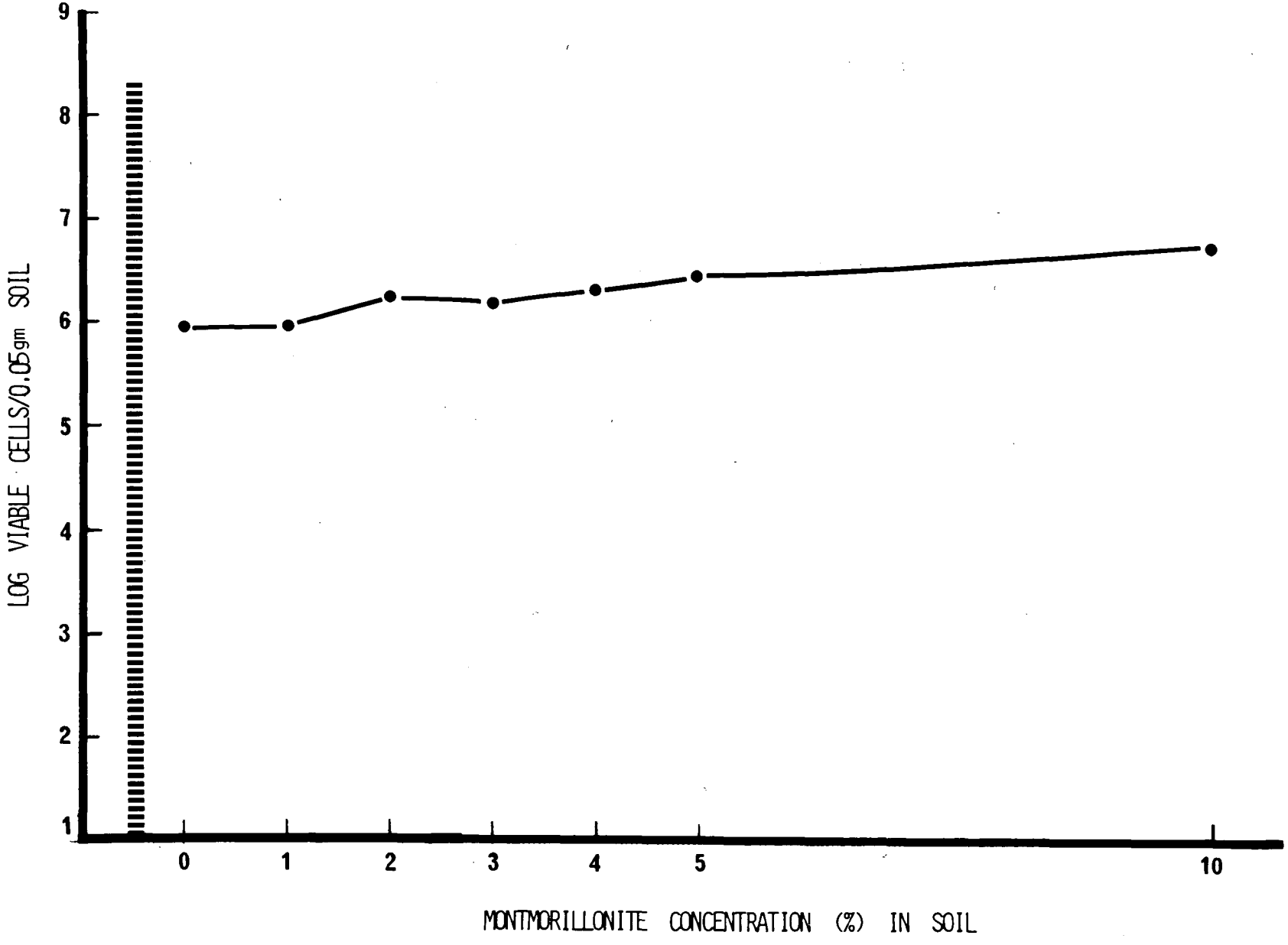
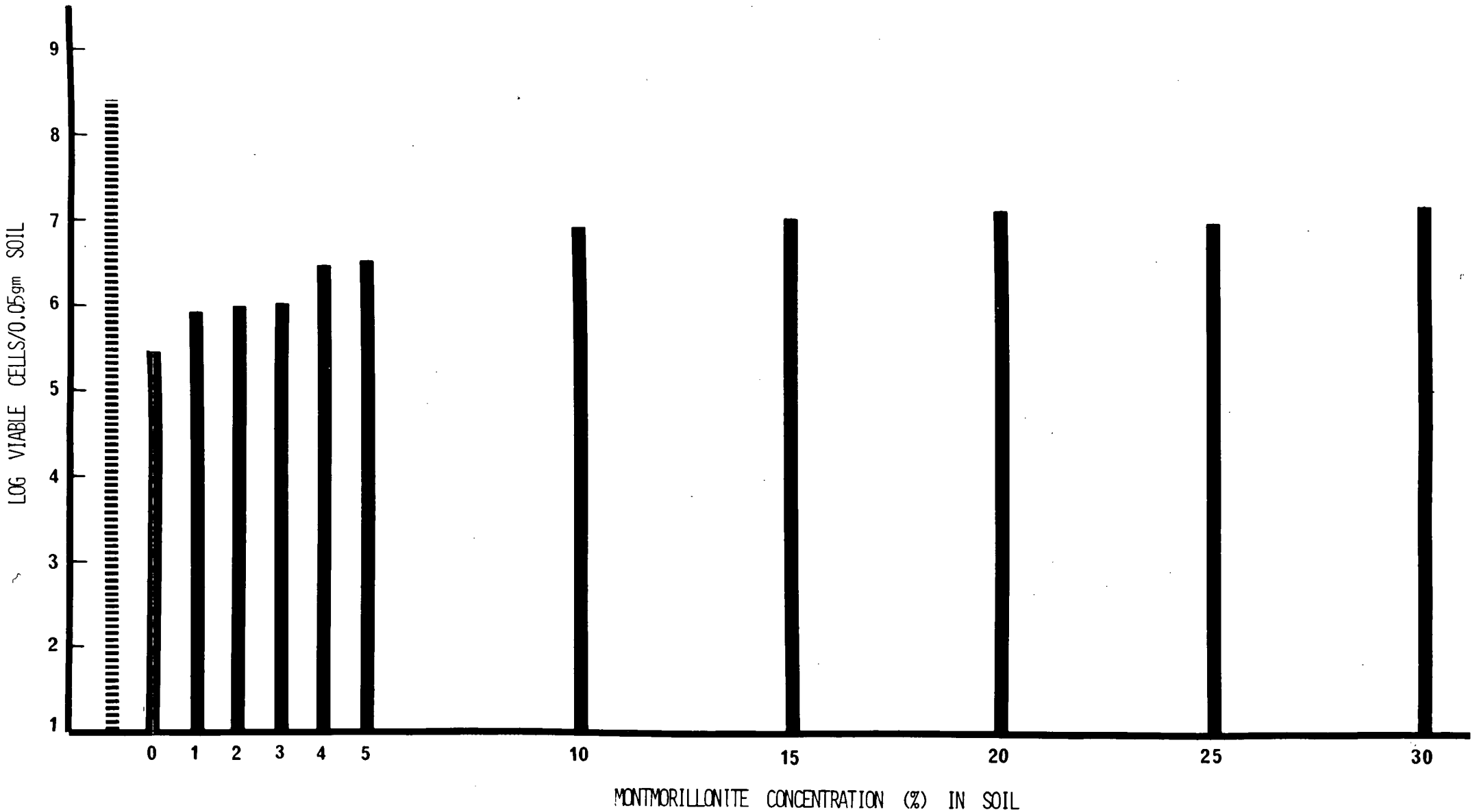

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
10
MONTMORILLONITE CONCENTRATION (%) IN SOIL

FIGURE 16

Effect of montmorillonite concentrations on the survival of desiccated *R.leguminosarum* TA101.

Detailed results and analysis of variance are presented in Appendix Table X.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
10
15
20
25
30
MONTMORILLONITE CONCENTRATION (%) IN SOIL

R.trifolii (figures 12 and 13). A larger proportion of Rhizobium sp. SU343 were able to withstand drying in the presence of maltose, however, the effect of PVP was not significantly different from the control (figure 14). The results of subsequent experiments (presented in figures 27 and 31) demonstrated that the result with PVP was anomalous, as the additive increased the ability of Rhizobium sp. SU343 to survive water loss.

As illustrated in figure 17, not all particulate soil amendments protected bacteria. Only the 2:1 lattice clays (montmorillonite and illite), afforded protection to R.trifolii SU297/31A, a result similar to that reported by Marshall (1964). The additives kaolinite, goethite and haematite significantly decreased ($P < 0.05$) the ability of this clover bacterium to survive desiccation. Marshall (1964) obtained similar results, with the exception that haematite was reported to protect the fast-growing rhizobium.

The results presented in figure 18, show that R.meliloti CC131 varied in the response to additions of montmorillonite to the soil. Significant protection ($P < 0.05$) was afforded by concentrations of 1,2,3,4 and 20% montmorillonite, but not at 5 or 10%. Both PVP and maltose protected this medic rhizobium from desiccation stress. This result was similar to that reported for the fast-growing rhizobia.

In marked contrast to the above results, the slow-growing root-nodule bacteria were not protected by montmorillonite at any concentration. This lack of protection was an unexpected

FIGURE 17

Effect of kaolinite, goethite, haematite, illite and montmorillonite used as soil amendments, on the survival of desiccated *R.trifolii* SU297/31A. All additive concentrations were 5% (w/w).

Detailed results and analysis of variance are presented in Appendix Table X1.

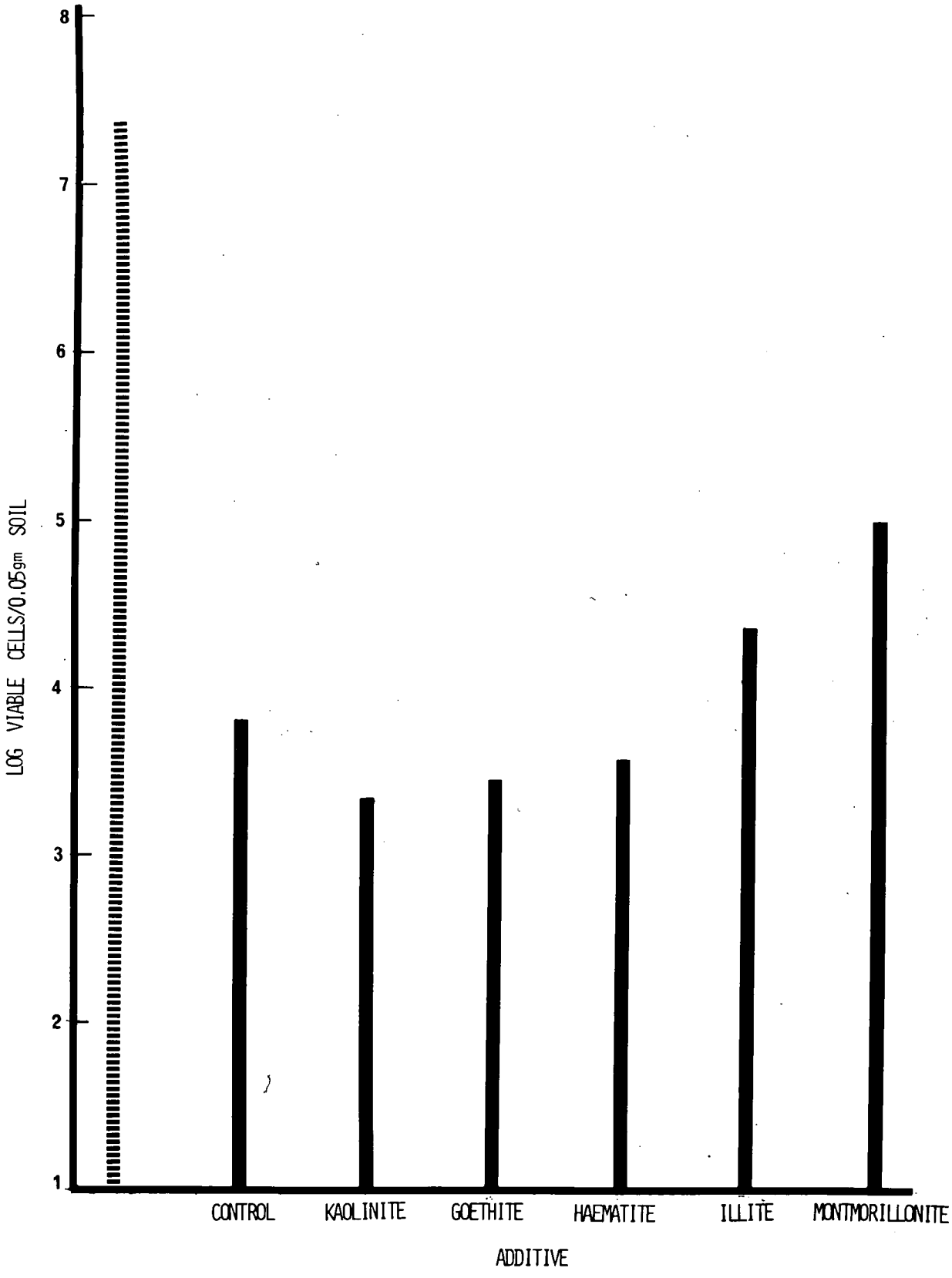
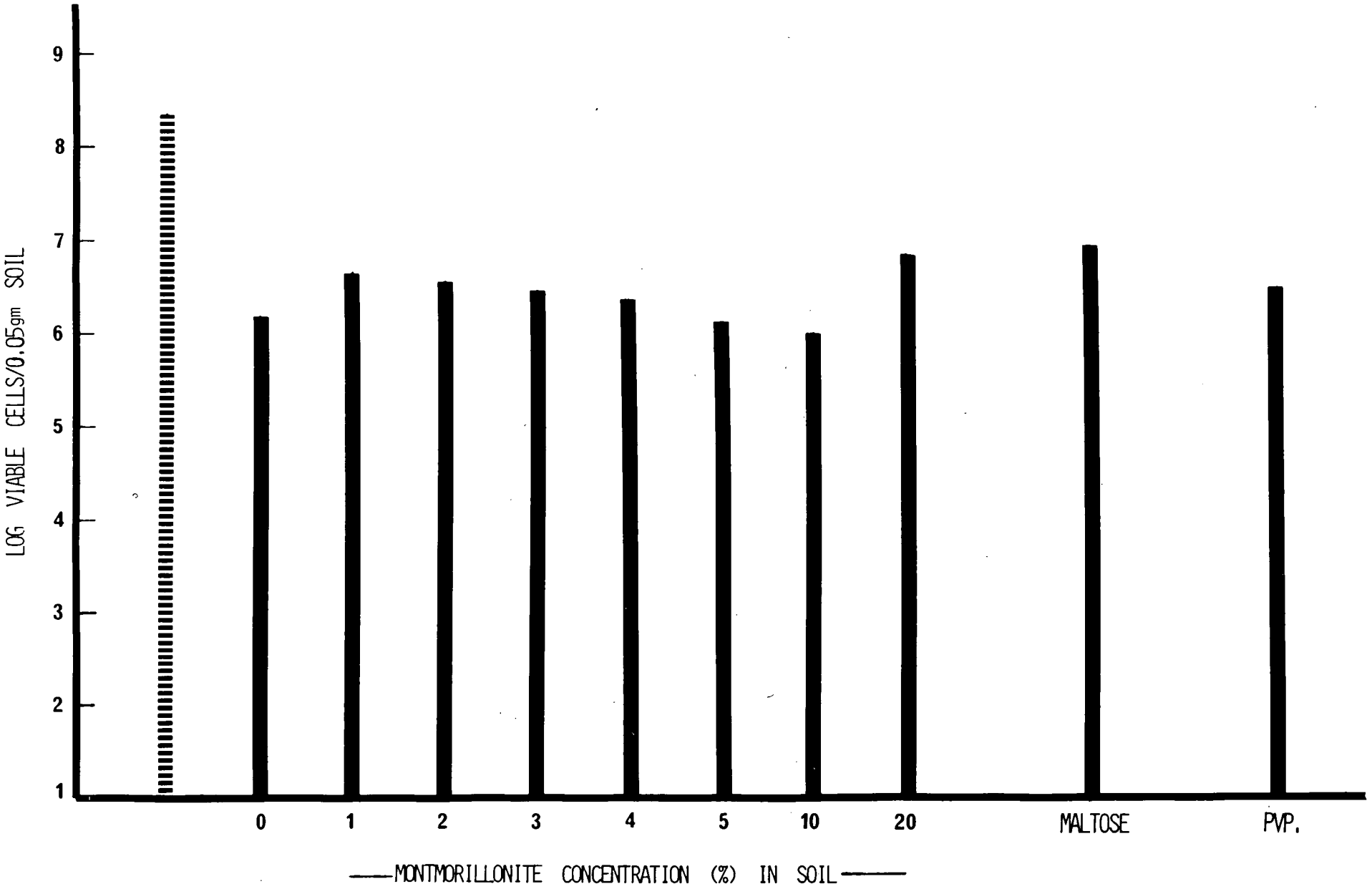

8
7
6
5
4
3
2
1
LOG VIABLE CELLS/0.05gm SOIL
CONTROL
KAOLINITE
GOETHITE
HAEMATITE
ILLITE
MONTMORILLONITE
ADDITIVE

FIGURE 18

Effect of maltose, PVP and various montmorillonite concentrations on the survival of desiccated R.meliloti CC131.

Detailed results and analysis of variance are presented in Appendix Table X11.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
10
20
MALTOSE
PVP.
——MONTMORILLONITE CONCENTRATION (%) IN SOIL——

result. In fact, the survival obtained when bacteria were dried on montmorillonite amended soil was sometimes less than the control. This finding is illustrated in figure 19 for R.japonicum QA372. The addition of powdered montmorillonite to the soil decreased the ability of this bacterium to survive dehydration. A similar result for R.lupini UT12 is presented in figure 20, although with the species, montmorillonite had no significant effect and did not decrease number of bacteria surviving desiccation. The ability of PVP and maltose to protect R.lupini UT12 is clearly demonstrated by the results presented in figure 20.

The results in figure 21, show survival of R.lupini UT12 in both amended and unamended soils, as a function of water lost from the samples. Once again, the lower final viable count in amended soils upon complete removal of water was evident. Death of R.lupini UT12 due to desiccation in the montmorillonite-amended soil, began at a lower percentage water loss than in the unamended samples (figure 21). This provided circumstantial evidence that, although the montmorillonite-amended samples retained more water than the unamended soil (see table 4), the availability of the water to desiccated R.lupini UT12 is lower than the water in the unamended samples.

Several experiments were carried out in which bacteria were mixed with aqueous suspensions of montmorillonite prior to the addition to soil and desiccation. The purpose of these

FIGURE 19

Effect of various montmorillonite concentrations on the survival of desiccated *R. japonicum* QA372.

Detailed results and analysis of variance are presented in Appendix Table XIII.

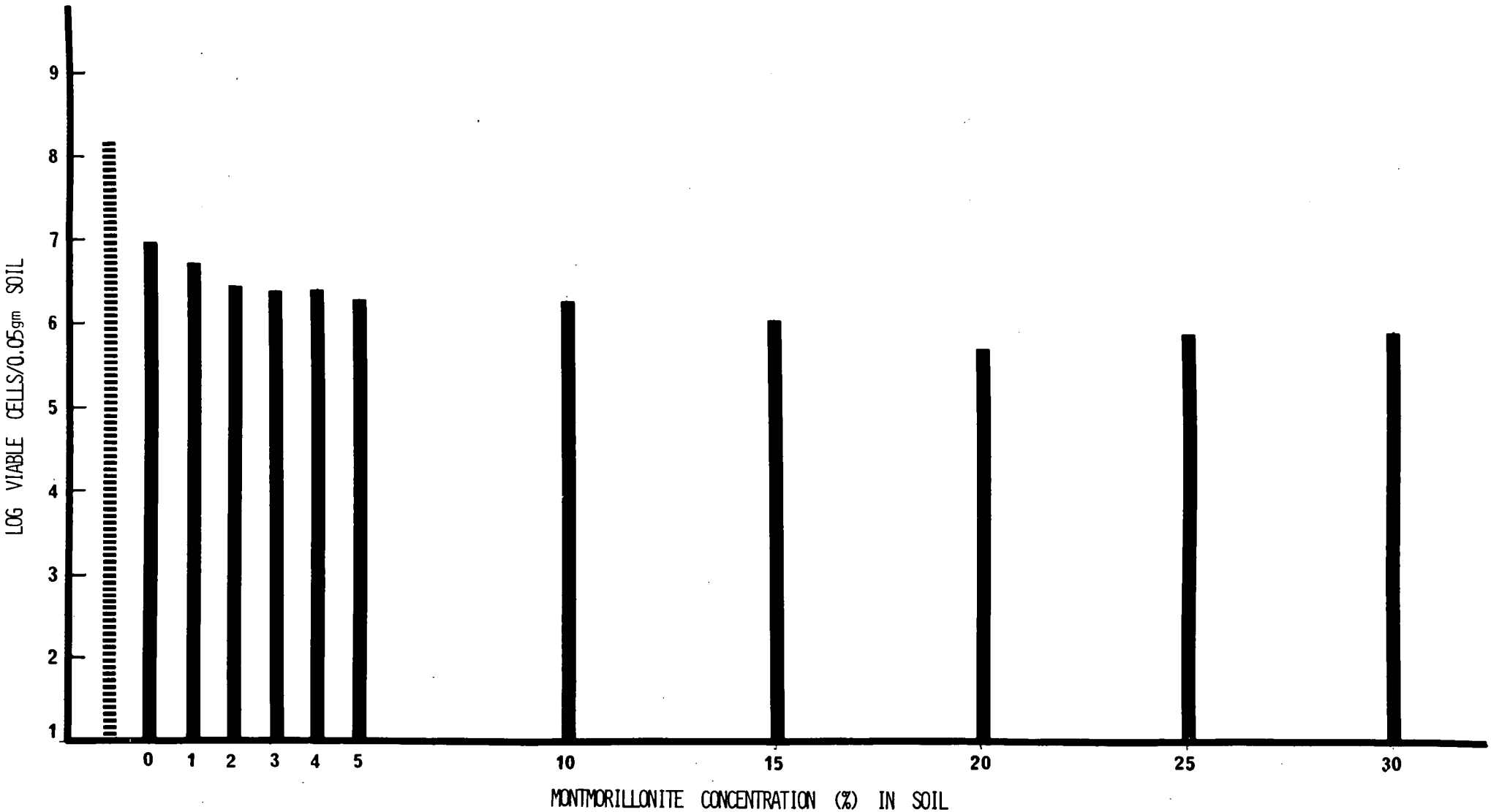

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
10
15
20
25
30
MONTMORILLONITE CONCENTRATION (%) IN SOIL

FIGURE 20

Effect of PVP, maltose and various montmorillonite concentrations on the survival of desiccated R.lupini UT12.

Detailed results and analysis of variance are presented in Appendix Table XIV.

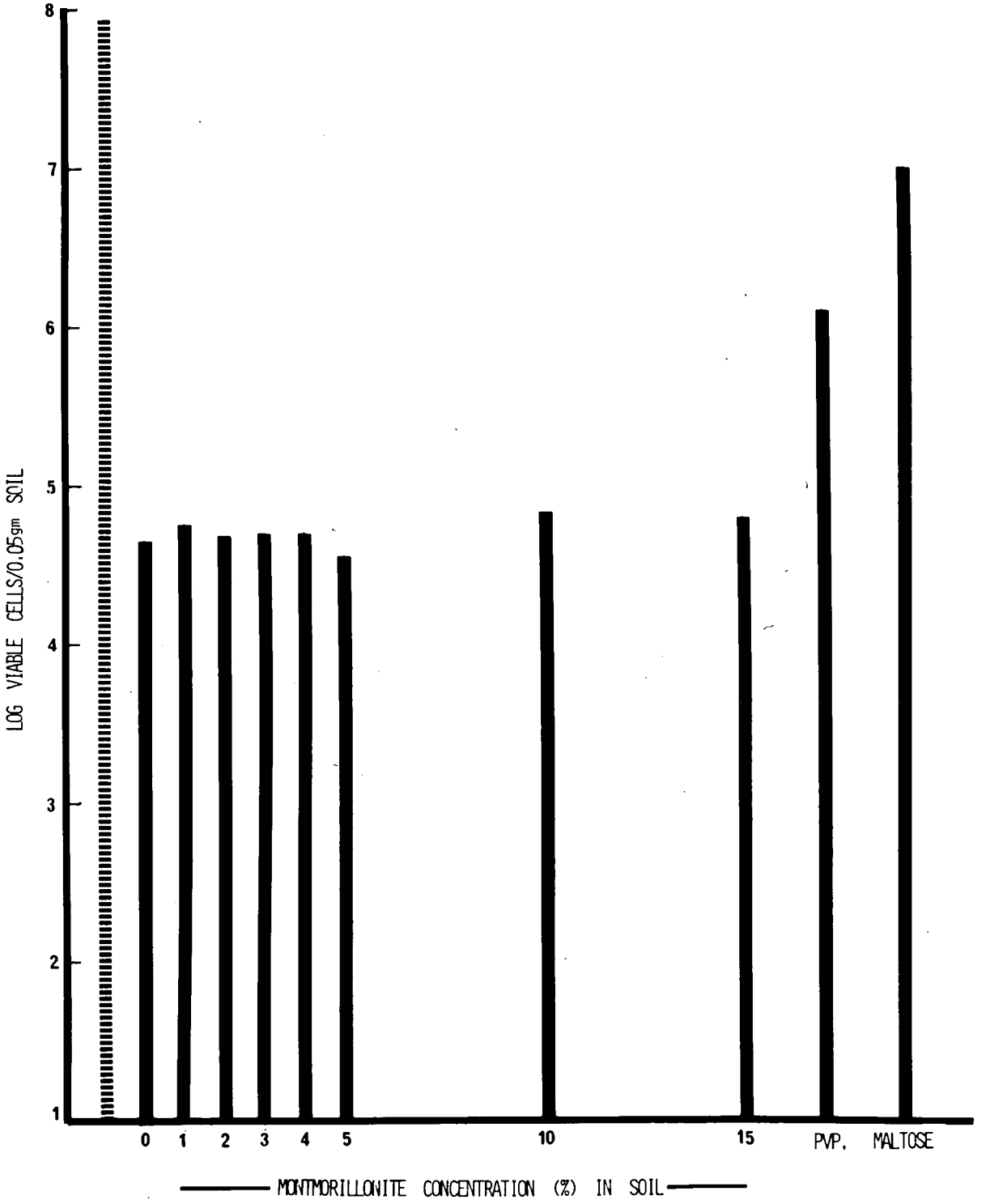
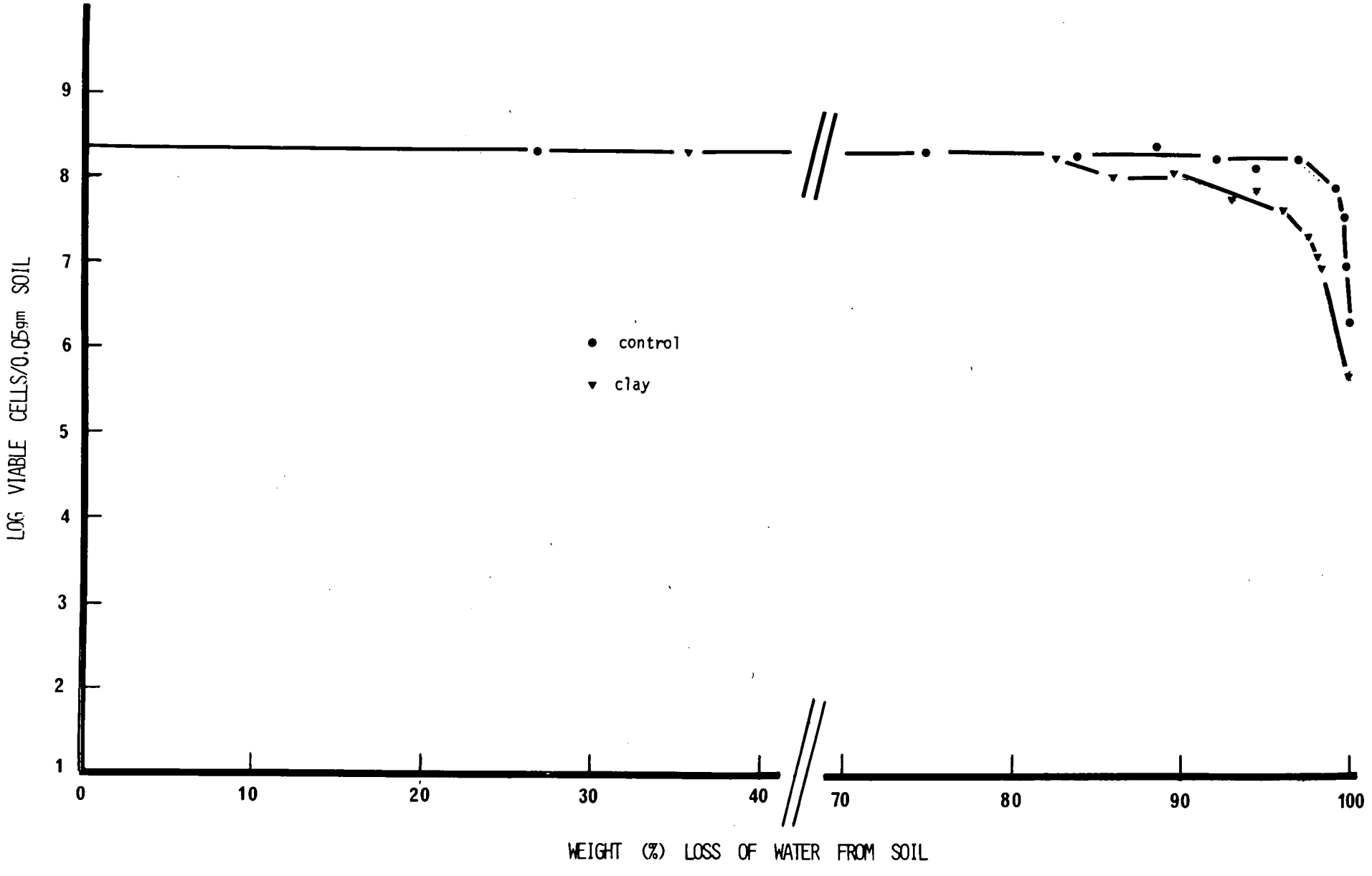

LOG VIABLE CELLS/0.05gm SOIL
8
7
6
5
4
3
2
1
0
1
2
3
4
5
10
15
PVP.
MALTOSE
MONTMORILLONITE CONCENTRATION (%) IN SOIL

FIGURE 21

Effect of the amount of water removed on the viable count of *R. lupini* UT12 in both montmorillonite-amended and unamended soils.

Detailed results and analysis of variance are presented in Appendix Table XVa and XVb.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
control
clay
0
10
20
30
40
70
80
90
100
WEIGHT (%) LOSS OF WATER FROM SOIL

investigations was to compare the effect of an hydrated suspension of montmorillonite on survival of desiccated rhizobia with the results obtained when soils were amended with the powdered clay. The results presented in figure 22 show the effect of various montmorillonite concentrations added as a suspension,and as a powder, on the survival of dried R.lupini UT12. The only treatment that significantly ($P < 0.05$) affected survival was desiccation of bacteria from a suspension of montmorillonite containing 17,500 µgm/ml. This decreased the ability of R.lupini to withstand desiccation. The results presented in figure 22 were in agreement with those reported in earlier graphs for the effect of montmorillonite on the survival of dried, slow-growing root-nodule bacteria.

Unexpected results were obtained for the effect of aqueous suspensions of montmorillonite on the fast-growing R.leguminosarum TA101 (figures 23, 24 and 25). The results presented in figures 24 and 25 clearly show the marked difference in the effect of montmorillonite added as a suspension compared with the effect of the clay when added as a powder. The ability of R.leguminosarum TA101 to survive desiccation was not affected by montmorillonite added as a suspension up to concentrations of 2000 µgm/ml (figure 23), or 1400 µgm/ml (figure 24). Occasionally, montmorillonite added as a suspension decreased the ability of this bacterium to survive the effects of desiccation (figure 25). The results in figure 24 for the 5% S treatment, were obtained as follows:- The addition of 14.3 ml of the stock suspension of montmorillonite

FIGURE 22

Effect on survival of desiccated R.lupini UT12 of montmorillonite added as a suspension and as a powder. The concentration of the clay in the suspension is given as µgm/ml., and the concentration of powdered montmorillonite added to the soil is given as a percentage (w/w).
(Four ml. of 17500 µgm. montmorillonite/ml. gave an amended soil containing 0.7% amendment).
Detailed results and analysis of variance are presented in Appendix Table XV1.

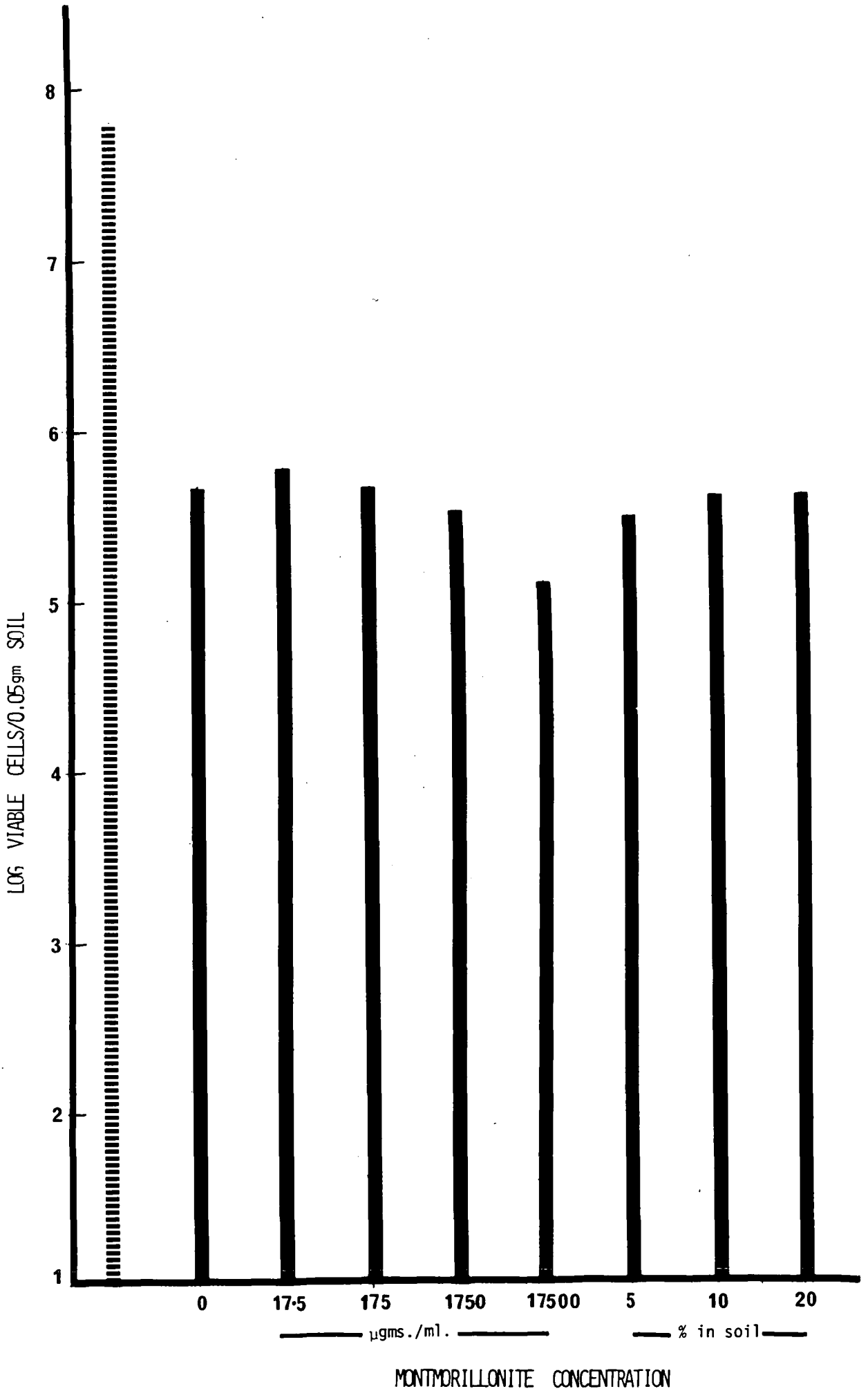

LOG VIABLE CELLS/0.05gm SOIL
8
7
6
5
4
3
2
1
0
17·5
175
1750
17500
5
10
20
µgms./ml.
% in soil
MONTMORILLONITE CONCENTRATION

FIGURE 23

Effect of various montmorillonite concentrations (added as a suspension,) on the survival of desiccated R.leguminosarum TA101.

Detailed results and analysis of variance are presented in Appendix Table XV11.

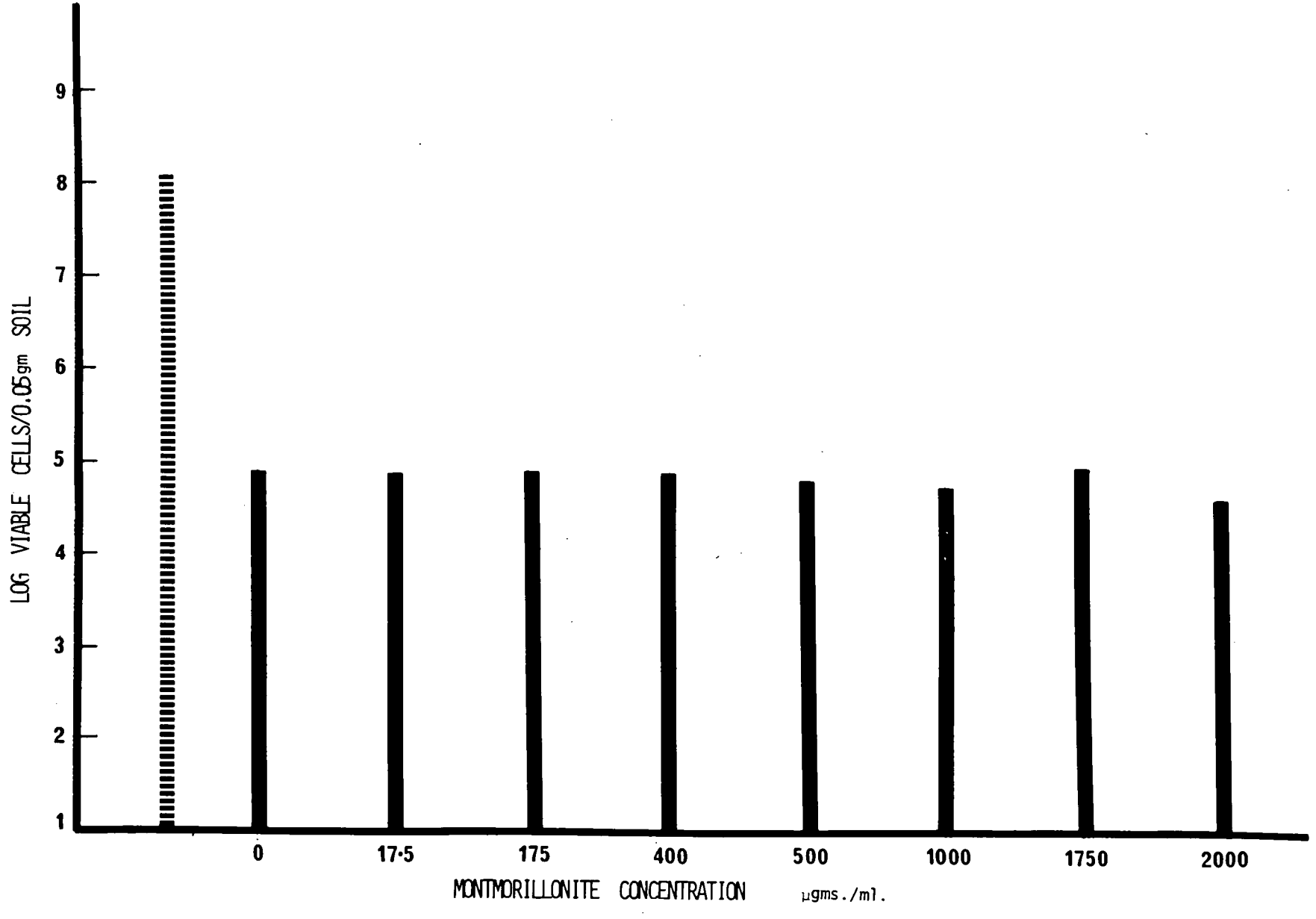

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
17·5
175
400
500
1000
1750
2000
MONTMORILLONITE CONCENTRATION µgms./ml.

FIGURE 24

Effect on survival of desiccated *R. leguminosarum* TA101 of montmorillonite added as a suspension and as a powder.
The concentration of the clay in the suspension is given as µgm./ml., and the concentration of the powdered montmorillonite added to the soil is given as a percentage (w/w).
(Four ml. of 1400 µgm. montmorillonite/ml. gave an amended soil containing 0.056% amendment).
5P%=montmorillonite added to soil as a powder.
5S%=montmorillonite added to soil as a suspension, then air-dried prior to the addition of rhizobia(see text).
Detailed results and analysis of variance are presented in Appendix Table XVIII.

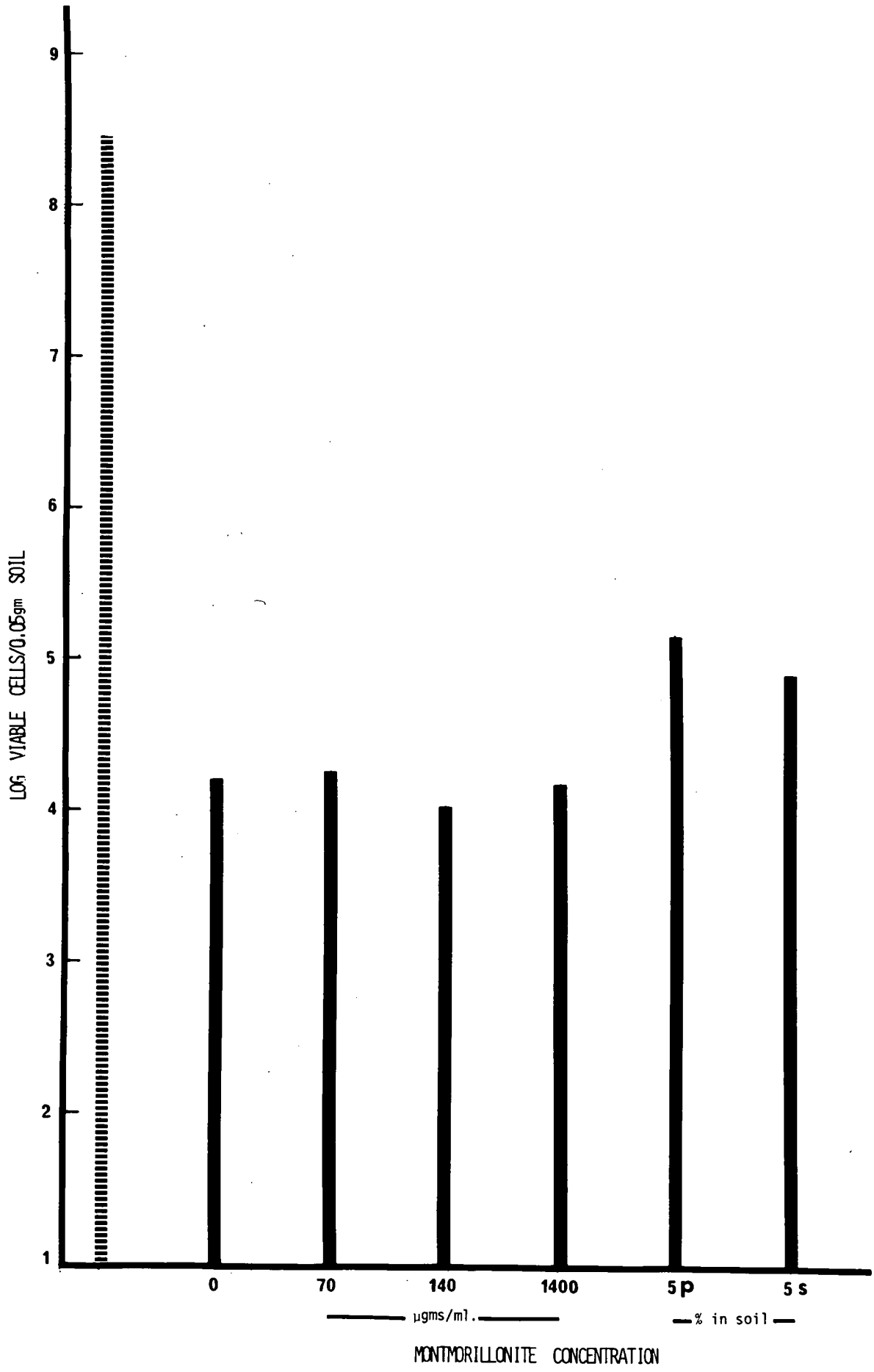
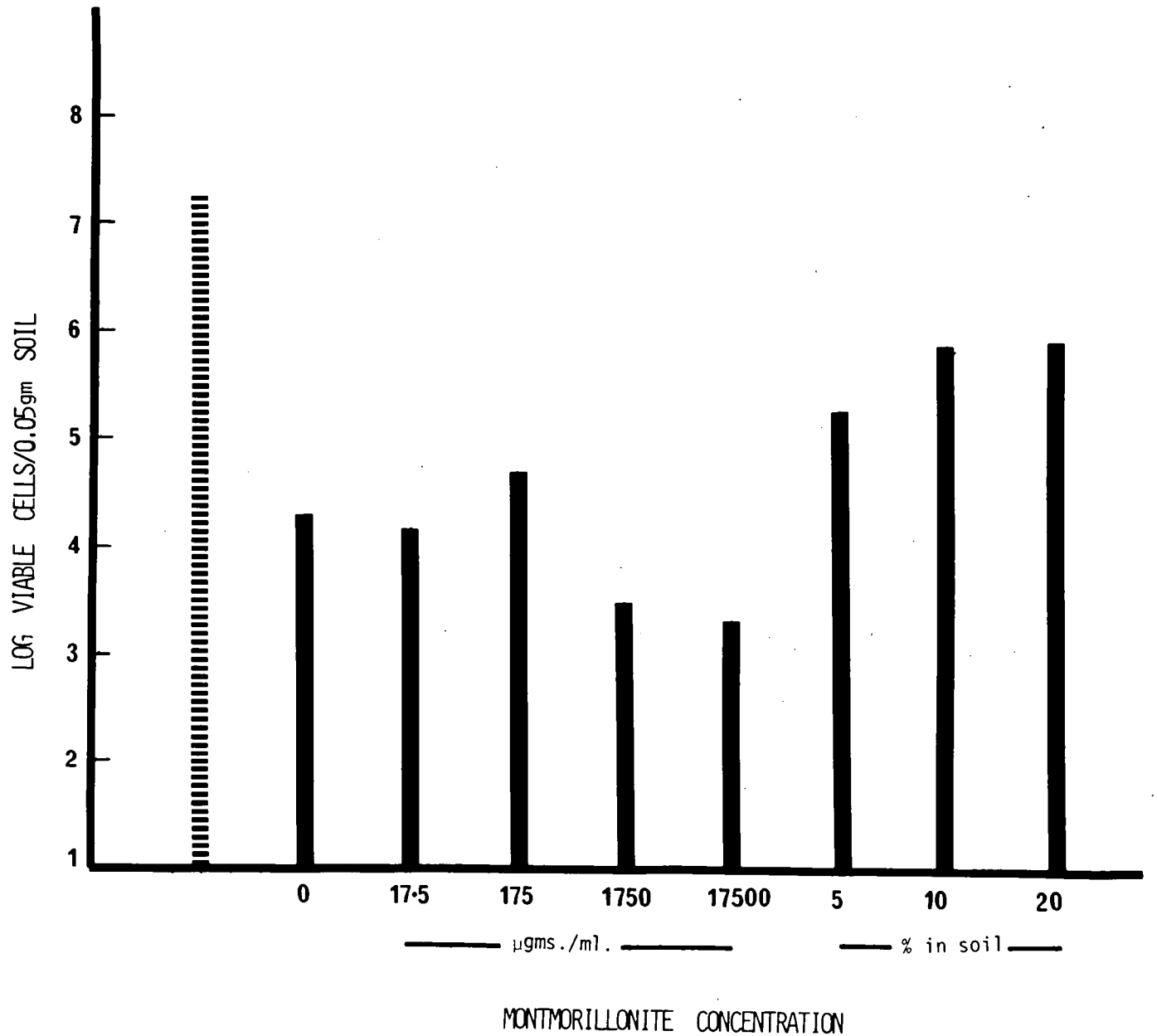

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
0
70
140
1400
5p
5s
µgms/ml.
% in soil
MONTMORILLONITE CONCENTRATION

FIGURE 25

Effect on survival of desiccated *R. leguminosarum* TA101 of montmorillonite added as a suspension and as a powder.
The concentration of the clay in the suspension is given as µgm/ml., and the concentration of the powdered montmorillonite added to the soil is given as a percentage (w/w).
(Four ml. of 17500 µgm. montmorillonite/ml. gave an amended soil containing 0.7% amendment).
Detailed results and analysis of variance are presented in Appendix Table XIX.


LOG VIABLE CELLS/0.05gm SOIL
8
7
6
5
4
3
2
1
0
17·5
175
1750
17500
5
10
20
µgms./ml.
% in soil
MONTMORILLONITE CONCENTRATION

to 95.0 gms. of unamended soil gave air dried soil samples containing 5% dried montmorillonite. The soil amended by this method was dried before the addition of bacteria, and the effect on survival of desiccated R.leguminosarum TA101 is presented in the column marked 5% S, figure 24. There was no significant difference ($P < 0.05$) between the enhanced survival obtained when the clay was added by this method, and when it was added as a dried powder (figure 24).

When montmorillonite amended soil samples were air dried prior to the addition of bacteria, a hard crust formed over the surface of the sample. It was thought that this might decrease the availability of montmorillonite for envelopment of added rhizobia and the protection of desiccated bacteria might be minimal. To test this, a series of experiments were carried out in which the montmorillonite amended samples were not air dried prior to the addition of bacteria i.e. the soil samples were moist. The results presented in figure 26 demonstrated that the ability of montmorillonite to protect desiccated R.leguminosarum TA101 was completely removed by this treatment. This result was similar to those for R.leguminosarum TA101 presented in figures 23, 24 and 25 when the bacteria were dried in the presence of montmorillonite added as a suspension.
The same trend was shown by the results for the fast-growing Rhizobium sp. SU343, presented in figure 27.

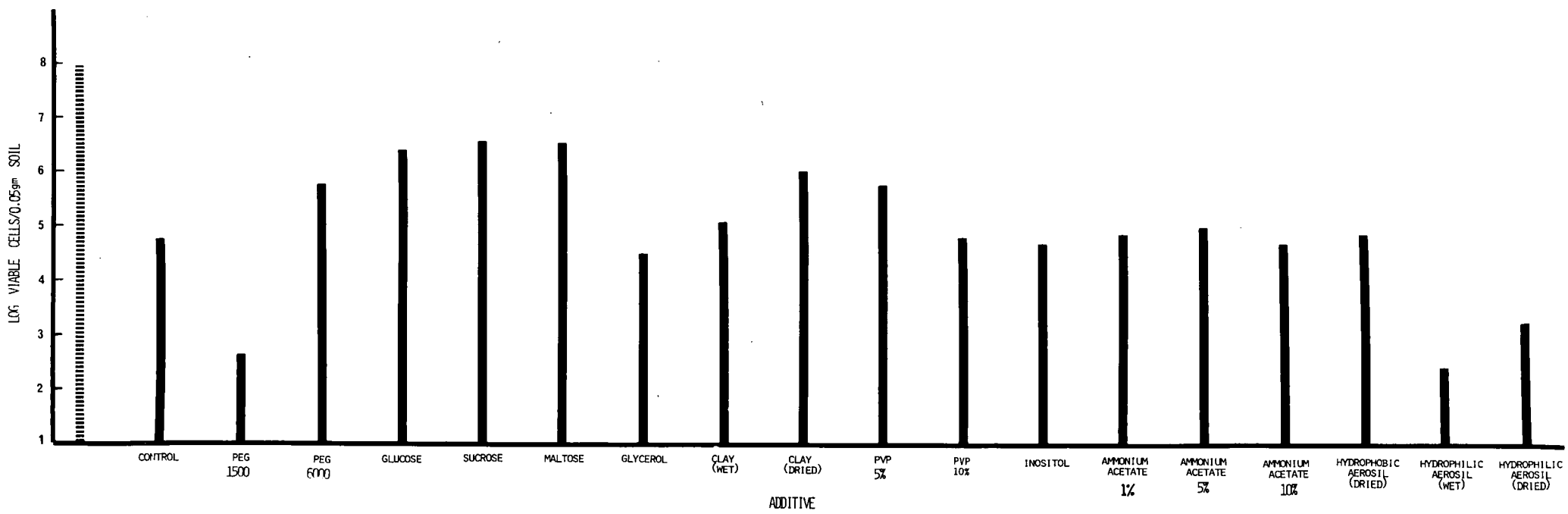
FIGURE 26

Effect of various additives on the survival of desiccated R.leguminosarum TA101.

PEG = polyethylene glycol with molecular weights of 1500 and 6000.

Clay = montmorillonite

Detailed results and analysis of variance are presented in Appendix Table XX.


LOG VIABLE CELLS/0.05gm SOIL
8
7
6
5
4
3
2
1
CONTROL
PEG 1500
PEG 6000
GLUCOSE
SUCROSE
MALTOSE
GLYCEROL
CLAY (WET)
CLAY (DRIED)
PVP 5%
PVP 10%
INOSITOL
AMMONIUM ACETATE 1%
AMMONIUM ACETATE 5%
AMMONIUM ACETATE 10%
HYDROPHOBIC AEROSIL (DRIED)
HYDROPHILIC AEROSIL (WET)
HYDROPHILIC AEROSIL (DRIED)
ADDITIVE

74/3/31

The conclusion drawn from this series of experiments was that in order to obtain protection of desiccated fast-growing rhizobia, montmorillonite must be added as a dried powder, not as an hydrated suspension.

Included in figures 26 and 27 are the results of the effects of a variety of additives on the survival of desiccated R.leguminosarum TA101 and Rhizobium sp. SU343, respectively. For both species, protection was afforded by the following substances:- PEG 6000; the sugars (glucose, maltose and sucrose); dried clay; and the macromolecule PVP. Other additives, such as the hydrophilic aerosil, PEG 400, PEG 1500 and glycerol, decreased the survival of these desiccated fast-growing rhizobia (figures 26 and 27). Such additives as inositol, ammonium acetate (at various concentrations), PVP at 10%, and the hydropholic aerosil, had no effect on the survival of desiccated R.leguminosarum TA101 (figure 26). Rhizobium sp. SU343 was not affected by the hydrophobic aerosil, but ammonium acetate (at various concentrations), and PVP (10%) significantly ($P<0.05$) increased the ability of this species to survive dehydration.

Presented in figures 28 and 29 are the results of experiments designed to determine the effects of various additives on survival of the slow-growing R.japonicum QA372 and R.lupini UT12, respectively. Protection from desiccation was given to both species by PVP and the sugars, (maltose glucose and sucrose). The additives ammonium acetate, glycerol, PEG400 and PEG1500, the hydrophilic aerosil, and

FIGURE 27

Effect of various additives on the survival of desiccated Rhizobium sp. SU343.
Detailed results and analysis of variance are presented in Appendix Table XXl.

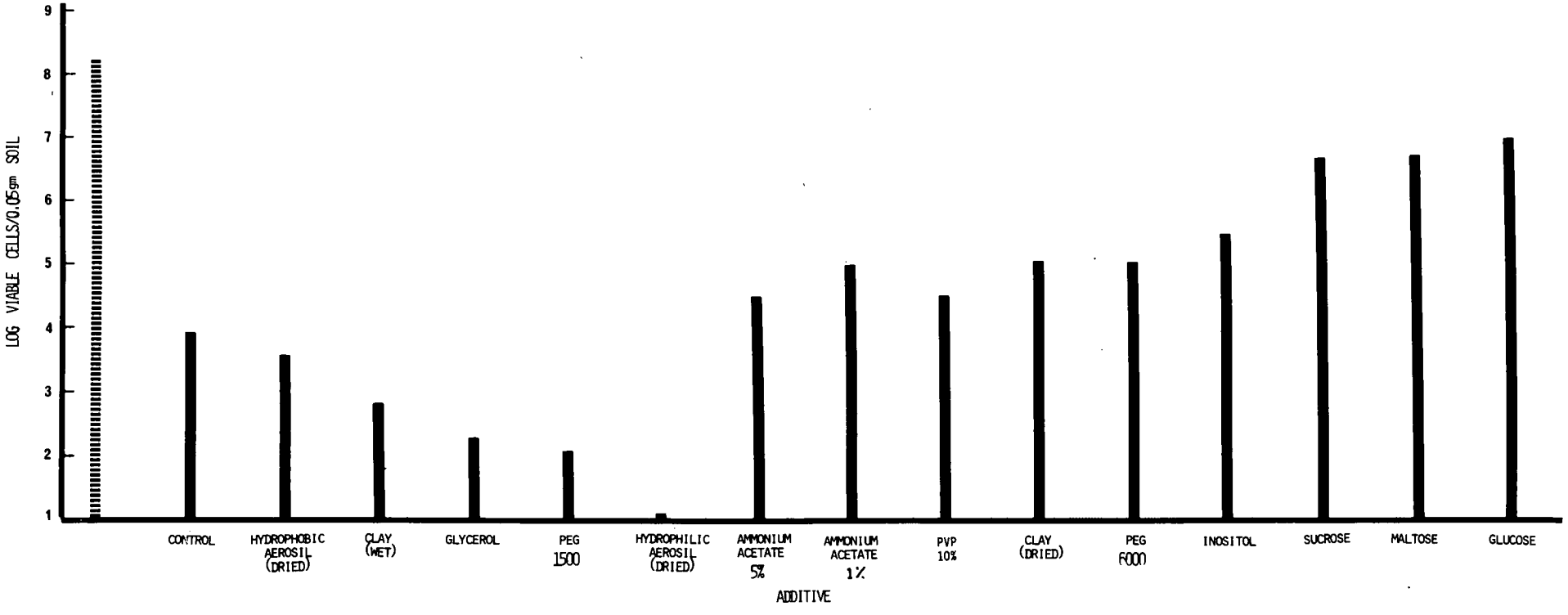

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
CONTROL
HYDROPHOBIC AEROSIL (DRIED)
CLAY (WET)
GLYCEROL
PEG 1500
HYDROPHILIC AEROSIL (DRIED)
AMMONIUM ACETATE 5%
AMMONIUM ACETATE 1%
PVP 10%
CLAY (DRIED)
PEG 6000
INOSITOL
SUCROSE
MALTOSE
GLUCOSE
ADDITIVE

FIGURE 28

Effect of various additives on the survival of desiccated R. japonicum QA372

Clay = montmorillonite

Detailed results and analysis of variance are presented in Appendix Table XX11.

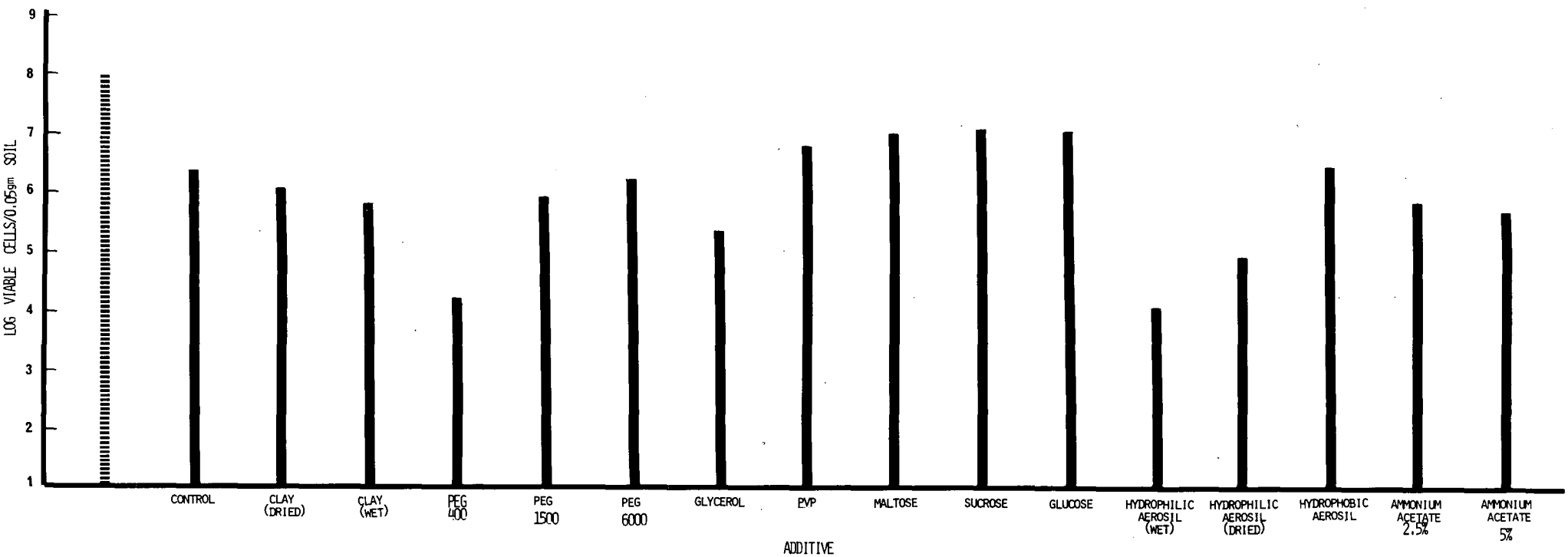
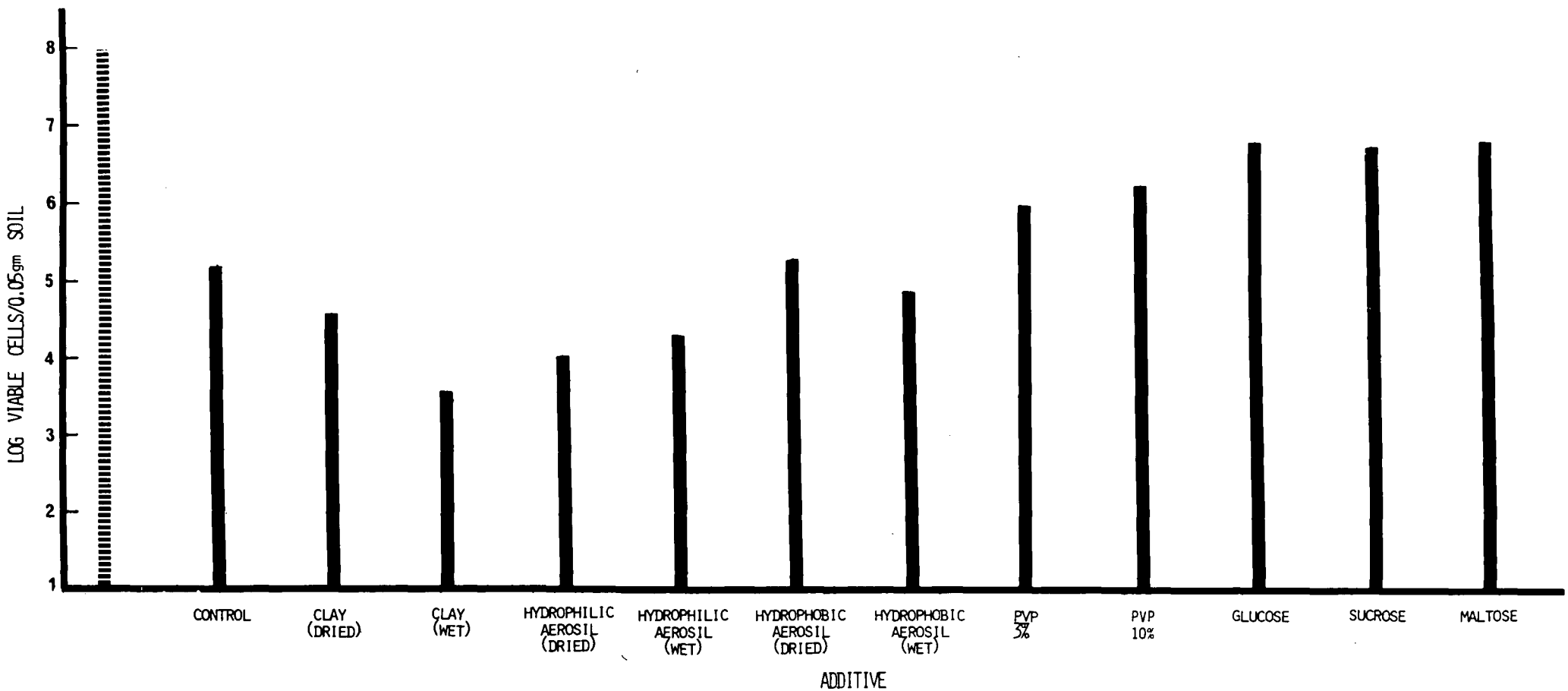

LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
CONTROL
CLAY (DRIED)
CLAY (WET)
PEG 400
PEG 1500
PEG 6000
GLYCEROL
PVP
MALTOSE
SUCROSE
GLUCOSE
HYDROPHILIC AEROSIL (WET)
HYDROPHILIC AEROSIL (DRIED)
HYDROPHOBIC AEROSIL
AMMONIUM ACETATE 2.5%
AMMONIUM ACETATE 5%
ADDITIVE

FIGURE 29

Effect of various additives on the survival of desiccated R. lupini UT12.
Clay = montmorillonite.
Detailed results and analysis of variance are presented in Appendix Table XXIII.


LOG VIABLE CELLS/0.05gm SOIL
8
7
6
5
4
3
2
1
CONTROL
CLAY (DRIED)
CLAY (WET)
HYDROPHILIC AEROSIL (DRIED)
HYDROPHILIC AEROSIL (WET)
HYDROPHOBIC AEROSIL (DRIED)
HYDROPHOBIC AEROSIL (WET)
PVP 5%
PVP 10%
GLUCOSE
SUCROSE
MALTOSE
ADDITIVE

bacteria added to moist soil samples amended with montmorillonite, all decreased the ability of R.japonicum QA372 to survive desiccation (figure 28). The hydrophobic aerosil, PEG6000, and bacteria added to air dried soil samples amended with montmorillonite, had no effect on rhizobium survival when desiccated. Decreased survival of R.lupini UT12 (figure 29) was obtained when it was desiccated with both the hydrophilic aerosil and montmorillonite, whereas soils amended with the hydrophobic aerosil did not have an effect on the survival of dried R.lupini UT12. When either R.japonicum QA372 (figure 28) or R.lupini UT12 (figure 29) was added to moist montmorillonite amended soil samples prior to desiccation, they were more susceptible to dehydration than when dried in similarly amended samples that had been air-dried prior to the addition of the bacteria. Similar results to this have been presented for the fast-growing bacteria in figures 26 and 27.

The different effect of PEG 6000 on the survival of desiccated fast-and slow-growing rhizobia is noteworthy. It was similar to the response of these two groups of microorganisms to the presence of montmorillonite, i.e. both PEG 6000 and montmorillonite protected the fast-growing root-nodule bacteria, but not the slow-growing rhizobia from the effects of drying. Another finding arising from results of all experiments dealing with the effects of additives on desiccated bacteria was that the sugars invariably gave the greatest protection to both groups of root-nodule bacteria.

Klotz (1965) reported that tetrabutylammonium bromide in solution was able to structure adjacent water molecules. The effect of this chemical upon survival of desiccated fast-growing rhizobia was determined. The results are presented in figures 30 and 31 for R.leguminosarum TA101 and Rhizobium sp. SU343, respectively. Both species died rapidly when dried with tetrabutylammonium bromide and, from Appendix tables XXIV and XXV, it can be seen that it was toxic to undried bacteria.

The remaining results in both figures 30 and 31 demonstrated previous conclusions, i.e. that PVP, montmorillonite, and maltose protected the fast-growing bacteria from the effects of desiccation. Because of this enhanced survival in the presence of PVP or montmorillonite, it was thought that bacteria dried with PVP and montmorillonite combined might be protected even further. This was not the result for R.leguminosarum TA101, as there was no significant difference ($P < 0.05$) between this treatment and the control (figure 30). The combined additives (PVP and montmorillonite) did protect Rhizobium sp. SU343 but the effect was not as great as for PVP alone (figure 31).

Figure 32 illustrates the results of the effects of various additives on survival R.japonicum QA372 desiccated for several days. Samples were taken at the time intervals shown in the graph and the viable count estimated. After 24 hrs. desiccation, maltose and PVP were the only additives

FIGURE 30

Effect of PVP, montmorillonite (clay), montmorillonite plus PVP, tetrabutylammonium bromide (T), and maltose on the survival of desiccated R.leguminosarum TA101.

Detailed results and analysis of variance are presented in Appendix Table XXIV.

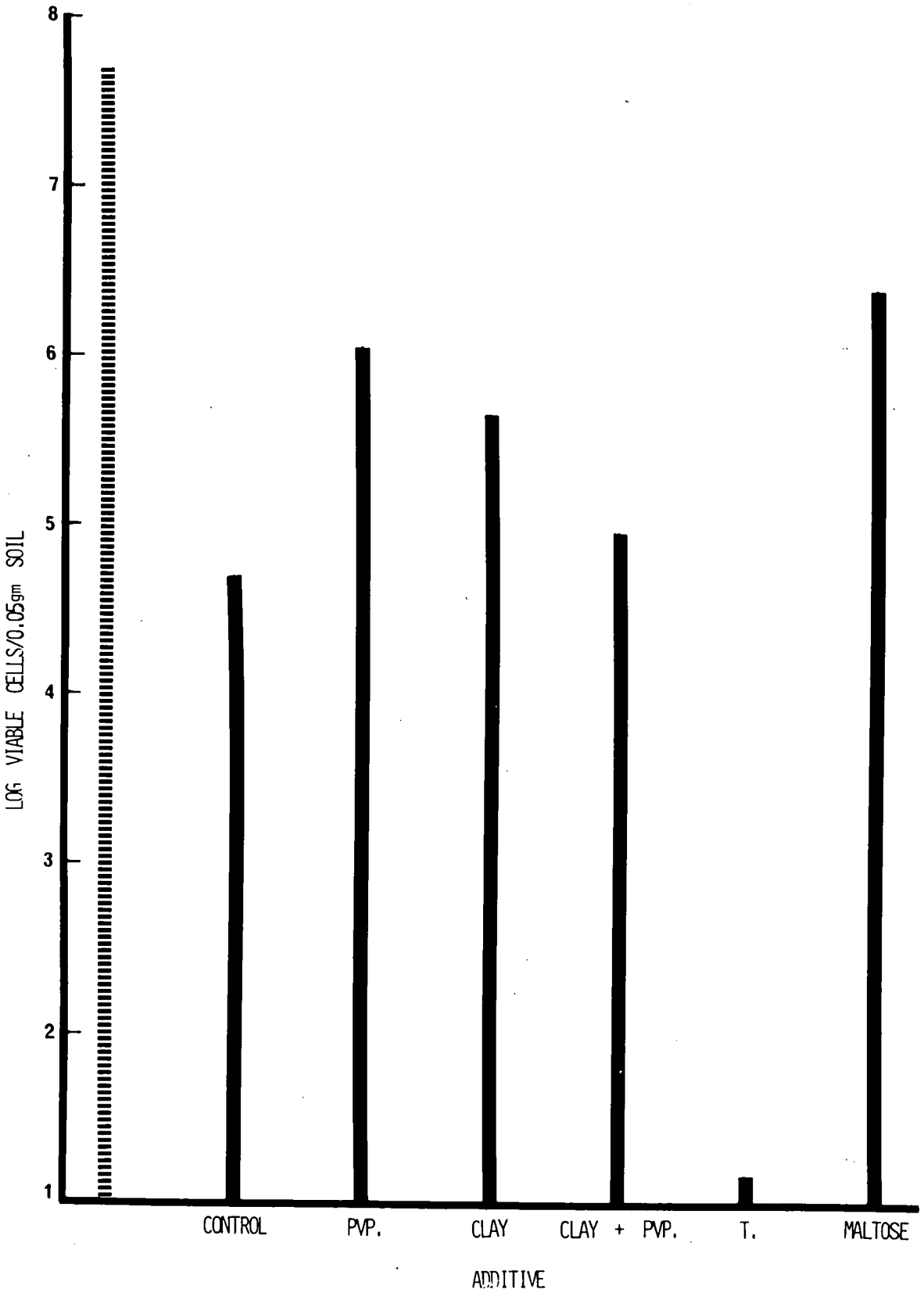
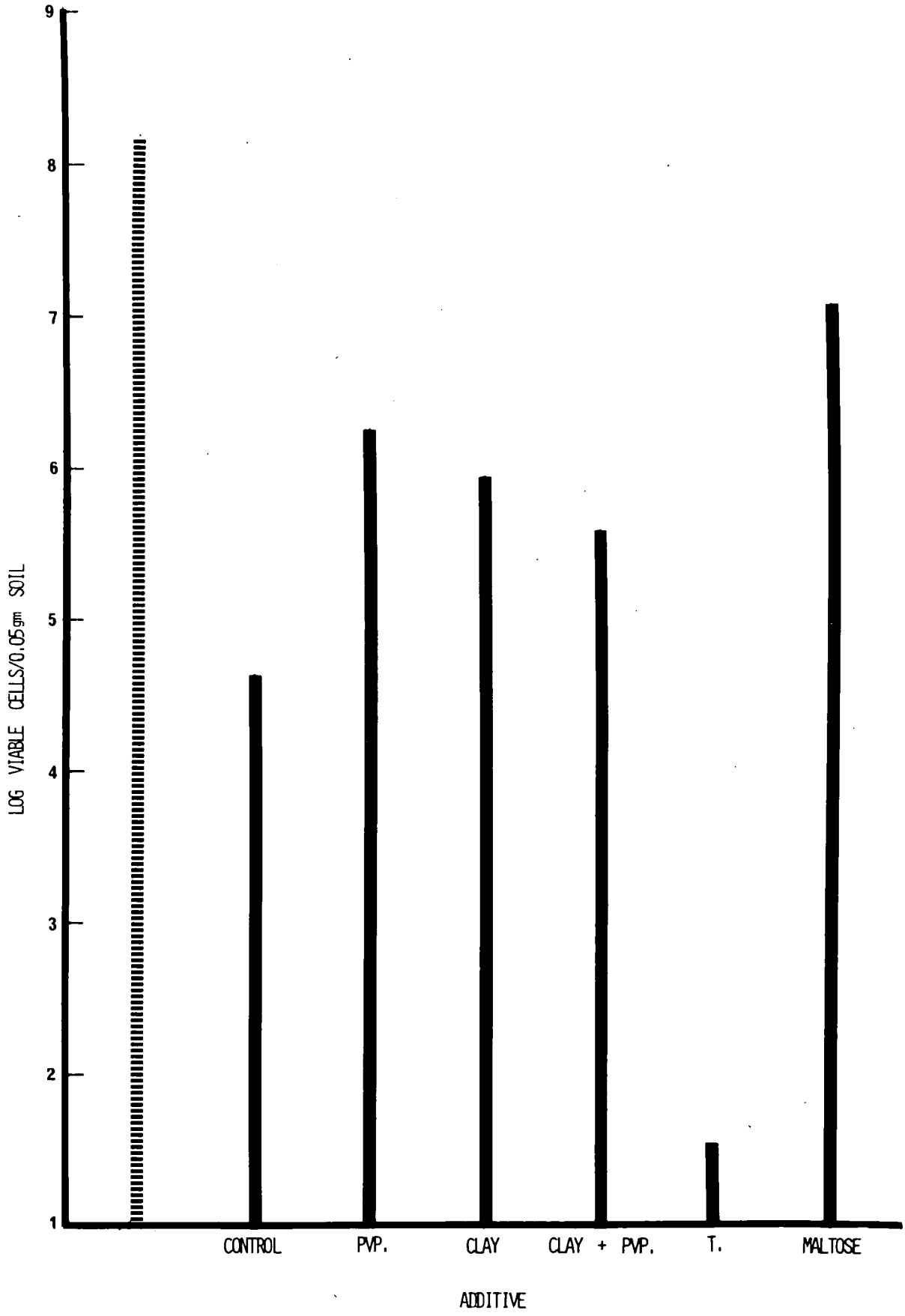

8
7
6
5
4
3
2
1
LOG VIABLE CELLS/0.05gm SOIL
CONTROL
PVP.
CLAY
CLAY + PVP.
T.
MALTOSE
ADDITIVE

FIGURE 31

Effects of PVP, montmorillonite (clay), montmorillonite plus PVP, tetrabutylammonium bromide (T), and maltose on the survival of desiccated Rhizobium sp. SU343.

Detailed results and analysis of variance are presented in Appendix Table XXV.


9
8
7
6
5
4
3
2
1
LOG VIABLE CELLS/0.05gm SOIL
CONTROL
PVP.
CLAY
CLAY + PVP.
T.
MALTOSE
ADDITIVE

that protected the strain of R. japonicum. The additives, montmorillonite, tetrabutylammonium bromide, and the combination of montmorillonite plus PVP, were all detrimental to the survival of desiccated R. japonicum QA372. This result is in agreement with those presented above for short term storage (24 hrs.) in the desiccated state. The results presented in figure 32, after a period of 24 hrs. desiccation were essentially the same as the results after the bacteria had been in the dried state for 120 hrs. A linear regression analysis was carried out on the data presented in figure 32, obtained after the initial 24 hr. period of dehydration for each of the additives. The regression coefficients and the significance of their differences are shown in table 5. The only result that was significantly different ($P<0.001$) from the control was that obtained when the bacteria were dried in the presence of maltose. The results of this analysis (table 5) suggested that the action of all the additives, except maltose, occurred during the period of dehydration (or perhaps rehydration) rather than during the period of storage at a low water content. Once dried, the rate of decrease of viable R. japonicum QA372 was constant whether or not an additive was present. This effect of maltose was similar to that described by Vincent et al. (1962) for R. trifolii, where considerable protection was obtained during both dehydration and storage.

FIGURE 32

Effect of montmorillonite (clay), PVP, maltose, tetrabutylammonium bromide, and montmorillonite plus PVP, on the survival of desiccated R. japonicum QA372 sampled over a period of 120 hrs. in the desiccated state.

Detailed results and analysis of variance are presented in Appendix Table XXVI.

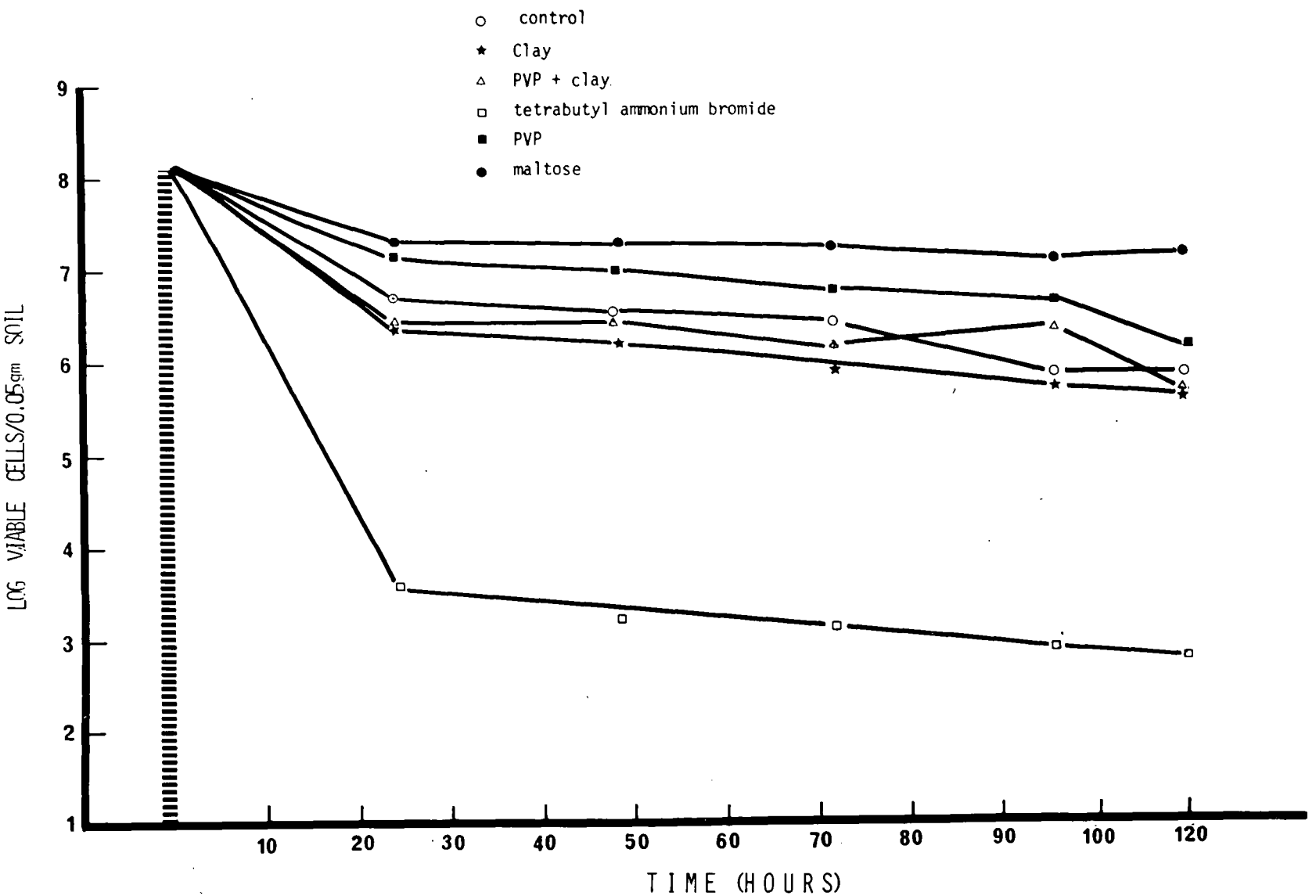

control
Clay
PVP + clay
tetrabutyl ammonium bromide
PVP
maltose
LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
120
TIME (HOURS)

Table 5

Regression coefficients calculated from the results presented in figure 32 for the decrease in the viable count of desiccated R. japonicum QA372 between 24 and 120 hours dehydration.

Additive	Regression coefficient	Significance of difference from control
Control	-0.0099	-
Maltose	-0.0023	xx
PVP	-0.0099	NS
Montmorillonite + PVP	-0.0070	NS
Montmorillonite	-0.0085	NS
Tetrabutyl-ammonium bromide	-0.0079	NS

The statistical significance of the regression lines from the control was estimated by the method of Steele and Torrie (1960), page 173.

D. Effect on survival of desiccation of rhizobia in atmospheres of nitrogen and air.

There have been many reports in the literature emphasising the toxic nature of oxygen in the air, to dehydrated microorganisms. The purpose of these experiments was to determine the effect on survival of dehydrating various rhizobia under atmospheres of nitrogen.

Because many investigations in the literature have reported enhanced survival of Escherichia coli when dehydrated in atmospheres of nitrogen, this microorganism was used in some experiments to test the functioning of the apparatus used and the procedure followed.

The results in table 6 and 7 show that Escherichia coli M13 and several species of rhizobia survived dehydration in atmospheres of nitrogen better than in air. The significant block effect of the results presented in table 6 was obtained because the results were obtained at different times using different cultures of E.coli (the initial numbers were probably different but were not estimated). The results presented in tables 6 and 7 suggested that oxygen may have been toxic to bacteria dried in air. It was not possible to determine whether R.trifolii SU297/31A was more susceptible to the toxic effects of air than R.japonicum QA372 because the initial numbers were not estimated and were probably different in each case.

Table 6

Effect of desiccation on the survival of Escherichia coli M13 in atmospheres of air and nitrogen.

Log viable count per 0.05 gm. soil.

Block Number	Replication Number	Air Dried	Mean	Nitrogen Dried	Mean
	1	5.06		6.07	
1	2	5.61	5.34	5.98	6.03
	3	5.12		6.04	
2	1	1.74		3.43	
	2	2.22	2.54	3.41	3.37
	3	2.63		3.27	

Analysis of variance on above log data.

Source of Variation	D.F	Sums of Squares	Mean Square	F
Replication	2	0.121	0.0605	
Block	1	24.596	24.596	286.05^{xxx}
Treatment	1	2.822	2.822	32.81^{xxx}
Error	7	0.602	0.086	
Total	11	28.141		

Table 7

Effect of desiccation on the survival of various rhizobia in atmospheres of air and nitrogen.

Bacterium	Log viable count per 0.05 gm. soil.					
	Air dried			Nitrogen dried		
	Replication No.			Replication No.		
	1	2	Mean	1	2	Mean
R.meliloti SU47	2.69	3.57	3.32	4.91	4.98	4.95
R.japonicum QA372	3.62	3.07	3.43	4.60	4.57	4.59
R.trifolii SU297/31A	5.47	5.13	5.33	5.73	5.76	5.75

Analysis of variance of above log data.

Source of Variation	D.F	Sums of Squares	Mean Square	F
Replication	1	0.0	0.0	
Bacterium	2	6.182	3.091	13.993^{xxx}
Treatment	1	4.083	4.083	18.48^{xxx}
Error	7	1.546	0.2209	
Total	11	11.811		

The results presented in tables 6 and 7 were obtained using two vacuum desiccators only. An experiment was carried out using R.leguminosarum TA101 desiccated in the presence and absence of montmorillonite, and under atmospheres of air and nitrogen. This involved the use of a greater number of desiccators which introduced larger errors. A summary of the results is presented in table 8. There was a trend towards enhanced survival under nitrogen, but, because of the large value of the error mean square between desiccators, this was not significant for R.leguminosarum TA101. There was a significant increase ($P < 0.005$) in survival in the presence of montmorillonite. However, there was no interaction between the presence or absence of the clay or the gas. This suggested that the protective effect of montmorillonite was independent of the presence or absence of air. Therefore, the mechanism of montmorillonite protection was not via the exclusion of air from dehydrated bacteria.

Apart from the variability of results obtained from different desiccators, there seemed to be a great deal of variability associated with the source of nitrogen. Frequently, the results obtained by the use of nitrogen from one cylinder varied greatly from those obtained when a different cylinder was used, even though the source was C.I.G. high-purity nitrogen in both instances.

To eliminate these complications, further purification of the nitrogen source would be required. Webb (1969) has

Table 8

Effect on survival of desiccation of R. leguminosarum TA101 in atmospheres of air and nitrogen, both in unamended and montmorillonite-amended soil samples.

The values in this table are the averages of replications. For more detailed results, see Appendix Table XXVII.

Log viable count per 0.05 gm. soil

Air-dried samples		Nitrogen-dried samples	
No. Montmorillonite	Plus Montmorillonite	No. Montmorillonite	Plus Montmorillonite
Desiccator A		Desiccator C	
4.11	4.69	5.28	5.87
Desiccator B		Desiccator D	
5.26	5.79	5.41	5.92

Log initial viable count per 0.05 gm. soil = 8.48

suggested that impurities in the nitrogen used to generate aerosols was a cause of the lack of agreement between many results reported in the literature. Because of the large error between desiccators, either many more would have to be used in subsequent experiments or, preferably, containers with larger volumes should be employed.

Because of the lack of equipment and the time required to purify and test the nitrogen gas it was not possible to improve the quality of these experiments. Consequently, the results obtained need to be interpreted with care. Nevertheless, there is the suggestion that dehydrated rhizobia were susceptible to the toxic effects of air.

E. The effects of water activity on rhizobia

(i) Growth of rhizobia in yeast mannitol broth adjusted to various water activities.

Chen and Alexander (1973) suggested that growth of microorganisms on media of low water activity (a_w) enhanced the ability of bacteria to survive desiccation. Before conducting experiments to determine the effect of growth at low a_w values on the ability of rhizobia to survive desiccation, it was necessary to determine the lowest a_w at which the various root-nodule bacteria would grow. Frequently, the results are presented as either a plus (indicating growth) or a minus (no growth) for a particular species of Rhizobium at a given a_w. However, results are also presented as the change of the absorbance reading after time had been allowed for growth. Some growth curves have been obtained for various rhizobia.

~~The results in~~ Table 9 presents data which indicates the presence or absence of growth for various bacteria at given a_ws. As a group, the fast-growing rhizobia were unable to grow at any a_w less than 0.999, which is the water activity of normal YMB. Growth of R.lupini UT12 (table 9), R.lupini UT2 (table 10) and R.japonicum QA372 (table 10) was consistent at a_w 0.997 and only marginal at a_w 0.995. The pigmented, slow-growing Rhizobium sp. CB736, consistently gave lower absorbance changes (table 10) relative to other members of the slow-growing group. This may be because Rhizobium sp. CB736 did not produce extracellular polysaccharide which would be a factor contributing to the absorbance changes measured for strains of R.lupini or R.japonicum. The results in table 10 indicated that growth of Rhizobium sp. CB736, R.lupini UT2 or R.japonicum QA372 was similar at a particular a_w whether the medium was adjusted by the use of either NaCl or KCl.

In contrast to the relatively poor growth of the fast- and slow-growing rhizobia at a_ws of 0.999 and 0.997, all strains of R.meliloti were able to grow at a_w levels of 0.980 (table 9). This was also typical of those cultures of Agrobacterium tested (table 9), which is not surprising because many authors (e.g. Vincent and Humphrey, 1970) have demonstrated that the agrobacteria and medic rhizobia are closely related to each other.

That R.trifolii SU297/32B and the slow-growing Rhizobium sp. 3C1f1 were sensitive to high salt concentrations as well as the water activities created by them, was demonstrated by the

Table 9

Growth of various species of Rhizobium in yeast mannitol broth of different water activities.

The presence or absence of growth was tested by the change in absorbance (at 600 nm) 140 hours after inoculation. The solute used to adjust the media was NaCl.

	Bacterium	Water activity 0.999	0.997	0.995	0.990	0.980	0.960
Fast-growing rhizobia	R.leguminosarum TA101	x	-	-	-	-	-
	R.trifolii UT6	x	-	-	-	-	-
	" " TA1	x	-	-	-	-	-
	" " UT48	x	-	-	-	-	-
	" " SU297/32B	x	-	-	-	-	-
Slow-growing rhizobium	R.lupini UT12	x	x	-	-	-	-
	R.meliloti SU45	x	x	x	x	x	-
Medic-rhizobia	" " CB112	x	x	x	x	x	-
	" " CC131	x	x	x	x	x	-
	" " SU47	x	x	x	x	x	-
	" " C1-n	x	x	x	x	x	-
	Agrobacterium sp. strain 10	x	x	x	x	x	nd
	Agrobacterium sp. strain 24	x	x	x	x	x	nd
	Agrobacterium sp. strain 150	x	x	x	x	x	nd
	Agrobacterium sp. strain 152	x	x	x	x	x	nd

x indicates that there was growth
- indicates that no growth took place
nd indicates that no test was done

Table 10

Growth of various rhizobia in yeast mannitol broth adjusted to different water activities with either NaCl or KCl.

The presence or absence of growth was tested by the change in absorbance (at 600 nm) 140 hours after inoculation of each water activity.

		Bacterial species		
		Rhizobium sp. CB736	R. lupini UT2	R. japonicum QA372
Water Activity	Solute[x]	△absorbance	△absorbance	△absorbance
0.999	Nil	0.128	1.084	0.705
0.997	NaCl	0.122	1.079	0.731
	KCl	0.148	1.026	0.661
0.995	NaCl	0.000	0.000	0.058
	KCl	0.030	0.000	0.057

x different solutes were used to adjust the a_w of the medium to the desired level.

results in table 11. When media were adjusted to different a_ws by the use of sucrose, adequate growth took place at all a_ws studied for both the fast-and slow-growing rhizobia. There was virtually no growth at any a_w value when the medium was adjusted by the use of a mixture of salts (table 11). This suggested that both R.trifolii SU297/32B and Rhizobium sp. 3C1f1 were very sensitive to high concentrations of salt mixtures. When glucose was used to adjust the media, growth was apparent at a_w 0.997 for both rhizobia, but did not occur at lower a_ws for R.trifolii SU297/32B. Some growth of Rhizobium sp. 3C1f1 was apparent at a_w 0.995 in glucose adjusted media, but none occurred at a_w 0.990. Rhizobium sp. 3C1f1 was able to grow at all a_w values when glycerol was used as the solute, growth of R.trifolii SU297/32B in glycerol adjusted media was not determined. These results emphasised that the solute used to adjust the a_w of the growth medium was an important factor involved in whether growth was possible at a particular a_w.

The results presented in figure 33 and 34 show the growth curves of strains of R.trifolii at various a_ws. Abundant growth of all strains was possible at a_w 0.999. However, only R.trifolii TA48 was able to grow at a_w 0.997 using NaCl to adjust the medium (figure 33). Neither R.trifolii strains SU297/31A or SU297/32B grew at any a_w lower than 0.999 (figure 34).

Table 11

Growth of various species of rhizobia in yeast mannitol broth adjusted to different water activities by the use of different solutes.

The presence or absence of growth was tested by the change in absorbance readings 140 hours after inoculation of each growth medium.

		Bacterial species	
		R. trifolii SU297/32B	*Rhizobium* sp. 3 C1f1
Water Activity	Solute	Δabsorbance	Δabsorbance
0.999	Nil	0.210	0.748
0.997	Sucrose	1.079	0.528
0.995		0.751	0.398
0.990		0.491	0.318
0.997	Salts[x]	0.000	0.044
0.995		0.000	0.000
0.990		0.000	0.000
0.997	Glucose	1.123	0.724
0.995		0.009	0.154
0.990		0.000	0.000
0.997	Glycerol	nd	0.727
0.995		nd	0.678
0.990		nd	0.589

x the salts were NaCl: KCl: Na_2SO_4 in the ratio 5:3:2, (see table 2). nd = not done.

FIGURE 33

Growth curve of R.trifolii UT48 at a_ws of 0.999, 0.997 and 0.995, obtained by the use of NaCl.

Detailed results are presented in Appendix Table XXVIII.

Absorbance values were multiplied by 10 prior to conversion to logarithms.

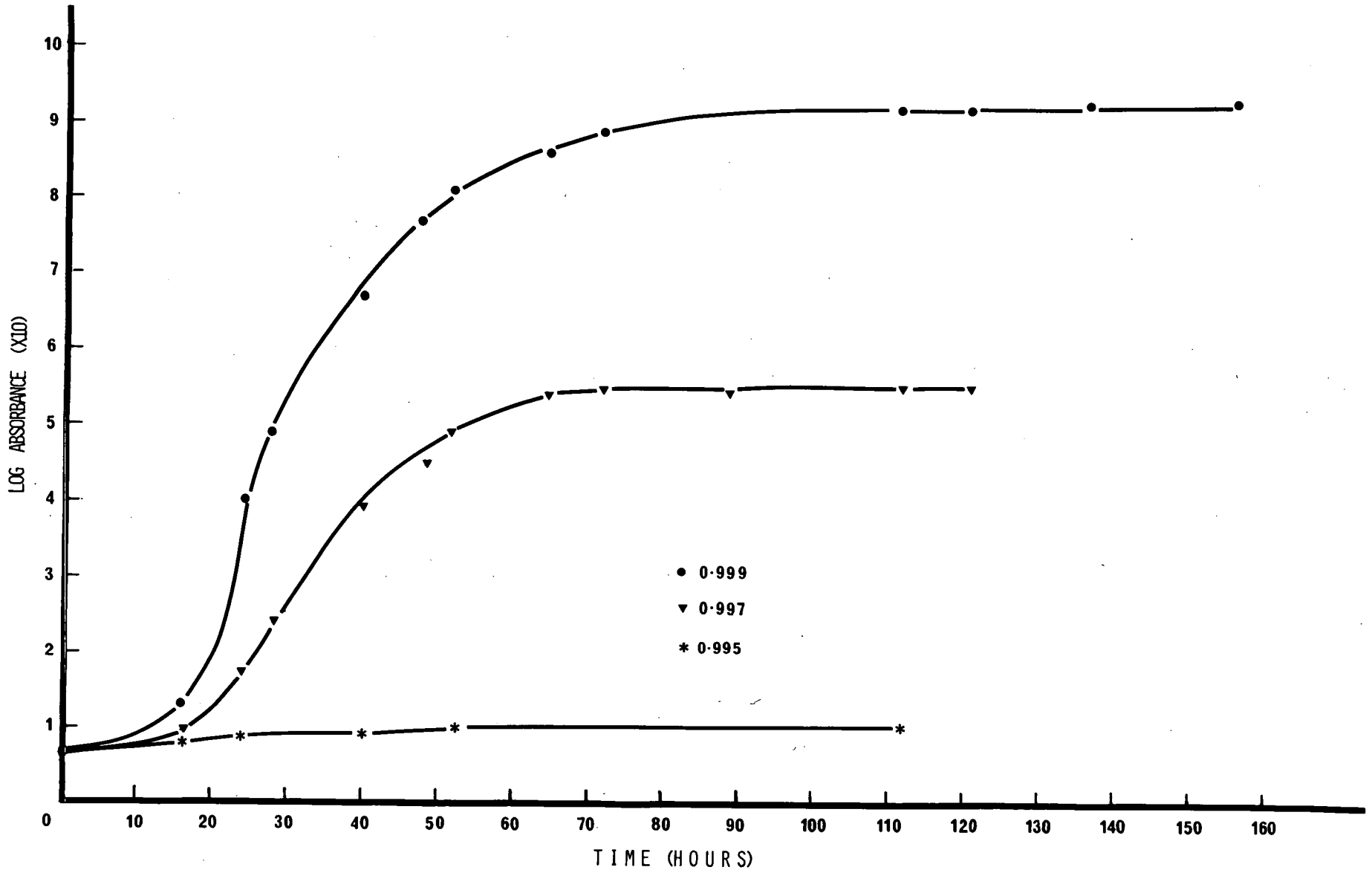
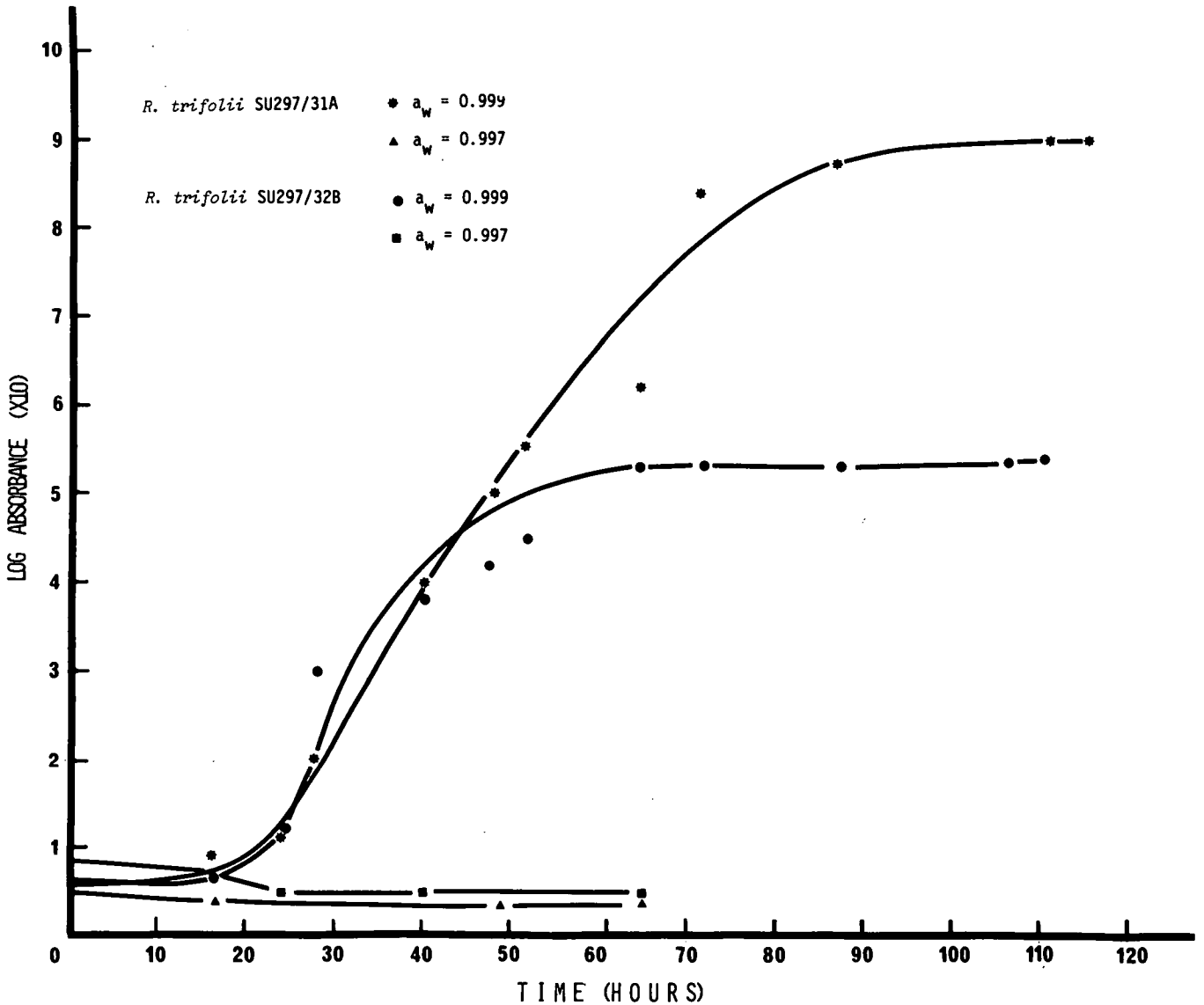

LOG ABSORBANCE (X10)
10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
TIME (HOURS)
● 0·999
▼ 0·997
* 0·995

FIGURE 34

Growth curves of two strains of *R. trifolii* at a_ws of 0.999 and 0.997, obtained by the use of NaCl.

Detailed results are given in Appendix Table XXIX.

Absorbance values were multiplied by 10 prior to conversion to logarithms.


R. trifolii SU297/31A
a_w = 0.999
a_w = 0.997
R. trifolii SU297/32B
a_w = 0.999
a_w = 0.997
LOG ABSORBANCE (X10)
TIME (HOURS)
0
10
20
30
40
50
60
70
80
90
100
110
120
1
2
3
4
5
6
7
8
9
10

The ability of the medic rhizobia to grow at low water activities obtained using NaCl, is shown for R.meliloti strains SU45 and SU47A in figures 35 and 36 respectively. As the results in table 9 indicated, growth of R.meliloti was possible at a_w 0.980. This was shown by the strain SU45 (figure 35), however growth was only marginal at this a_w and was not apparent for R.meliloti SU47A (figure 36). Growth at all the water activities tested above 0.980 was abundant and was frequently as prolific as at a_w 0.999. The results in figures 35 and 36 indicate that the lag and log phases of growth of R.meliloti were only affected by a_ws 0.990 and below. Above these a_w values, the growth curves during the log phase are essentially parallel to the curve obtained in the normal YMB. At a_w 0.980 R.meliloti SU45 exhibits a longer lag phase, decreased growth rate and the final biomass obtained is much lower than the yield from growth in higher a_w values.

Results of growth at various a_w values (obtained using NaCl) of strains of the slow-growing R.lupini are presented in figures 37 to 39, and of R.japonicum QA372 in figure 40. Rhizobium lupini UT2 was able to grow at a_ws 0.999 and 0.997 (figure 37), whereas R.lupini UT12 exhibited marginal growth at a_w 0.995 (figure 38). This was also typical of R.japonicum QA372 (figure 40), as it grew adequately at a_w 0.997 but only marginally at a_w 0.995. More abundant growth at a_w 0.995 was evident for R.lupini 1A425 than was shown by the other slow-

FIGURE 35

Growth curves of R.meliloti SU45 in YMB at a_ws of 0.999, 0.997, 0.995, 0.990 and 0.980, obtained by the use of NaCl.

Detailed results are given in Appendix Table XXX.

Absorbance values were multiplied by 10^3 prior to conversion to logarithms.

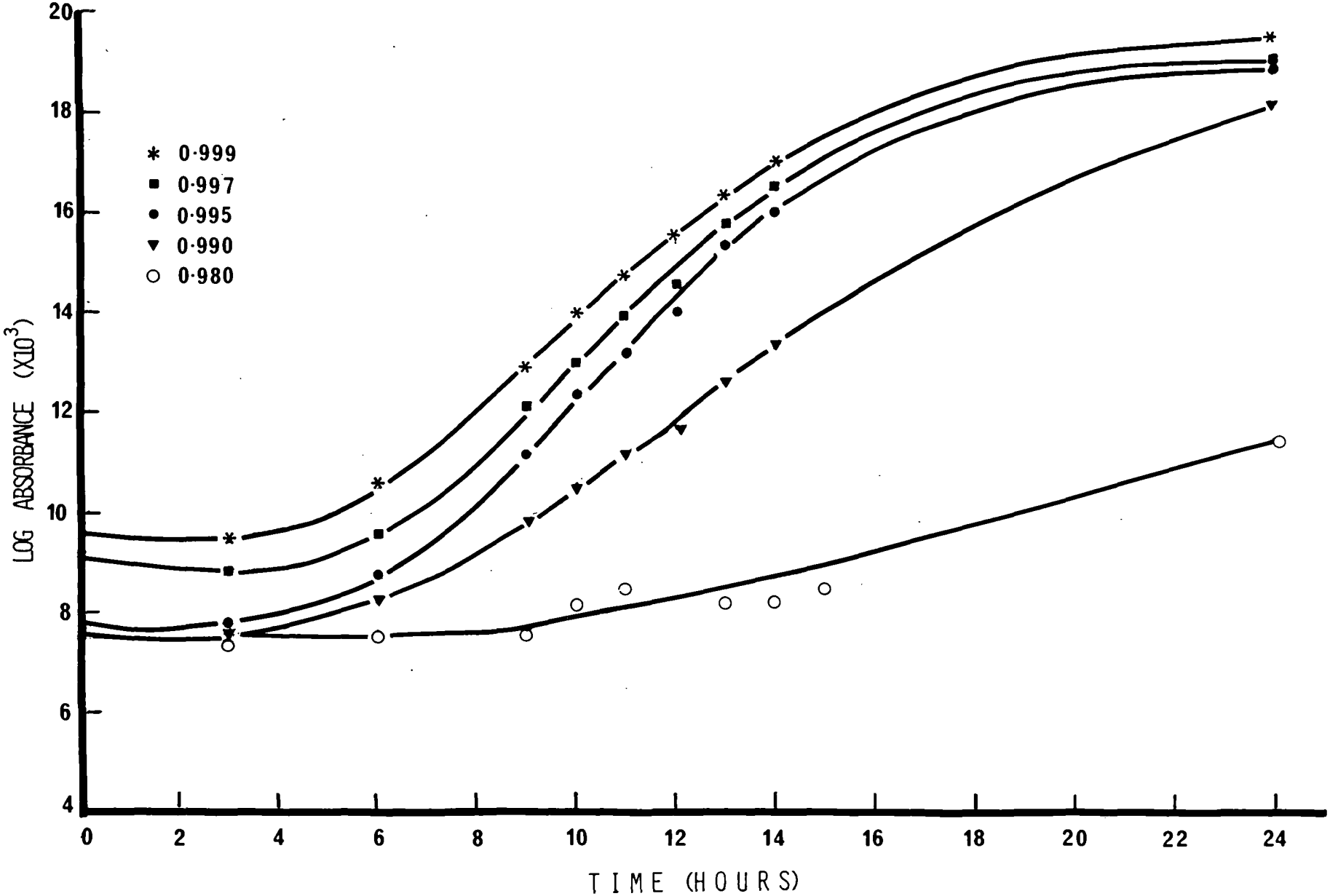
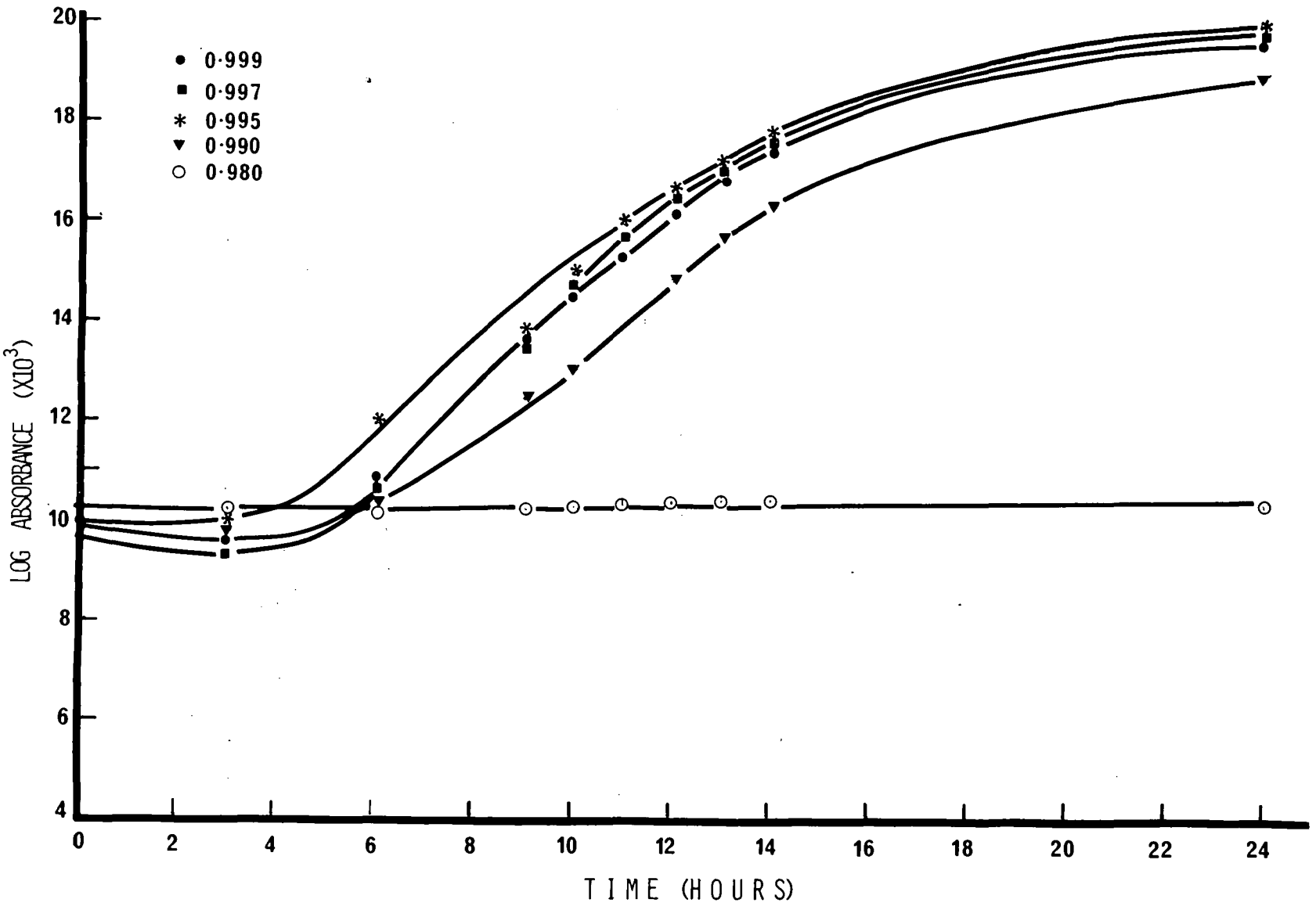

* 0·999
■ 0·997
● 0·995
▼ 0·990
○ 0·980
LOG ABSORBANCE (X10^3)
20
18
16
14
12
10
8
6
4
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (HOURS)

FIGURE 36

Growth curves of R.meliloti SU47A in YMB at a_ws of 0.999, 0.997, 0.995, 0.990 and 0.980, obtained by the use of NaCl.

Detailed results are presented in Appendix Table XXXI.

Absorbance values were multiplied by 10^3 prior to conversion to logarithms.


20
18
16
14
12
10
8
6
4
0·999
0·997
0·995
0·990
0·980
LOG ABSORBANCE (X10^3)
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (HOURS)

growing rhizobia (figure 39), however, the reasons for this are not known. Generally, the effect of a_w on the growth of the slow-growing rhizobia was to decrease the growth rate below that in unadjusted YMB. This is particularly evident for R.lupini UT12 (figure 38) and R.japonicum QA372 (figure 40).

The results from this section indicated that:-

1. if NaCl was used to adjust the a_w of YMB, the fast-growing root-nodule bacteria would not grow at any of the a_ws tested below 0.999. If other solutes, such as glucose or sucrose were used, then growth was possible at lower a_w values.
2. the slow-growing rhizobia were able to grow at a_w 0.997 and generally showed growth at a_w 0.995 (when NaCl was used as the solute to adjust the a_w of the YMB growth medium).
3. the medic rhizobia grew adequately at all a_ws down to 0.990 and often exhibited marginal growth at a_w 0.980 (NaCl used). It is not known why these rhizobia were able to grow at markedly lower a_w values than the other root-nodule bacteria, it may be related to tolerance of such factors as high osmotic pressure and/or high salt concentrations.

FIGURE 37

Growth of R. lupini UT2 in YMB of a_ws 0.999, 0.997 and 0.995, obtained by the use of NaCl.

Detailed results are given in Appendix Table XXXII.

Absorbance values were multiplied by 10 prior to conversion to logarithms.

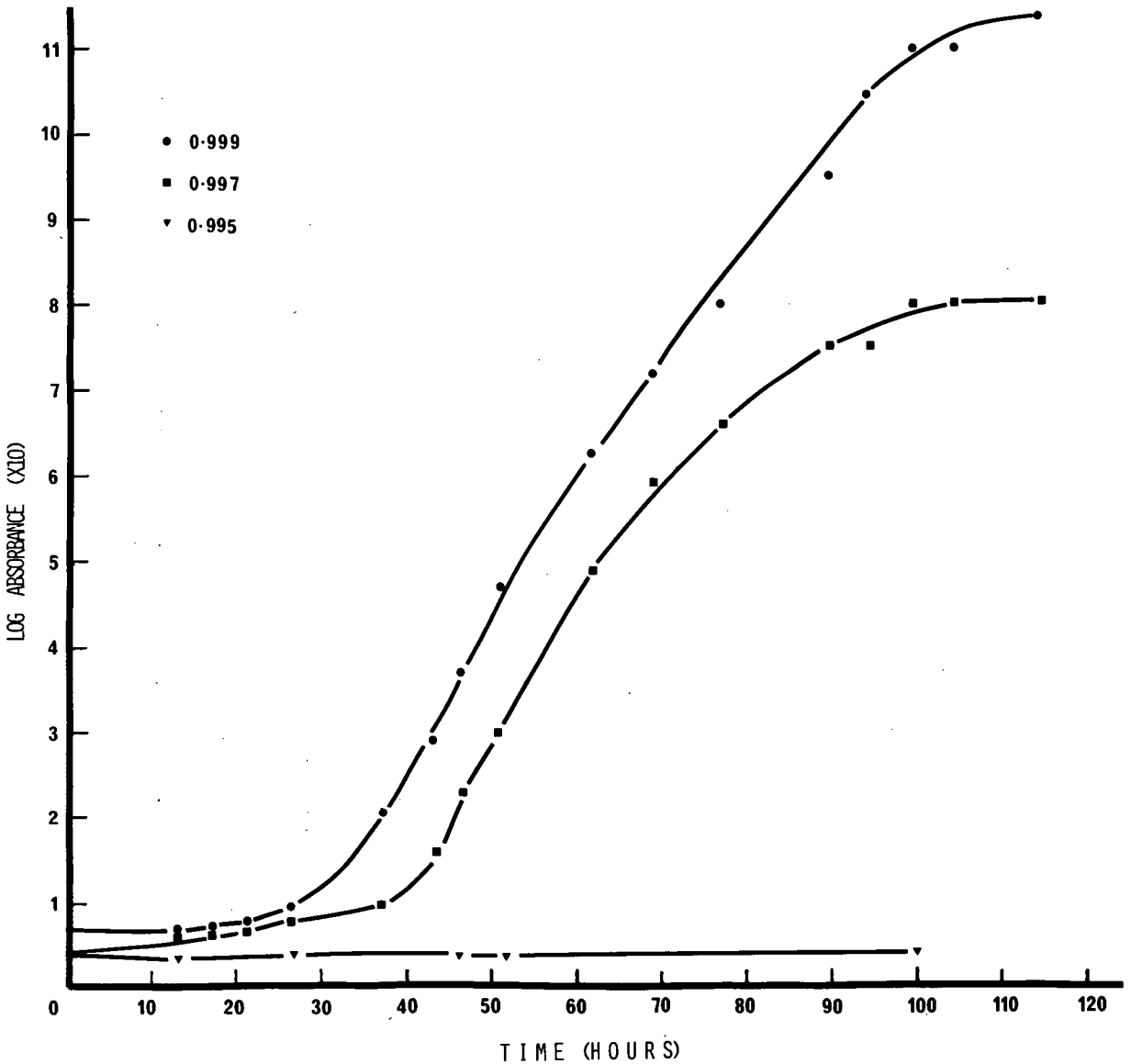

LOG ABSORBANCE (X10)
TIME (HOURS)
0·999
0·997
0·995
0
10
20
30
40
50
60
70
80
90
100
110
120
1
2
3
4
5
6
7
8
9
10
11

FIGURE 38

Growth curves of R.lupini UT12 in YMB of a_ws 0.999, 0.997, 0.995 and 0.990, obtained by the use of NaCl.

Detailed results are given in Appendix Table XXXIII.

Absorbance values were multiplied by 10^2 prior to conversion to logarithms.

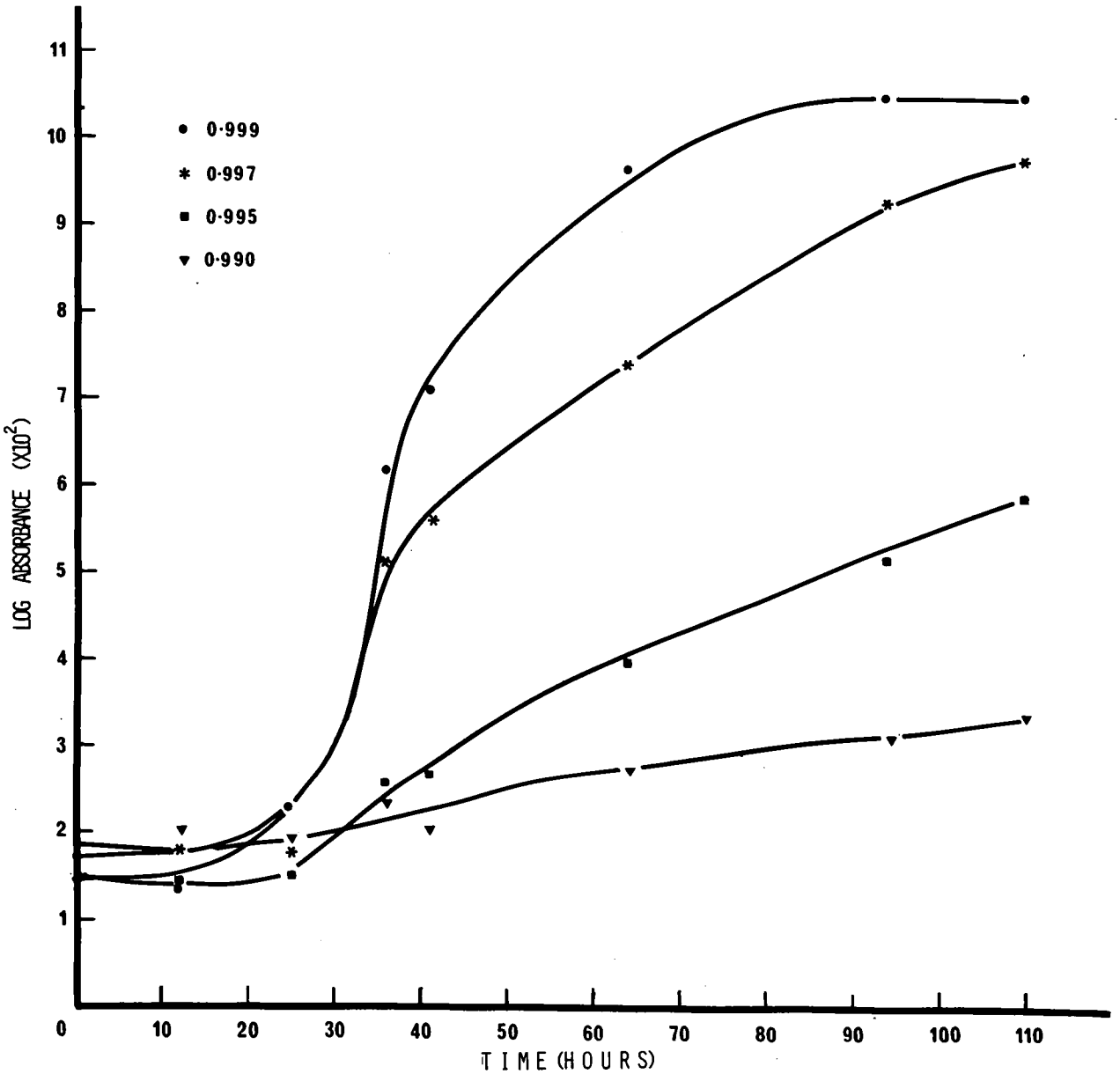

LOG ABSORBANCE (X10²)
TIME (HOURS)
0·999
0·997
0·995
0·990
0
10
20
30
40
50
60
70
80
90
100
110
1
2
3
4
5
6
7
8
9
10
11

FIGURE 39

Growth curves of R.lupini 1A425 in YMB of a_ws 0.999, 0.997 and 0.995, obtained by the use of NaCl.

Detailed results are given in Appendix Table XXXIV.

Absorbance values were multiplied by 10 prior to conversion to logarithms.

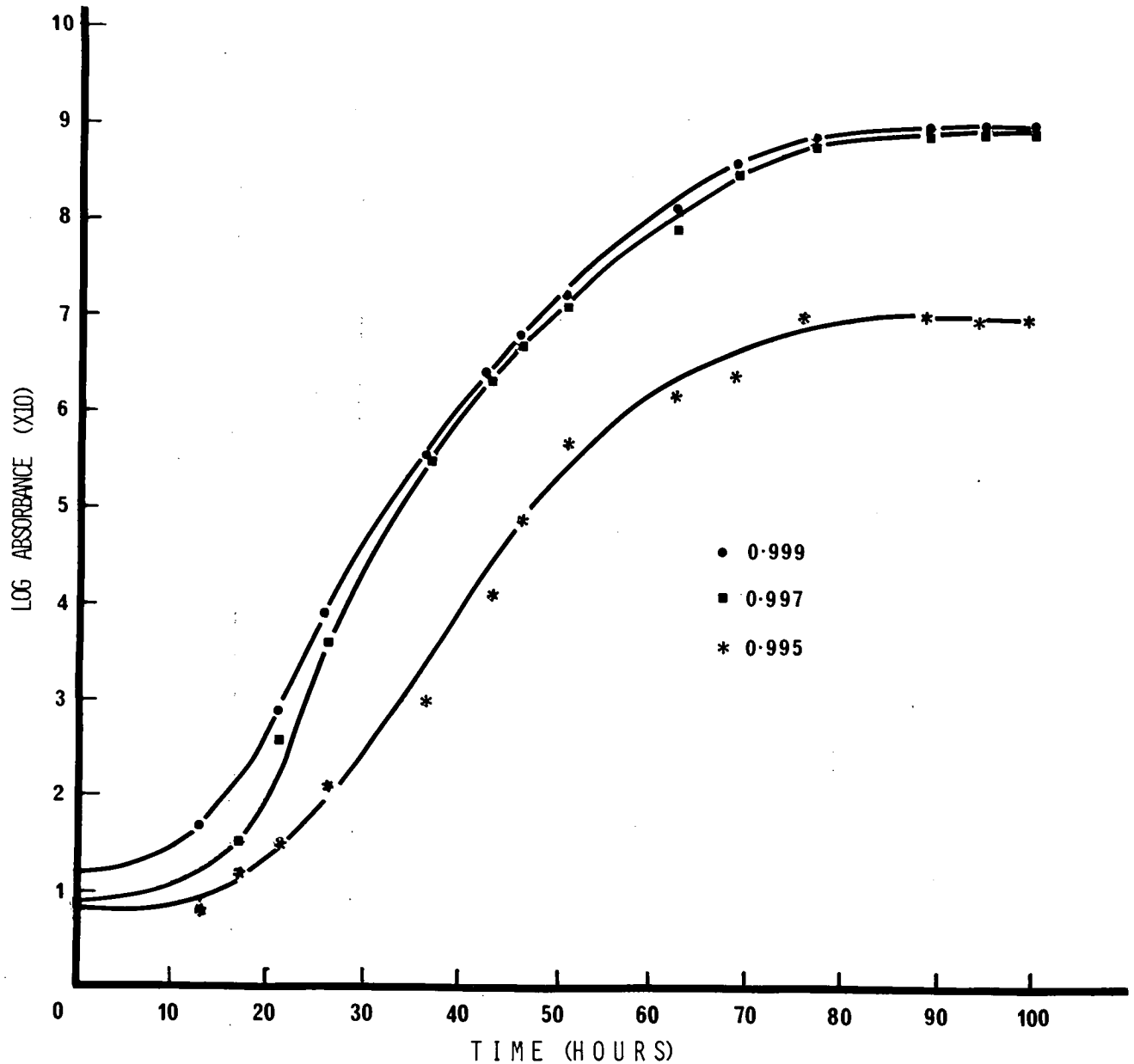
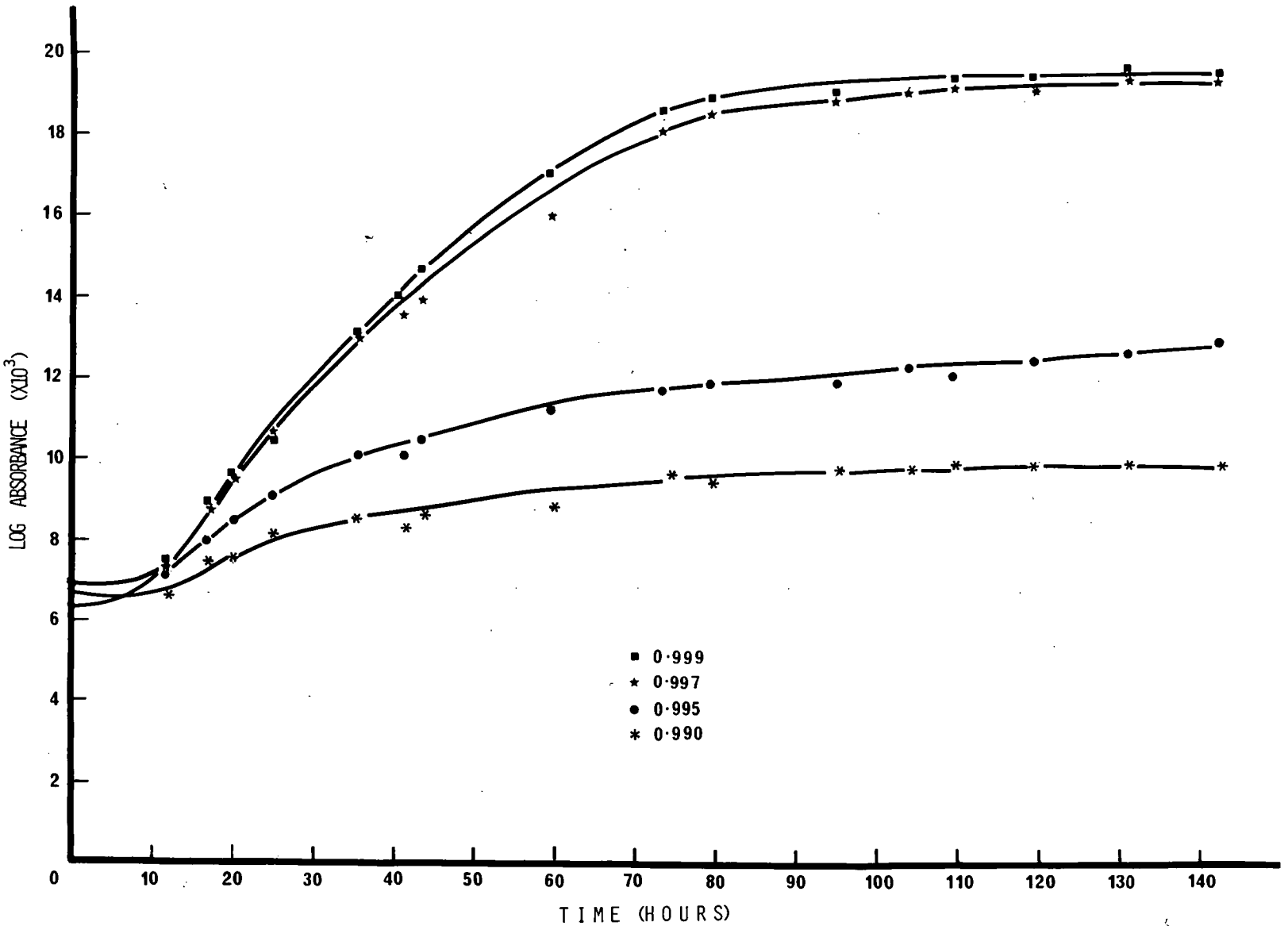

LOG ABSORBANCE (X10)
TIME (HOURS)
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
6
7
8
9
10
• 0·999
■ 0·997
* 0·995

FIGURE 40

Growth curves of R. japonicum QA372 in YMB of a_ws 0.999, 0.997, 0.995 and 0.990, obtained by the use of NaCl.

Detailed results are given in Appendix Table XXXV.

Absorbance values were multiplied by 10^3 prior to conversion to logarithms.


LOG ABSORBANCE (X10^3)
20
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
TIME (HOURS)
0·999
0·997
0·995
0·990

E(ii) Effect of the water activity of the growth medium upon the ability of rhizobia to survive desiccation.

Many attempts were made to adapt representatives of the fast-and slow-growing rhizobia, and the medic-bacteria, to growth at water activities lower than the minimum values reported for growth of each group in the previous section. It was not possible to obtain substantial growth of any rhizobia at such lower water activities. Consequently, it was not possible to determine the effect of a_w of the growth medium for strains of R.trifolii or R.leguminosarum. However, it was possible to compare the survival of desiccated R.japonicum QA372 grown on media at a_w 0.999 with the survival of bacteria grown on a_w 0.997. Similarly, comparisons were made between the effects of the a_w of the growth medium on the ability of R.meliloti to survive desiccation.

The results presented in table 12 compare the survival of desiccated R.japonicum QA372 which had been grown at a_w 0.999 with bacteria grown on media with a_w 0.997 (the a_w of the media was adjusted using NaCl). The results indicate that, after having taken into account the differences in the initial viable count, the decrease in the viable count caused by desiccation was less when R.japonicum QA372 was grown at a_w 0.997, than when grown at a_w 0.999. However, this result was not consistent because the results in table 13 suggest the opposite, i.e. bacteria grown at a_w 0.999 survived desiccation better than rhizobia grown at a_w 0.997.

Table 12

Effect of growth in media of different water activity upon the ability of R. japonicum QA372 to survive desiccation in sandy soil.

Replication Number	Log viable count per 0.05 gm soil. Water activity of initial growth medium	
	0.999	0.997
1	7.02	6.36
	7.03	6.26
	6.95	6.32
	6.99	6.40
2	7.03	6.34
	7.00	6.34
	6.95	6.34
	6.90	6.36
Means	6.98 ± 0.047	6.34 ± 0.04

Log initial viable counts were:-

(1) for growth on a_w 0.999 medium = 8.53 ± 0.04

(2) " " " a_w 0.997 " = 8.15 ± 0.04

Table 13

Effect of growth in media of different water activities upon the ability of R. japonicum QA372 to survive desiccation in sandy soil.

Replication Number	Log viable count per 0.05 gm soil. Water activity of the initial growth medium	
	0.999	0.997
	5.43	4.96
1	5.48	4.94
	5.30	4.91
	5.42	4.94
	5.45	5.02
2	5.43	5.00
	5.38	4.95
	5.20	4.96
	5.51	4.92
3	5.48	4.97
	5.56	4.83
	5.52	4.91
	5.48	4.85
4	5.51	4.84
	5.57	4.91
	5.49	4.93
Means	5.46 ± 0.0947	4.93 ± 0.061

Log initial viable count was:-

(1) for cells grown on medium with water activity of 0.999= 7.92 ± 0.079

(2) for cells grown on medium with water activity of 0.997= 7.19 ± 0.079

Similar experiments were carried out with strains of R.meliloti. The results in table 14 indicate that, after having taken into account the differences in the initial viable count, R.meliloti CC131 grown on media with a_w 0.980 survived dehydration better than when grown at a_w 0.999. However, the results in table 15 demonstrate that survival of R.meliloti SU47A grown at a_w 0.990 was the same as when grown at a_w 0.999.

The conclusions derived from the results presented in tables 12 to 15 are that the effect of the a_w of the growth medium upon the ability of rhizobia to survive dehydration is negligible. Although statistical differences were obtained, the effect of the a_w of the growth medium was not consistent from one experiment to another. Statistically significant differences may not necessarily reflect biological significance, and it is suggested that because of the very low standard deviations involved in these experiments, the statistical differences obtained did not reflect real biological effects.

E(iii) Effect of rehydration of dehydrated rhizobia in media of different water activities.

Cox (1966a;b; 1967; 1970; 1971), Cox and Baldwin (1966) and Leach and Scott (1959) have emphasised the importance of the rate and degree of rehydration, as well as the composition of the rehydration medium, as factors involved in the survival of bacteria, i.e. dehydration resulted in the death of many microorganisms, but the method of rehydration employed could result in the death of many more bacteria.

Table 14

Effect of growth in media of different water activities upon the ability of R.meliloti CC131 to survive desiccation in sandy soil.

Replication Number	Log viable count per 0.05 gm soil Water activity of the initial growth medium.	
	0.999	0.980
	4.88	4.83
	4.90	4.95
1	4.76	4.80
	4.90	4.76
	4.89	4.75
2	4.87	4.72
	4.85	4.76
	4.90	4.76
	4.85	4.76
3	4.89	4.90
	4.90	4.81
	4.86	4.72
	4.88	4.90
4	4.81	4.83
	4.92	4.81
	4.89	4.72
Means	4.87 ± 0.040	4.80 ± 0.059

Log initial viable count:-
(1) bacteria grown on a_w 0.999 medium = 7.92 ± 0.053
(2) " " " a_w 0.980 " = 7.47 ± 0.053

Table 15

Effect of growth in media of different water activities upon the ability of R.meliloti SU47A to survive desiccation in soil.

Replication Number	Log viable count per 0.05 gm soil. Water activity of the initial growth medium. 0.999	0.990
	5.30	5.15
	5.30	5.15
1	5.42	4.95
	5.34	4.95
	5.30	5.38
2	5.34	5.34
	5.26	5.28
	5.26	5.38
	5.38	5.42
3	5.38	5.36
	5.42	5.40
	5.43	5.40
	5.32	5.30
4	5.00	5.26
	4.90	5.32
	5.04	5.42
Means	5.30	5.38

Log initial viable count :-
(1) bacteria grown on a_w 0.999 medium = 8.35
(2) " " " a_w 0.990 " = 8.30

Because of the similarity of the initial viable counts and the means of the viable counts after dehydration, no analysis of variance was carried out.

Therefore, the effect of the water activity of the rehydration medium on the viability of desiccated rhizobia has been studied.

The results presented in figure 41 were obtained when air-dried R.japonicum QA372 and R.trifolii 297/32B were rehydrated in media with different water activities obtained by the use of a variety of solutes. Rehydration of R.japonicum QA372 in media adjusted to different water activities by the use of glycerol, showed no effect of a_w, however, high concentrations of salts such as NaCl and KCl were detrimental. The means of log numbers of R.japonicum QA372 surviving rehydration show a graded range of differences (Appendix Table XXXVIa) and it is difficult to determine true biological differences under such circumstances. Probably, the only real differences are between the log survivors at the extreme a_w values obtained using NaCl. Rehydration at water activities between these values did not significantly decrease the viable count of R.japonicum QA372 (figure 41).

A similar result was obtained with R.trifolii SU297/32B (figure 41), although there was a consistent statistically significant decrease (relative to rehydration in water, a_w = 1.000)of the viable count with glycerol adjusted media below a_w 0.995. This suggested that the water availability of the rehydration medium was important in determining the survival of this fast-growing root-nodule bacterium. However, this was probably minimal because there was no significant difference between survival of desiccated bacteria, rehydrated at a_w 0.995 or a_w 0.860 in glycerol adjusted media (figure 41).

FIGURE 41

Survival of air-dried R.japonicum QA372 and R.trifolii SU297/32B rehydrated in media adjusted to different water activities with different solutes.

Letters above each histogram denote the water activity i.e.

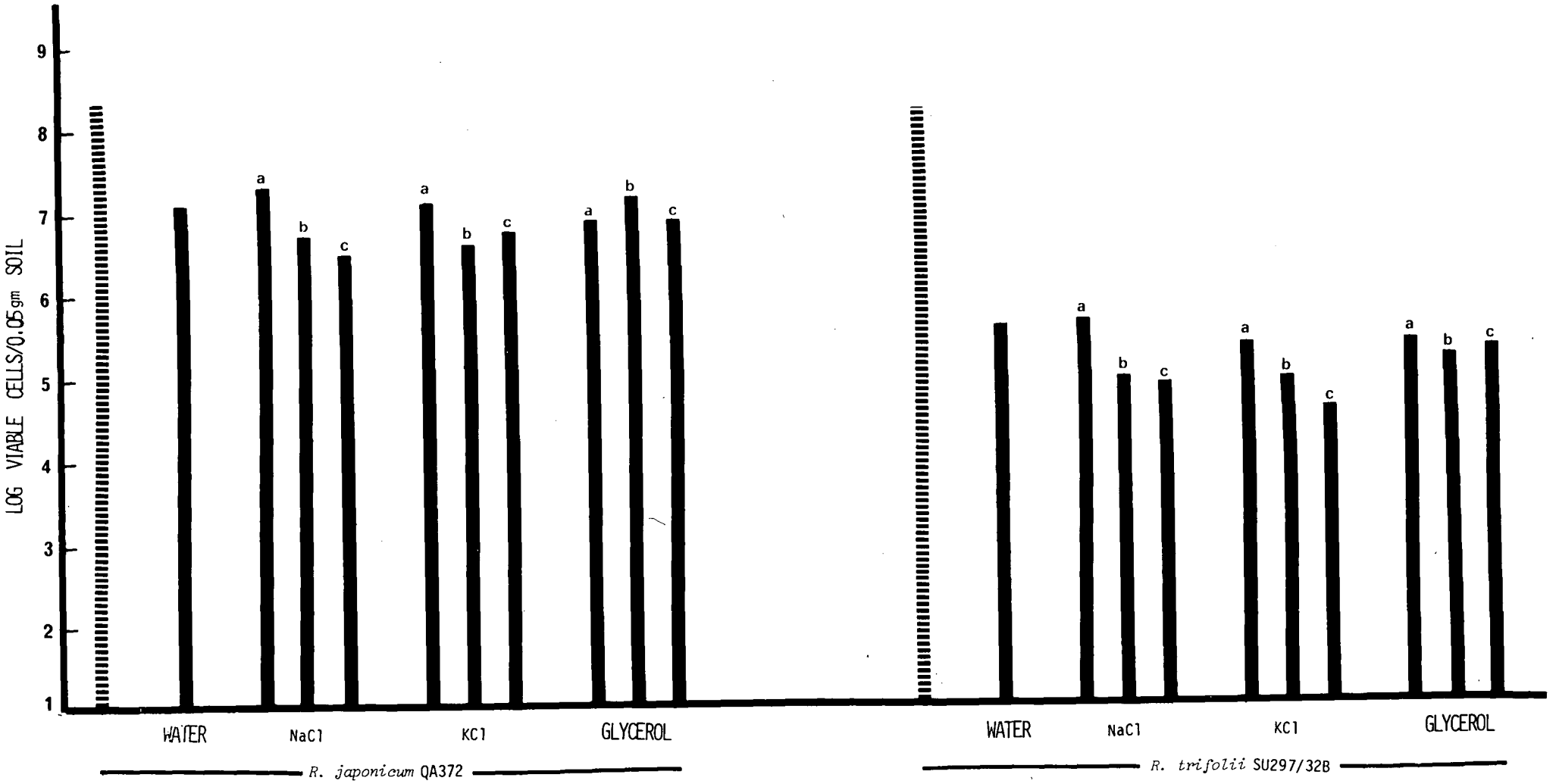
a = a_w 0.995

b = a_w 0.940

c = a_w 0.860

water = a_w 1.000

Detailed results and appropriate analysis of variance are shown in Appendix, tables XXXVIa and XXXVIb.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
a
b
c
WATER
NaCl
KCl
GLYCEROL
R. japonicum QA372
R. trifolii SU297/32B

Rehydration of R.trifolii SU297/32B in media of a_w 0.995 when either NaCl or KCl were used to adjust the medium did not have a significant effect upon survival. However, rehydration in media with a_w values of 0.940 and 0.860, obtained by the use of high concentrations of NaCl or KCl, were detrimental to this fast-growing rhizobium (figure 41). Therefore, as with R.japonicum QA372, the solute used to adjust the a_w of the rehydration medium was an important factor involved in the survival of desiccated fast-growing rhizobia.

The results presented in figures 42 and 43 were obtained by rehydration of freeze-dried bacteria in media of different a_w values obtained by the use of a variety of solutes. The effect of rehydration of R.trifolii SU297/31A at a_ws 0.995 and 0.940 was not significantly different from rehydration in water when the solutes used were either sucrose or glycerol (figure 42). Similarly, rehydration in NaCl adjusted media at a_w 0.995 did not affect the viable count. However, at a_w 0.860 in sucrose and glycerol adjusted media, there was a significant decrease, and media adjusted with NaCl to a_ws 0.940 and 0.860 were detrimental to the survival of rehydrated R.trifolii SU297/31A (figure 42). The results in figure 42 for this fast-growing rhizobium demonstrated that it was susceptible to the toxic effects of high NaCl concentrations at a_w values of 0.940 and 0.860. This was demonstrated by the significantly lower viable count obtained when the bacteria were rehydrated in these salt adjusted media, compared to the viable count when rehydrated in

FIGURE 42

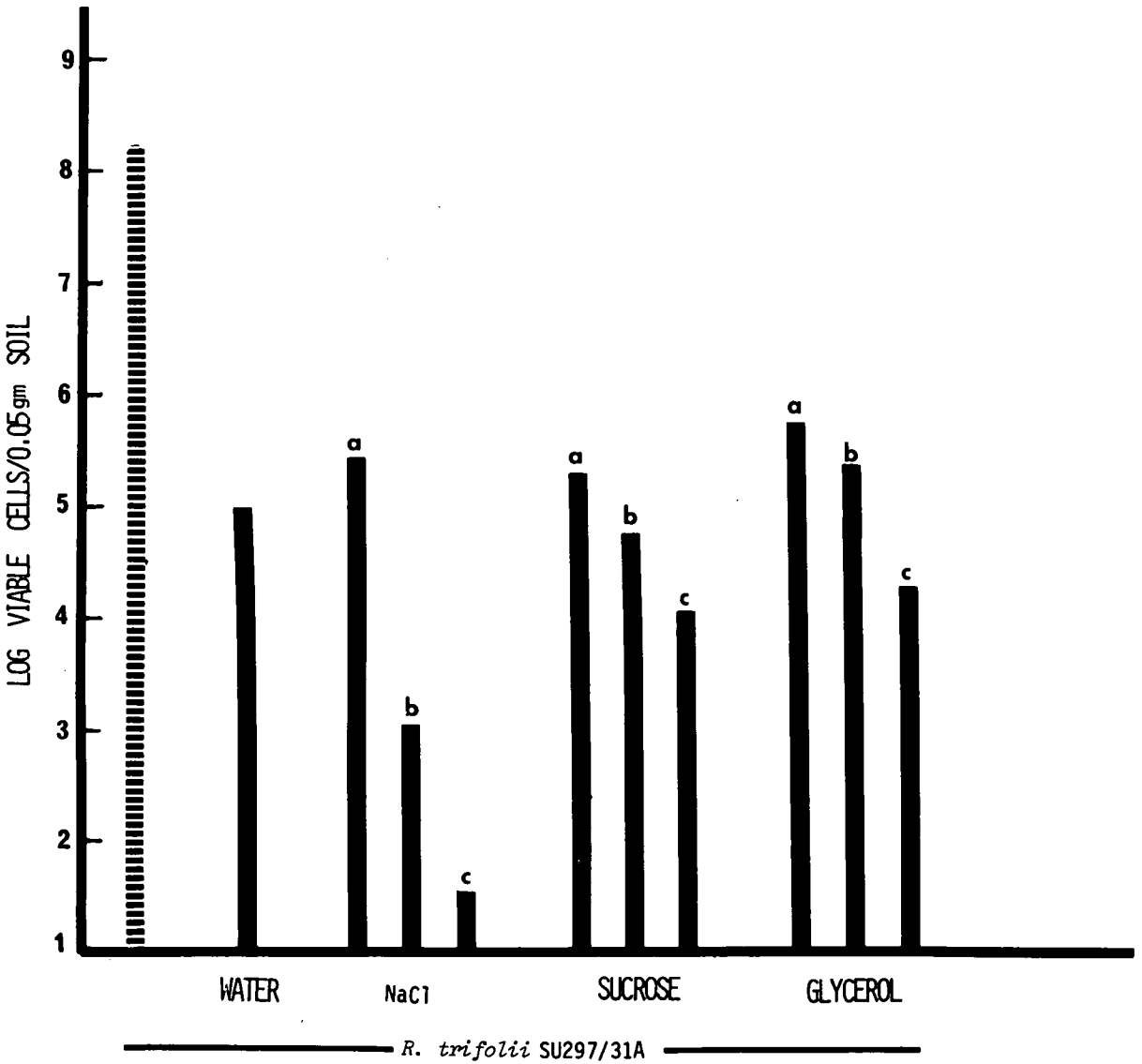
Survival of freeze-dried R.trifolii SU297/31A rehydrated in media adjusted to different water activities with different solutes. Letters above each histogram denotes the water activity i.e.

a = a_w 0.995

b = a_w 0.940

c = a_w 0.860

Detailed results and analysis of variance are presented in Appendix Table XXXVII.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
a
b
c
a
b
c
a
b
c
WATER
NaCl
SUCROSE
GLYCEROL
R. trifolii SU297/31A

media with corresponding a_ws obtained by the use of sucrose or glycerol.

The results presented in figure 43 illustrate the effects of the a_w of the rehydration medium on freeze-dried R.trifolii SU297.31A, R.lupini UT12 and Rhizobium sp. CB736. Rehydration of Rhizobium sp. CB736 in media with a_w 0.940 had no effect (relative to the control), regardless of the solute used to obtain this a_w value. Statistically significant increases were obtained for a_w 0.995 (glycerol, sucrose and NaCl used as solutes). However, because of the low error variance in these experiments (Appendix table XXXVIIIc), the statistical significance probably did not reflect biologically significant differences. That this was the case, is suggested by the similarity of the histograms for Rhizobium sp. CB736 at a_w 0.995 (figure 43). Therefore, it is concluded that except for the toxic effects of high NaCl concentrations in media with a_w 0.860, the water availability of the rehydration medium had no effect upon survival of the dried, pigmented Rhizobium sp.CB736.

The slow-growing R.lupini UT12 was more susceptible to the a_w of the rehydration medium than Rhizobium sp. CB736, as rehydration in media with a_w 0.940 or below, decreased survival relative to survival at a_w 1.000 (figure 43). The toxic effect of NaCl on R.lupini UT12 at a_w 0.860 was again obvious. Rehydration at a_w 0.995 had no effect upon survival of these slow-growing bacteria no matter what the solute used.

A qualitatively similar result to that reported for R.lupini UT12, was obtained for R.trifolii SU297/31A, as

FIGURE 43

Survival of freeze dried R.trifolii SU297/31A, R.lupini UT12 and Rhizobium species CB736 rehydrated in media adjusted to different water activities with different solutes.

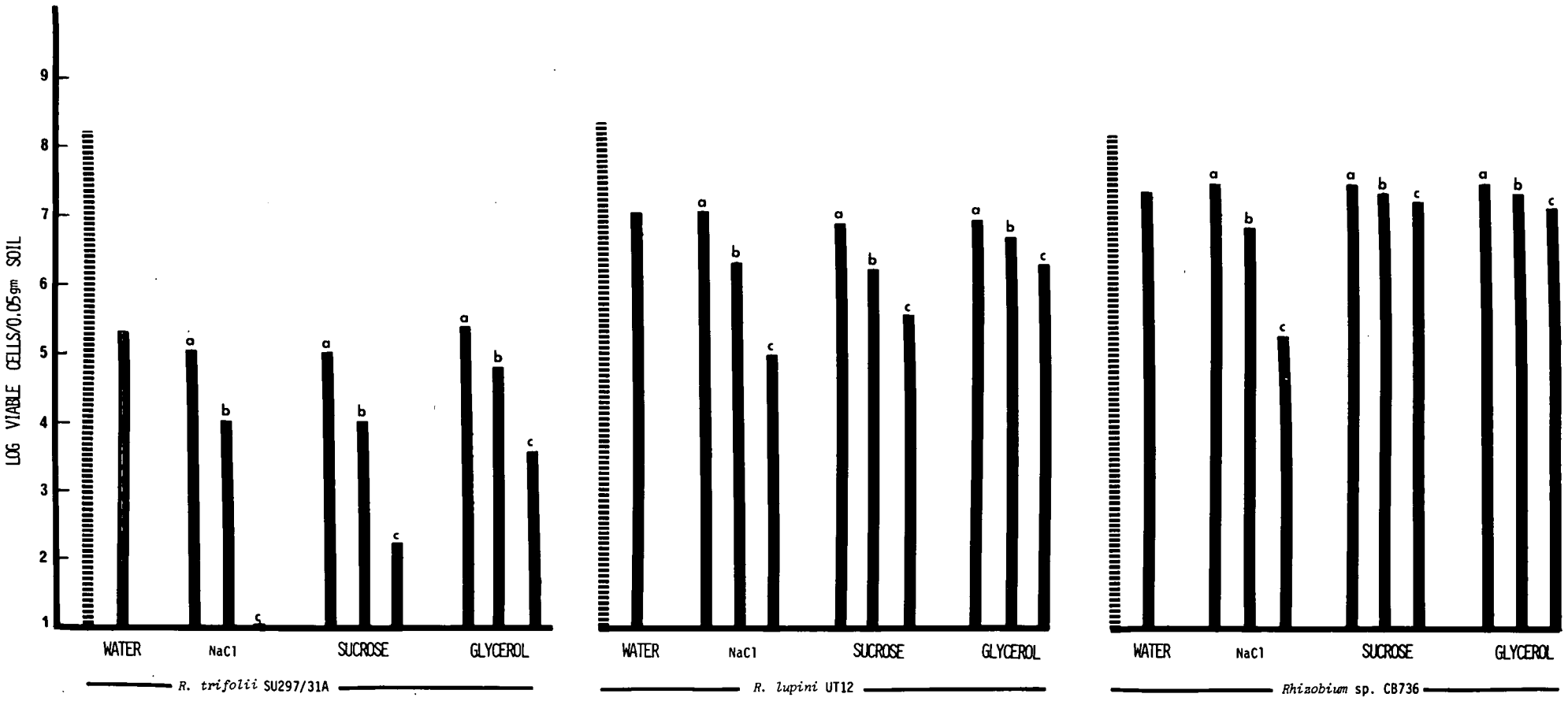
Letters above each histogram denote the water activity ie.

a = a_w 0.995

b = a_w 0.940

c = a_w 0.860

Detailed results and analysis of variance are in the Appendix tables XXXVIIIa, b and c.


LOG VIABLE CELLS/0.05gm SOIL
9
8
7
6
5
4
3
2
1
a
b
c
WATER
NaCl
SUCROSE
GLYCEROL
R. trifolii SU297/31A
R. lupini UT12
Rhizobium sp. CB736

rehydration of the desiccated bacteria in media with a_w values below 0.995 decreased survival (figure 43). The effect of salt toxicity on R.trifolii SU297/31A at a_w 0.860 in NaCl adjusted media was very obvious as there was virtually no survival. There was no significant effect of rehydration in a_w 0.995 regardless of the solute used.

From all the results in figures 41 to 43, it is apparent that at all a_ws, no matter which solute was used, the slow-growing rhizobia survived rehydration better than the fast-growing bacteria. Generally, rehydration at water tensions below 1,000 and certainly below 0.995, were detrimental to survival of strains of R.trifolii, especially when NaCl was the solute used to adjust the a_w of the medium. This has been attributed to the toxicity of high salt concentrations. Frequently, when dried fast-and slow-growing rhizobia were rehydrated in media with a_w 0.860 using sucrose, the decrease in the viable count was greater than when glycerol was employed. From the effects of NaCl, KCl, sucrose and glycerol, therefore, it can be said that the solute used to adjust the a_w of the rehydration medium was an important factor determining the survival of dried rhizobia rehydrated in media with different water availabilities.

F. Effect of desiccation on the surface characteristics of rhizobia.

F(i) Fluorescence of fast-and slow-growing rhizobia with 1-anilino-8-naphthalene sulphonate.

Radda and Vanderkooi (1972) have stated that the probe 1-anilino-naphthalene sulphonate (ANS) was bound at polar/non-polar sites in membranes. These sites may include lipid-protein, lipid-water or lipid-protein-water contacts. More specifically, Radda and Vanderkooi (1972) suggested that ANS was bound at polar/non-polar regions within membranes that contained charged groups as well as water. Therefore, the amount of ANS bound to biological material depends on the charge distribution of the adsorbing surface. Radda and Vanderkooi (1972) concluded that ANS was most useful for making structural comparisons rather than for obtaining absolute morphological data. The probe was used in the present investigations to give an indication of relative, rather than absolute, differences of the surface features of the fast-and slow-growing rhizobia and the medic rhizobia.

Initial microscopic examination of the fluorescence of dried and undried bacteria suspended in an ANS solution gave the data in table 16. None of the undried slow-growing or the medic rhizobia fluoresced. Following desiccation, however, all bacteria tested fluoresced when rehydrated in a solution of ANS. In contrast, all the fast-growing rhizobia

Table 16

Effect of desiccation on the fluorescence under the microscope of various bacteria when mixed with ANS.

Bacterial Group	Bacterium	Not Dried	Dried
Slow-growing rhizobia	*Rhizobium* sp. CB736	-	x
	Rhizobium sp. 3C1f1	-	x
	Rhizobium sp. 3I1b	-	x
	Rhizobium sp. UT35	-	x
	Rhizobium sp. CB756	-	x
	Rhizobium sp. UT53	-	x
	R.lupini 1A8	-	x
	R.lupini UT12	-	x
	R.lupini UT2	-	x
	R.japonicum QA372	-	x
Medic rhizobia	*R.meliloti* SU45	-	x
	R.meliloti SU47A	-	x
Fast-growing rhizobia	*R.trifolii* UT48	x	x
	R.trifolii SU297/31A	x	x
	SU297/32B	x	x
	SU298/533A	x	x
	SU298/534C	x	x
	SU298/536D	x	x
	TA1	x	x
	R.leguminosarum TA101	x	x
	Escherichia coli M13	x	x

x designates fluorescence under the microscope
- designates no fluorescence under the microscope

(as well as Escherichia coli M13, table 16) fluoresced both before and after desiccation.

The results presented in tables 17 and 18 confirmed the observations that undried medic or slow-growing rhizobia did not fluoresce when reacted with ANS. The intensity of fluorescence of undried R.trifolii and R.leguminosarum was 4 to 5 times greater than either the medic or the slow-growing rhizobia. It is noteworthy that the undried, fast-growing Rhizobium sp. SU343 isolated from Lotus corniculatus L. fluoresced as intensely as undried strains of R.trifolii and R.leguminosarum.

Several experiments were carried out to test the change of fluorescence of rhizobia after desiccation. The results presented in table 19 show that at any bacterial concentration the intensity of fluorescence of both dried and undried R.trifolii SU297/32B was similar. The results in tables 20 and 21 also demonstrated the similarity of fluorescence of both dried and undried clover bacteria. The fluorescence of desiccated slow-growing or medic rhizobia, however, was very different from the intensity of fluorescence of the undried bacteria (tables 20 and 21). Although the fluorescence of undried R.meliloti, R.japonicum and R.lupini was marginal, it increased dramatically after dehydration and was similar to that of the untreated fast-growing rhizobia (tables 20 and 21). This finding demonstrated that removal of intracellular water drastically altered the surface properties of the slow-growing and medic rhizobia. Similar changes to the surface of fast-

Table 17

Relative fluorescence in the fluorometer of variance species of rhizobia when mixed with ANS.

Bacterial Group	Bacterium	Relative Fluorescence (%)
Slow-growing rhizobia	*Rhizobium* sp. 3C1f1	8.0
	Rhizobium sp 3I1b	5.0
	Rhizobium sp. CB756	5.0
	Rhizobium sp. CB421	0.0
	Rhizobium sp. UT35	0.0
	Rhizobium sp. UT53	5.0
	Rhizobium sp. CB736	0.0
	R. japonicum QA372	0.0
Medic rhizobia	*R. meliloti* CB112	0.0
	" " CC131	0.0
	" " SU47A	0.0
	" " SU47B	0.0
Fast-growing rhizobia	*R. trifolii* SU297/31A	25.0
	" " SU297/32B	30.0
	" " UT6	25.0
	R. leguminosarum TA101	20.0

The total count for all bacteria was adjusted to 5.10^9 bacteria/ml. Volume of bacterial suspension used was 0.5 mls. Volume of buffer used was 1.5 mls. Volume of ANS used was 0.2 mls.

Table 18

Relative fluorescence in fluorometer of various species of rhizobia in the presence of ANS.

Bacterial Group	Bacterium	Volume bacterial suspension (mls)	Volume of buffer (mls.)	Relative Fluorescence (%)
Slow-growing rhizobia	*Rhizobium japonicum* QA372	1.0	1.0	6.0
		2.0	0.0	12.0
	R.lupini UT12	1.0	1.0	8.0
		2.0	0.0	16.0
Fast-growing rhizobia	*R.trifolii* SU297/32B	1.0	1.0	34.0
		2.0	0.0	62.0
	R.trifolii SU297/31A	1.0	1.0	39.0
		2.0	0.0	70.0
	R.trifolii TA1	2.0	0.0	50.0
	Rhizobium sp.SU343	1.0	1.0	33.0
		2.0	0.0	66.0
	R.leguminosarum TA101	2.0	0.0	56.0

Total count for all cells was adjusted to 2 to 3 x 10^9 bacteria/ml.
Volume of ANS stock solution used was 0.2 ml. in all cases.

Table 19

Relative fluorescence in fluorometer of various concentrations of dried and undried R.trifolii SU297/32B at several concentrations of ANS.

Log total Bacterial Concentration per ml.	Treatment	Relative Fluorescence (%) Volume ANS stock solution (ml) 0.0	0.1	0.2	0.3	0.4	0.5	0.6
8.48	Not dried	2.1	>100	>100	>100	>100	>100	>100
	Dried	2.1	>100	>100	>100	>100	>100	>100
7.48	Not dried	0.0	20	40	42	40	40	40
	Dried	0.0	20	40	42	41	40	42
6.48	Not dried	0.0	1.0	22	31	32	33	29
	Dried	0.0	1.0	20	31	31	30	30
5.48	Not dried	0.0	0.0	16	20	28	27	26
	Dried	0.0	0.0	16	21	28	27	26

Table 20

Effect of desiccation on the relative fluorescence in fluorometer with ANS of various species of rhizobia.

Bacterium	Treatment	Relative Fluorescence (%)
	Not dried	7.0
	Not dried	7.0
R.japonicum QA372		
	Dried	32.0
	Dried	35.0
	Not dried	11.0
	Not dried	12.0
R.lupini UT12		
	Dried	23.0
	Dried	21.0
	Not dried	52.0
	Not dried	50.0
R.trifolii SU297/32B		
	Dried	49.0
	Dried	51.0

(a) Total count for all bacteria were adjusted to 2.10^9 cells/ml.

(b) Volume of bacterial suspension = 0.5 ml.
Volume of phosphate buffer = 1.5 ml.
Volume of ANS = 0.2 ml.

Table 21

The effect of desiccation on the relative fluorescence in fluorometer of various rhizobia when mixed with ANS.

Bacterium	Treatment	Volume of bacterial suspension (mls)	Volume of buffer (mls)	Relative Fluorescence (%)
R.japonicum QA372	Not dried	1.0	1.0	13.0
	Not dried	2.0	0.0	26.0
	Dried	1.0	1.0	60.0
R.lupini UT12	Not dried	1.0	1.0	7.0
	Not dried	0.25	1.75	<0
	Dried	1.0	1.0	55.0
R.meliloti SU47B	Not dried	1.0	1.0	19.0
	Not dried	1.0	1.0	19.0
	Dried	1.0	1.0	41.0
R.trifolii SU297/31A	Not dried	1.0	1.0	>100
	Not dried	0.25	1.75	23.0
	Not dried	0.1	1.9	12.0
	Dried	1.0	1.0	>100
	Dried	0.5	1.5	>100
	Dried	0.25	1.75	30.0
R.trifolii SU297/32B	Not dried	1.0	1.0	82.0
	Not dried	0.25	1.75	26.0
	Not dried	0.1	1.9	11.0
	Dried	1.0	1.0	98.0

Total count for all bacteria was adjusted to 7.10^9 bacteria/ml.

Volume of ANS stock solution used in all cases was 0.2 ml.

growing rhizobia probably occurred, however the high fluorescence intensity of these undried bacteria, masked any changes that might have been induced by desiccation.

Occasionally, the fluorescence of the medium (water) used to rehydrate desiccated rhizobia, was measured after the removal of bacteria by centrifugation. Invariably, the fluorescence was high (50 to 60%) relative to the fluorescence of water. The results have not been reported because very few experiments were carried out. However, the increased fluorescence of the rehydrating medium after the removal of bacteria suggests that, desiccation induced leakage of internal contents of bacteria.

F(ii) Estimation of desiccation-induced repairable damage to Escherichia coli and various species of Rhizobium.

The purpose of this investigation was to determine whether there was a difference in the stability of the cell envelope of either the fast-or slow-growing root-nodule bacteria to dehydration. Damage to the cell envelope (or lipopolysaccharide layer of the cell wall) caused by drying was estimated by induced susceptibility to agents such as lysozyme, to which the undried bacteria were resistant. The extent to which this induced susceptibility was removed has been attributed to the action of active repair mechanisms.

Repairable damage has been reported for aerosolized, frozen and freeze-dried Escherichia coli. Consequently, this

organism was used as a check that the techniques employed were capable of detecting such damage to the bacteria.

The results in figures 44 to 46 demonstrate that, immediately after rehydration, E.coli M13 was very sensitive to lysozyme. However, within 0.5 to 0.75 hr. most of this lysozyme-sensitive damage had been repaired. As shown in figure 45, rehydrated bacteria were not equally susceptible to all selective agents. Thus, the effect of deoxyribonuclease on the viable count was much less than the effect of lysozyme. The results in figures 44 to 46 indicate that, at room temperature all repair was completed within 0.75 hr. When rehydrated at low temperature ($10^{o}C$) however, repair took much longer to reach completion (figure 47). The results in figure 47 show that when rehydrated at $10^{o}C$ it took 2.0 hrs. to complete the repair of dehydration-induced damage of E.coli M13. This was the case whether the selective agent was lysozyme or actinomycin D, even though their effects on the viable count immediately after rehydration were different (figure 47). The results presented in figure 47 suggested that physiological activity was required for rapid repair.

The medium used to rehydrate E.coli M13 was that suggested by Hambleton (1971). Because the medium used to rehydrate rhizobia was yeast mannitol broth two experiments were carried out which E.coli M13 was rehydrated in YMB. The purpose of this was to ensure that lysozyme was not inactivated by the yeast mannitol broth. The results presented in figures 48 and 49 indicate that E.coli M13 was sensitive to lysozyme when

FIGURE 44

Desiccation-induced susceptibility to lysozyme and repair of damage by *Escherichia coli* M13.

Detailed results and analysis of variance is given in Appendix Table XXXIX.

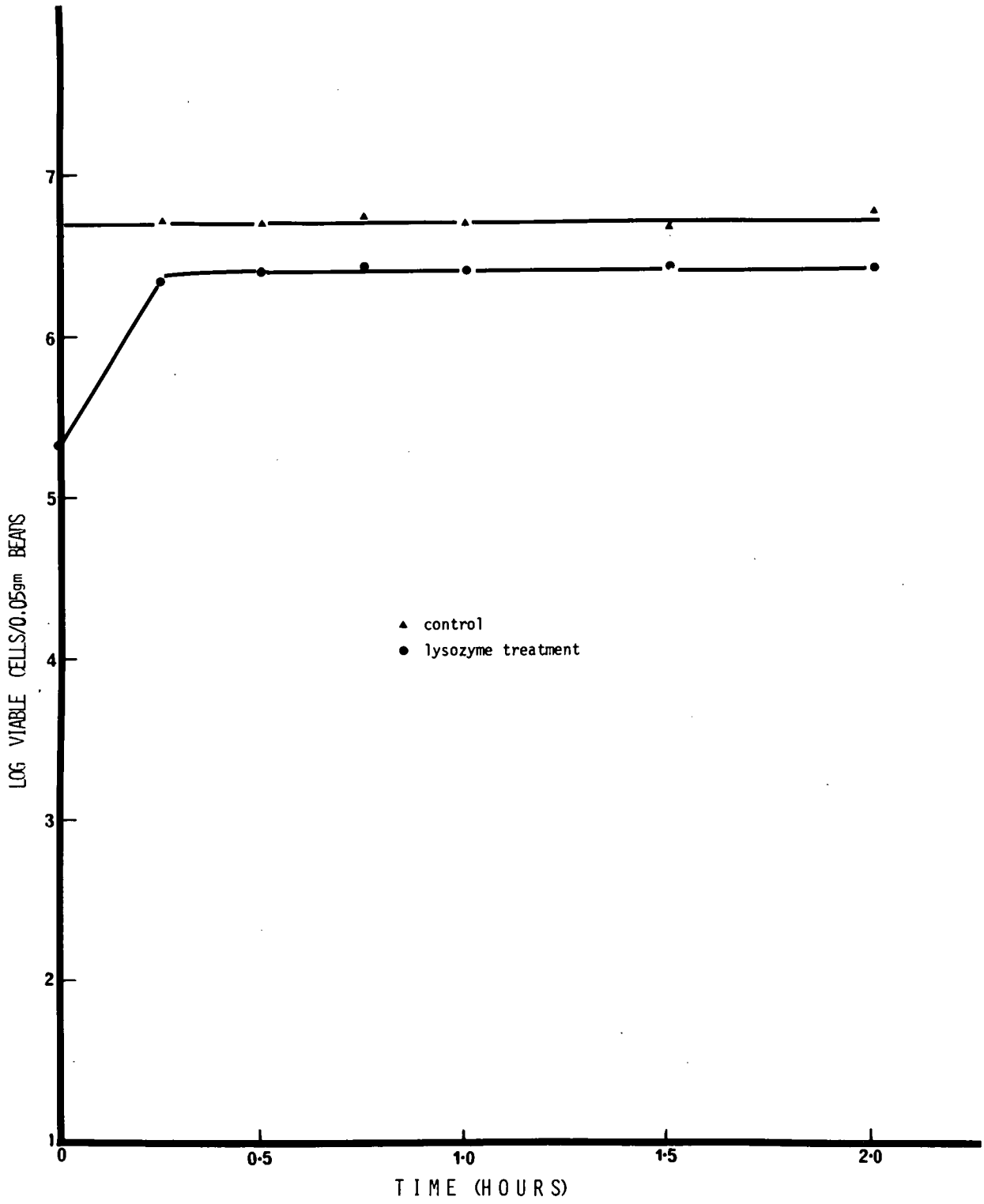

LOG VIABLE CELLS/0.05gm BEANS
7
6
5
4
3
2
1
0
0·5
1·0
1·5
2·0
TIME (HOURS)
▲ control
● lysozyme treatment

FIGURE 45

Desiccation-induced susceptibility to lysozyme and deoxyribonclease, and repair of the damage by Escherichia coli M13.

Detailed results and analysis of variance, Appendix Table XL.

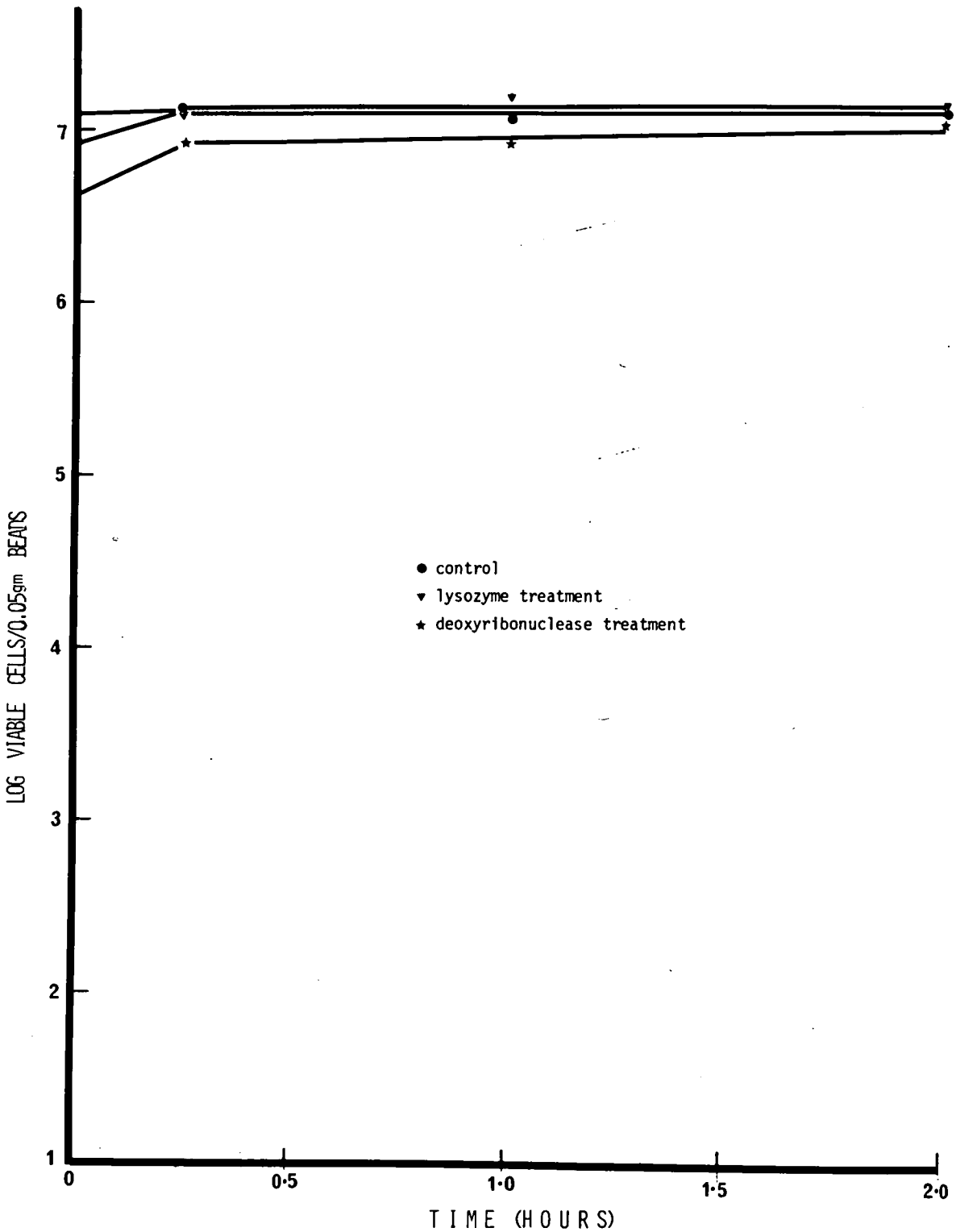
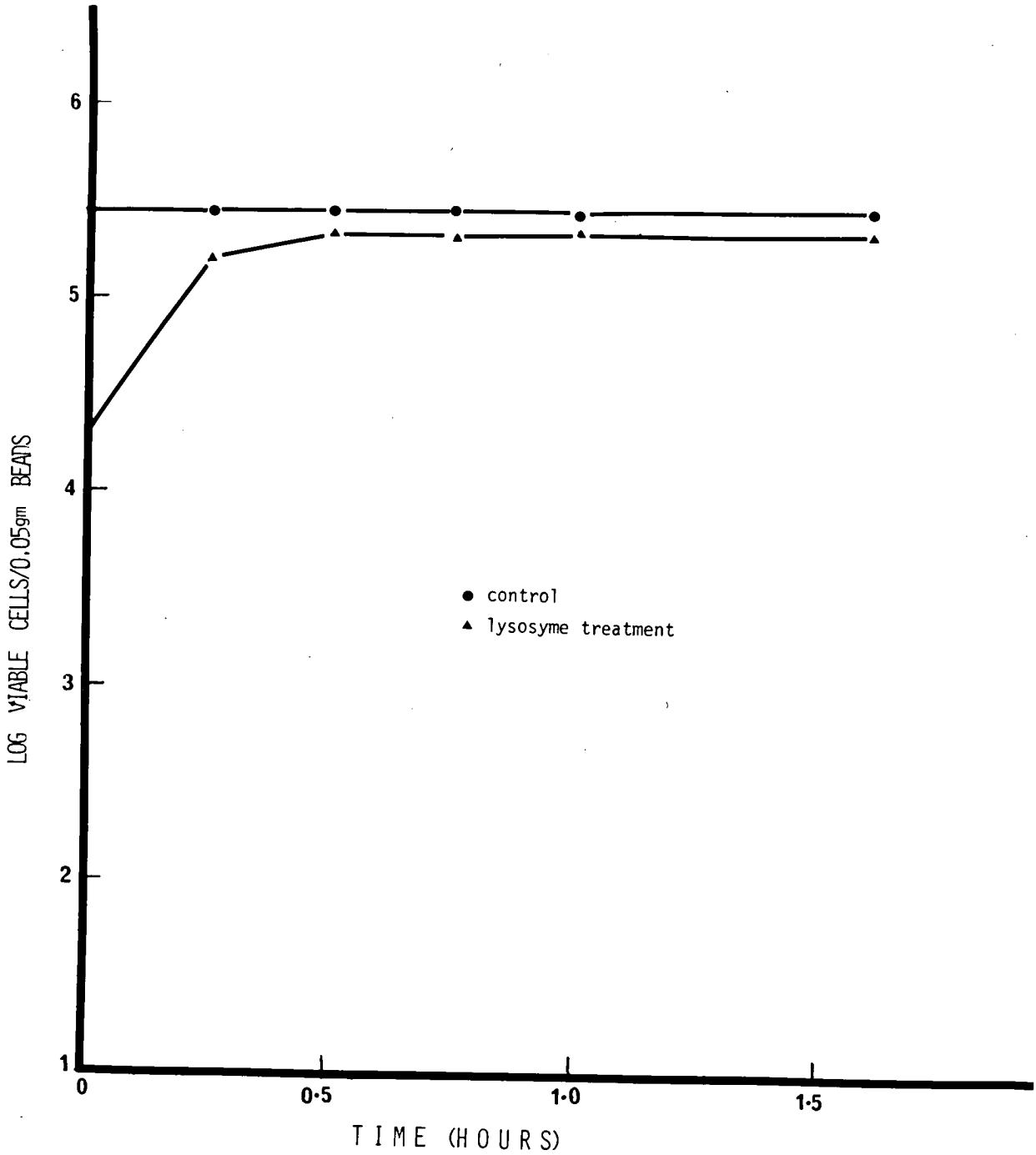

LOG VIABLE CELLS/0.05gm BEADS
7
6
5
4
3
2
1
0
0·5
1·0
1·5
2·0
TIME (HOURS)
● control
▼ lysozyme treatment
★ deoxyribonuclease treatment

FIGURE 46

Desiccation-induced susceptibility to lysozyme and repair of the damage by Escherichia coli M13.

Detailed results and analysis of variance are presented in Appendix Table XLI.

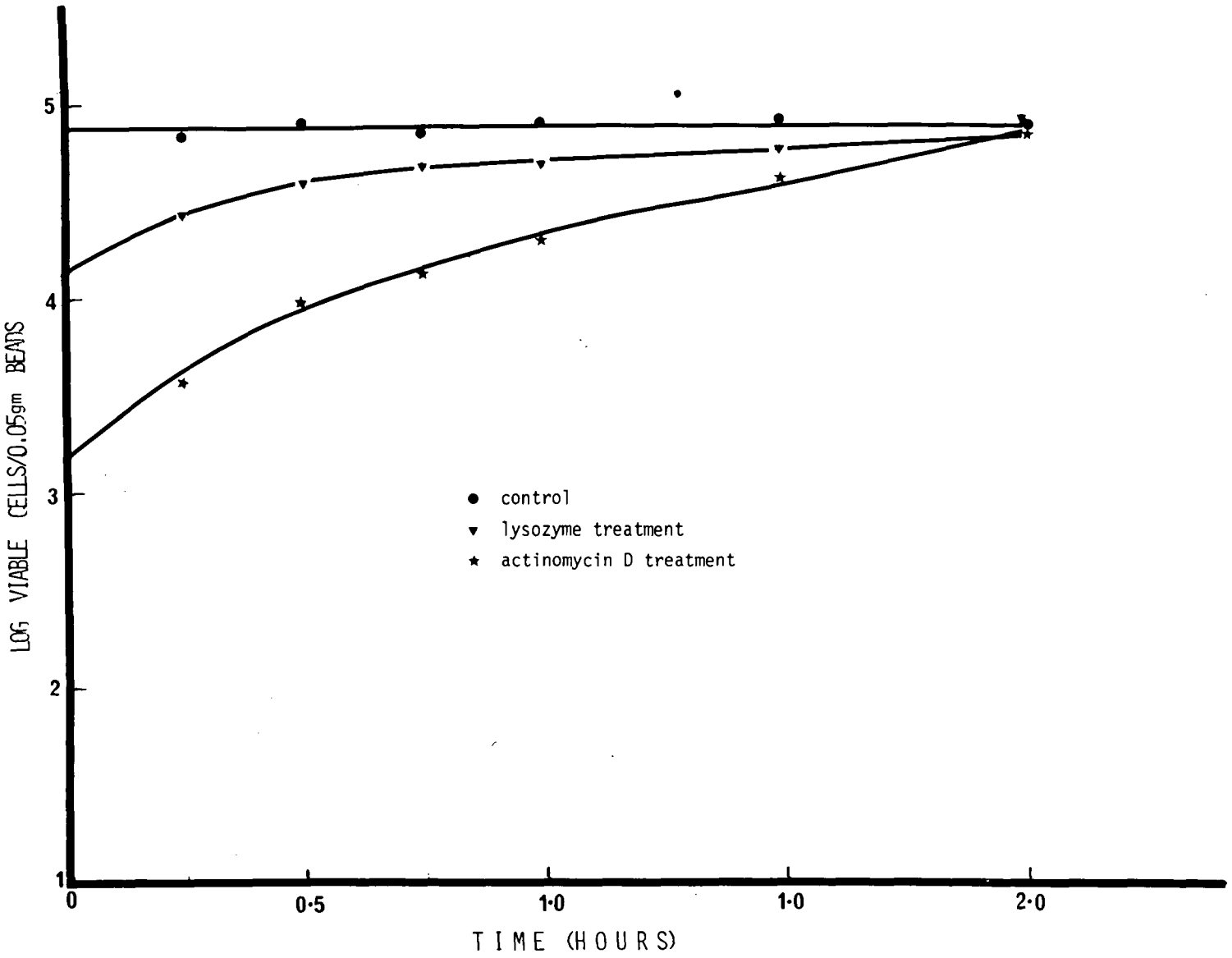

6
5
4
3
2
1
LOG VIABLE CELLS/0.05gm BEATS
control
lysosyme treatment
0
0·5
1·0
1·5
TIME (HOURS)

7413/28

FIGURE 47

Effect of low temperature (10°C) on the repair of desiccation-induced susceptibility of Escherichia coli M13 to lysozyme and actinomycin D.

Detailed results are presented in Appendix Table XLII.


LOG VIABLE CELLS/0.05gm BEADS
5
4
3
2
1
0
0·5
1·0
1·0
2·0
TIME (HOURS)
control
lysozyme treatment
actinomycin D treatment

rehydrated in YMB. Therefore, the activity of the lysozyme was not affected by yeast mannitol broth. The results in figures 48 and 49 indicate that the time taken to complete the repair of damaged E.coli M13 was 1.0 to 2.0 hours. This is longer than the time taken when desiccated bacteria were rehydrated in Hambleton's (1971) medium.

The conclusion from the experiments presented in figures 44 to 49 is that desiccation-induced repairable susceptibility of E.coli M13 to lysozyme (and other selective agents) probably occurs by alterations of the cell envelope. The techniques used were able to detect the damage and follow the time course of its repair.

The results presented in figures 50, and 51 for Rhizobium leguminosarum TA101, rehydrated in YMB, suggest that the effect of dehydration on bacterial susceptibility to lysozyme was small. Immediately after rehydration, some bacteria were susceptible to lysozyme ($P < 0.05$). However, this was overcome within 1.0 to 2.0 hours by repair mechanisms. When R.leguminosarum TA101 was rehydrated in the medium suggested by Hambleton (1971) for Escherichia coli, the results were essentially the same (figure 52), i.e. typically low desiccation-induced susceptibility to lysozyme occurred.

When desiccated R.trifolii SU297/32B was rehydrated in YMB and exposed to lysozyme, the bacteria were unaffected by the enzyme (tables 22 and 23). It was concluded, therefore, that desiccation of R.leguminosarum or R.trifolii did not

FIGURE 48

Repair of dehydration-induced susceptibility to lysozyme by Escherichia coli M13 when rehydrated in yeast mannitol broth instead of Hamblerton's (1971) medium.

Detailed results and analysis of variance are presented in Appendix Table XLIII.

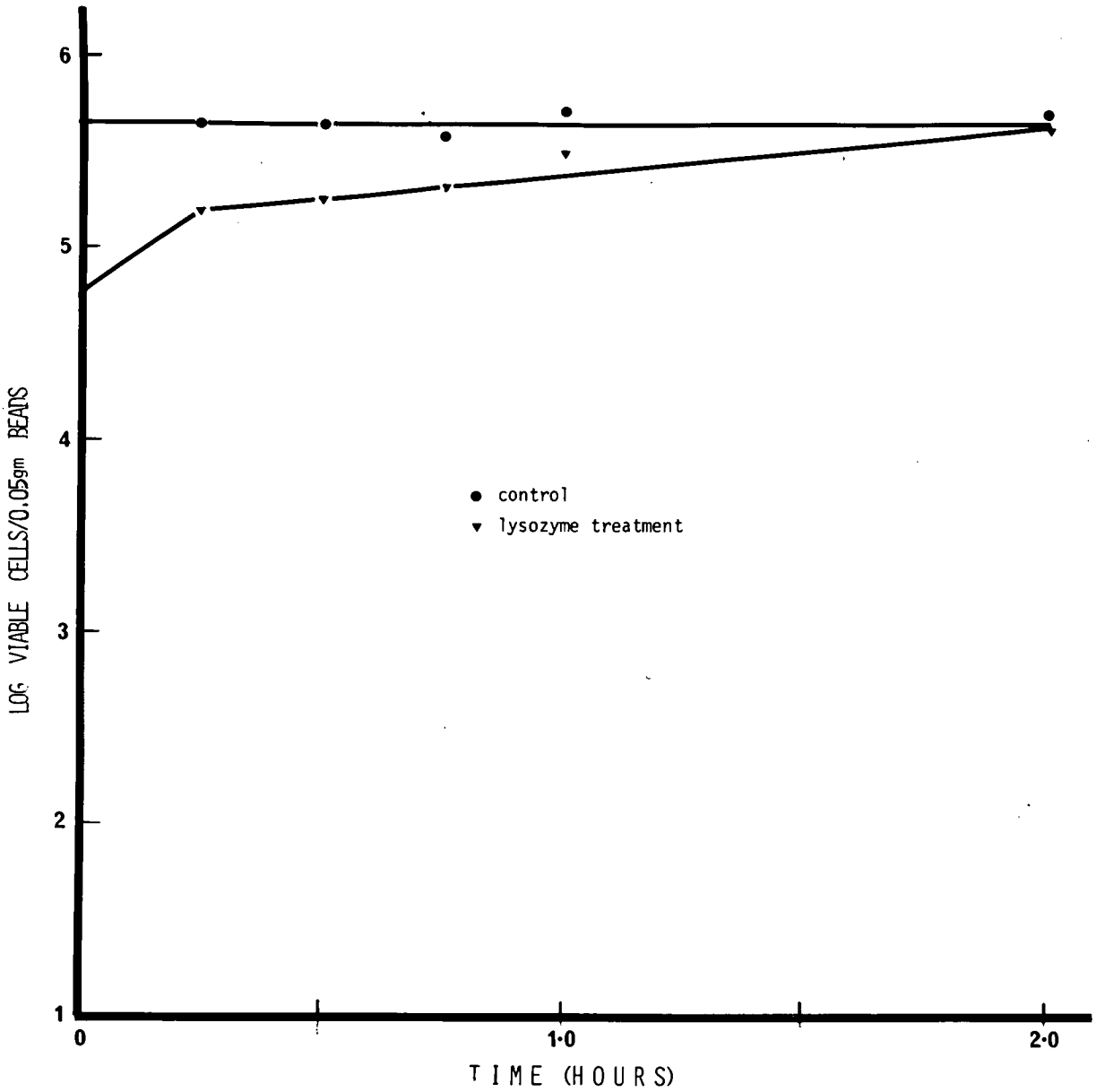

6
5
4
3
2
1
LOG VIABLE CELLS/0.05gm BEADS
control
lysozyme treatment
0
1·0
2·0
TIME (HOURS)

FIGURE 49

Repair of desiccation-induced susceptibility to lysozyme, by Escherichia coli M13 rehydrated in yeast mannitol broth instead of Hambleton's (1971) medium.

Detailed results and analysis of variance are presented in Appendix Table XLIV.

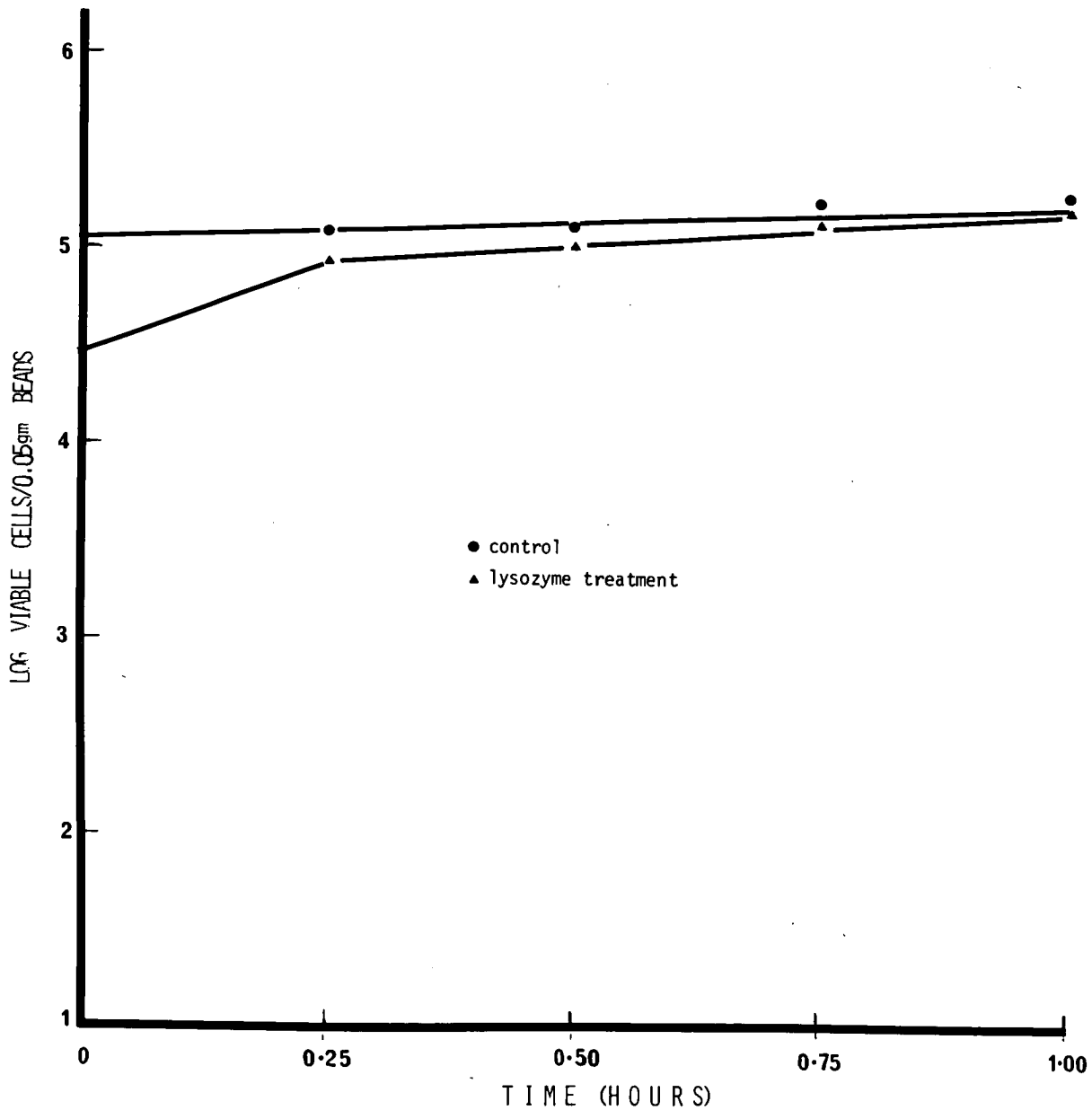

LOG VIABLE CELLS/0.05gm BEADS
6
5
4
3
2
1
0
0·25
0·50
0·75
1·00
TIME (HOURS)
control
lysozyme treatment

FIGURE 50

Repair of desiccation-induced susceptibility to lysozyme by *R. leguminosarum* TA101.

Detailed results and analysis of variance are given in Appendix Table XLV.

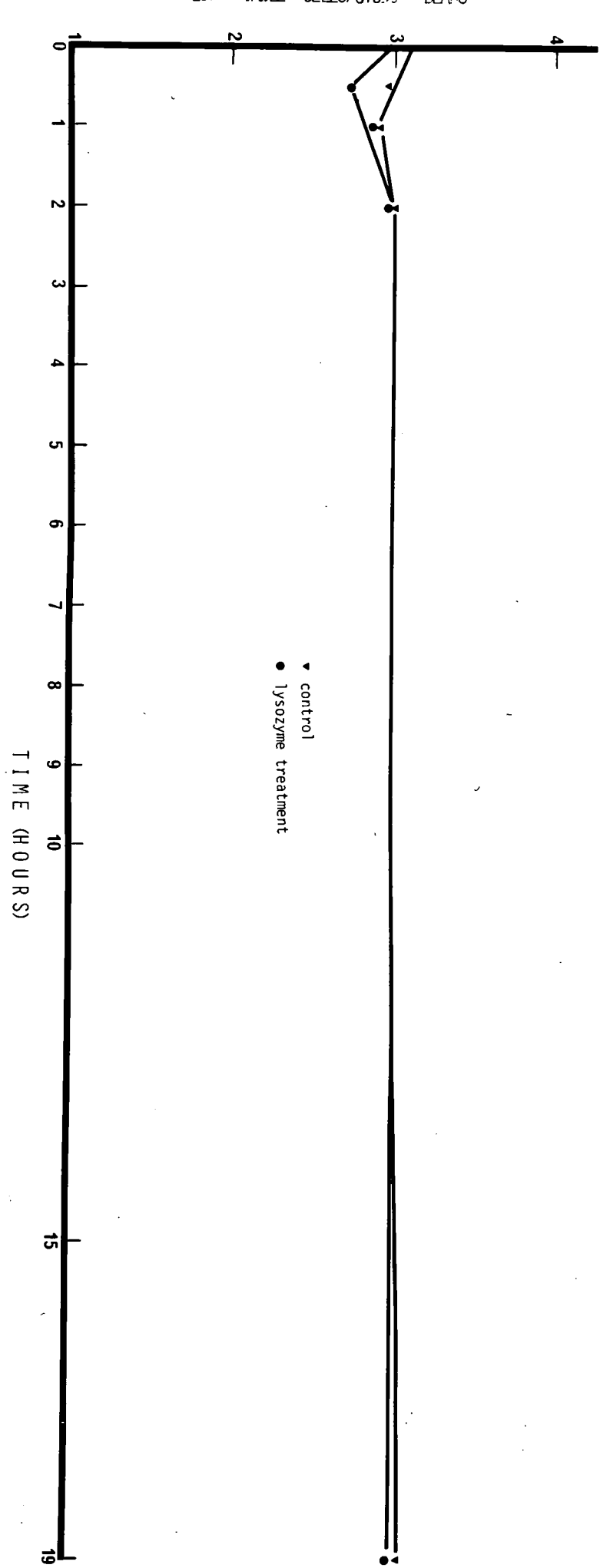

LOG VIABLE CELLS/0.05gm BEADS
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10
15
19
TIME (HOURS)
control
lysozyme treatment

FIGURE 51

Repair of desiccation-induced susceptibility to lysozyme by *R. leguminosarum* TA101.

Detailed results and analysis of variance are presented in Appendix Table XLVI.

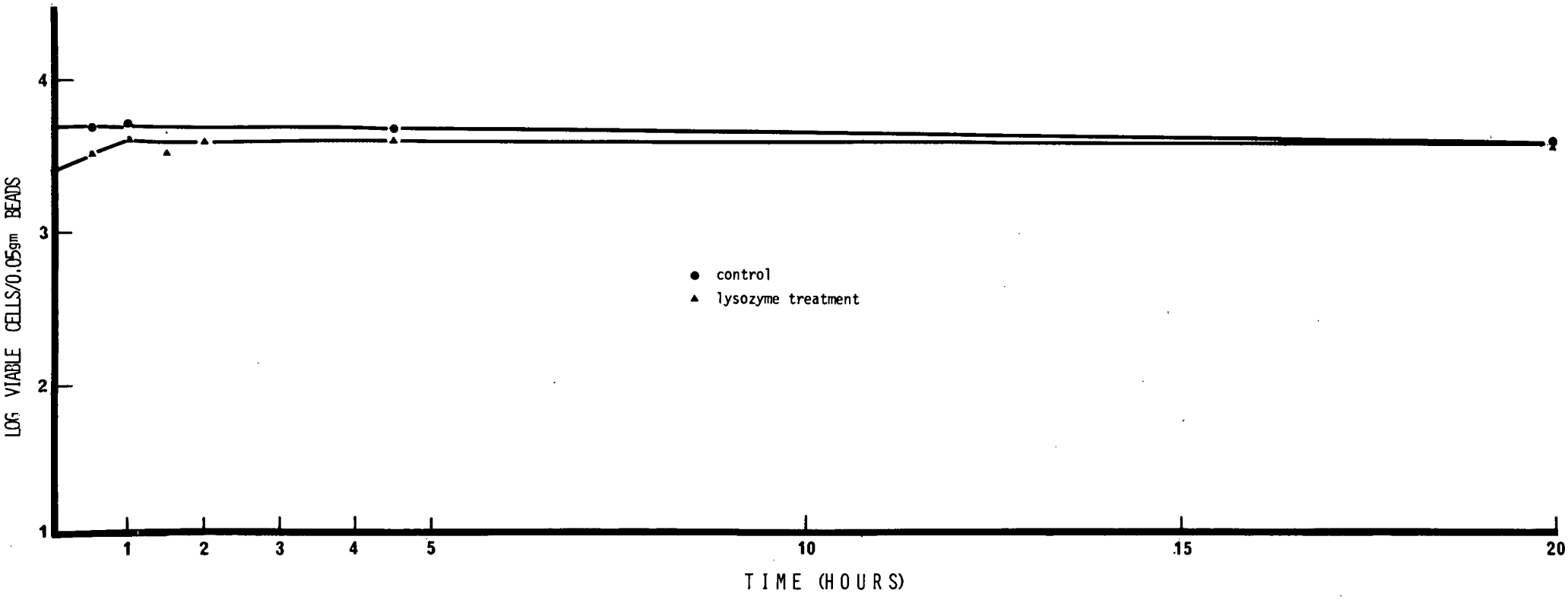
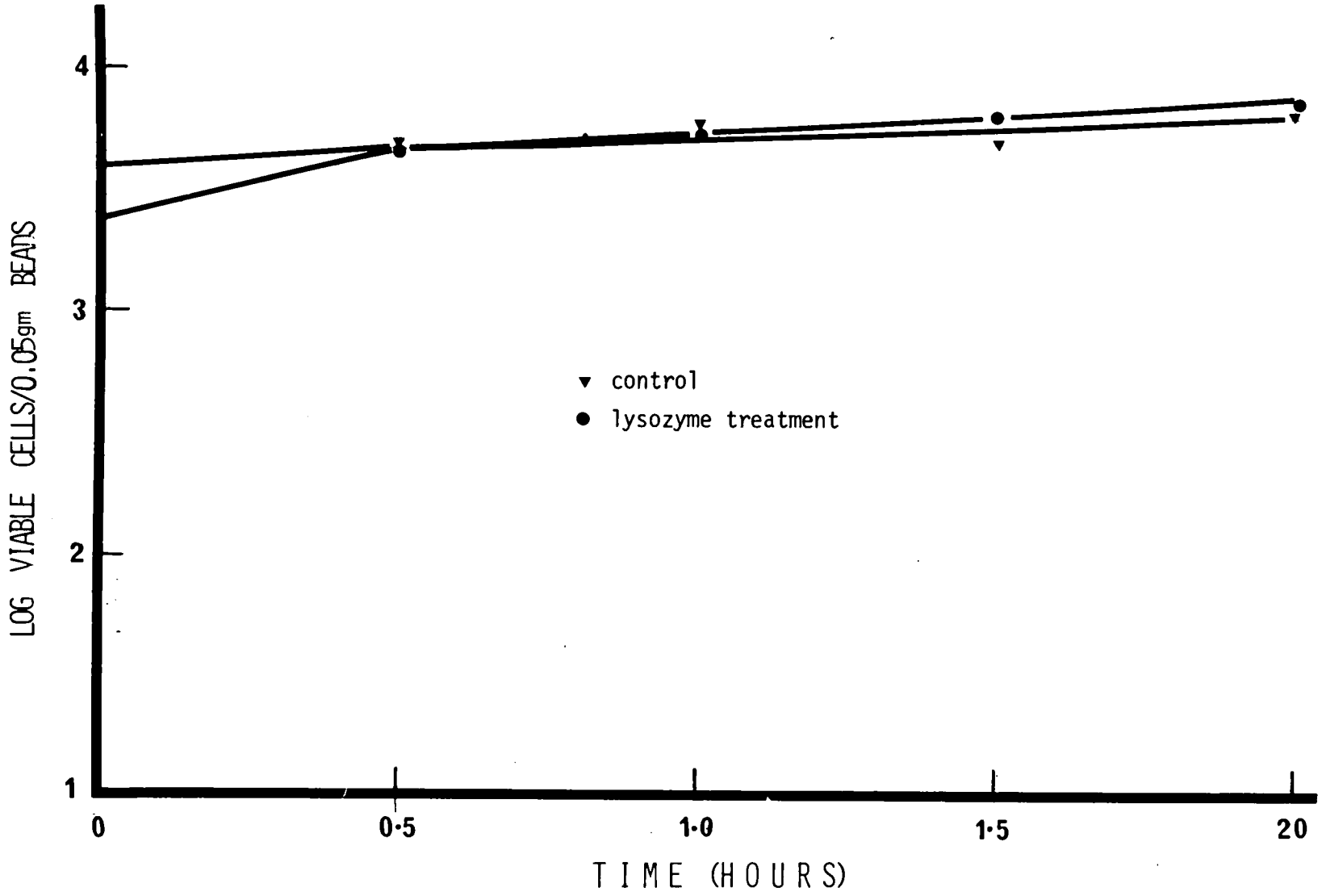

LOG VIABLE CELLS/0.05gm BEADS
4
3
2
1
control
lysozyme treatment
1
2
3
4
5
10
15
20
TIME (HOURS)

FIGURE 52

Repair of desiccation-induced susceptibility to lysozyme by R. leguminosarum TA101. The rehydration medium was that suggested by Hambleton (1971) for Escherichia coli.

Detailed results and analysis of variance are presented in Appendix Table XLVII.


4
3
2
1
LOG VIABLE CELLS/0.05gm BEADS
▼ control
● lysozyme treatment
0
0·5
1·0
1·5
20
TIME (HOURS)

74/3/32

Table 22

Repair of desiccation-induced susceptibility to lysozyme by R.trifolii SU297/32B.

Time after rehydration (hours)	Log viable count per 0.05 gm glass beads. Control Replication No. 1	2	Lysozyme Replication No. 1	2
0	5.50	6.12	5.99	5.66
0.5	5.56	6.17	5.26	6.12
1.0	5.65	6.31	5.55	6.36
1.5	5.64	6.23	5.64	6.29
2.0	5.65	6.35	5.68	6.22
2.5	5.56	6.38	5.68	6.20

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	2.0709	2.07	43.40^{xxx}
Lysozyme	1	0.0092	0.009	0.193
Time	5	0.1489	0.0298	0.624
Lysozyme x Time	5	0.0266	0.0053	0.111
Error	11	0.5249	0.0477	
Total	23	2.7805		

Table 23

Repair of desiccation-induced susceptibility to lysozyme by R.trifolii SU297/32B.

Time after rehydration (hours)	Log viable count per 0.05 gm. glass beads. Control Replication No. 1	2	Lysozyme Replication No. 1	2
0	5.34	5.23	5.40	5.19
0.5	5.56	5.37	5.42	5.25
1.0	5.66	5.56	5.64	5.40
2.0	5.65	5.56	5.68	5.21
3.0	5.54	5.63	5.40	5.55

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.0898	0.0898	6.01[x]
Lysozyme	1	0.0460	0.0460	3.085
Time	4	0.2105	0.0526	3.524
Lysozyme x Time	4	0.0167	0.00418	0.2799
Error	9	0.1344		
Total	19	0.04975		

drastically affect the permeability of the lipopolysaccharide layer of the cell wall to lysozyme.

The results presented in figure 53 for R.meliloti CC131 and figure 54 for R.japonicum QA372, suggested similar results to the above, i.e. that rehydration of these bacteria did not drastically alter the permeability of the lipopolysaccharide layer to lysozyme.

The low desiccation induced susceptibility to lysozyme did not imply that the stressed cells were not made sensitive to other chemicals. Therefore, various substances were screened for their toxicity to rhizobia after desiccation. These experiments were designed to screen for various chemicals to which bacteria demonstrated desiccation-induced repairable damage, therefore replicates were not included and statistical analysis of the results was not possible. The results for R.japonicum QA372 and R.trifolii SU297/32B are presented in tables 24 and 25, respectively. Desiccated R.japonicum QA372 did not become susceptible to chloramphenicol, streptomycin, congo red, penicillin or polymyxin (table 24). Similarly the effects of rose bengal, congo red, polymysin or penicillin on desiccated R.trifolii (table 25) did not warrant further investigation as the results were similar to the effect of lysozyme. Thus, the conclusions drawn above still applied, i.e. desiccation of the rhizobia does not drastically alter the permeability to various anti bacterial agents.

FIGURE 53

Repair of desiccation-induced susceptibility to lysozyme by R. meliloti CC131.

Detailed results and analysis of variance are presented in Appendix Table XLVIII.

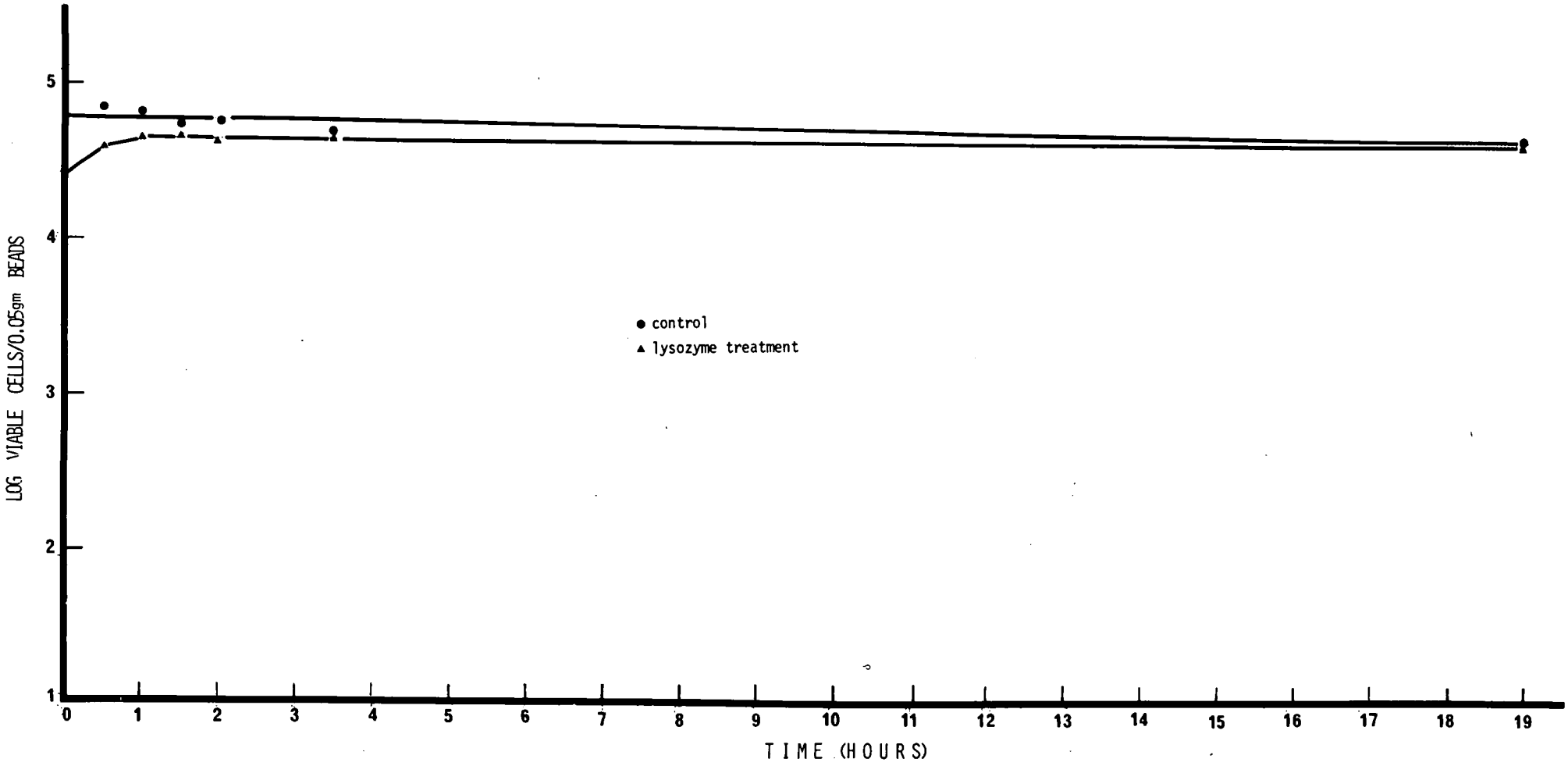

LOG VIABLE CELLS/0.05gm BEADS
5
4
3
2
1
● control
▲ lysozyme treatment
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
TIME (HOURS)

FIGURE 54

Repair of desiccation-induced susceptibility to lysozyme by R. japonicum QA372.

Detailed results and analysis of variance are presented in Appendix Table XLIX.

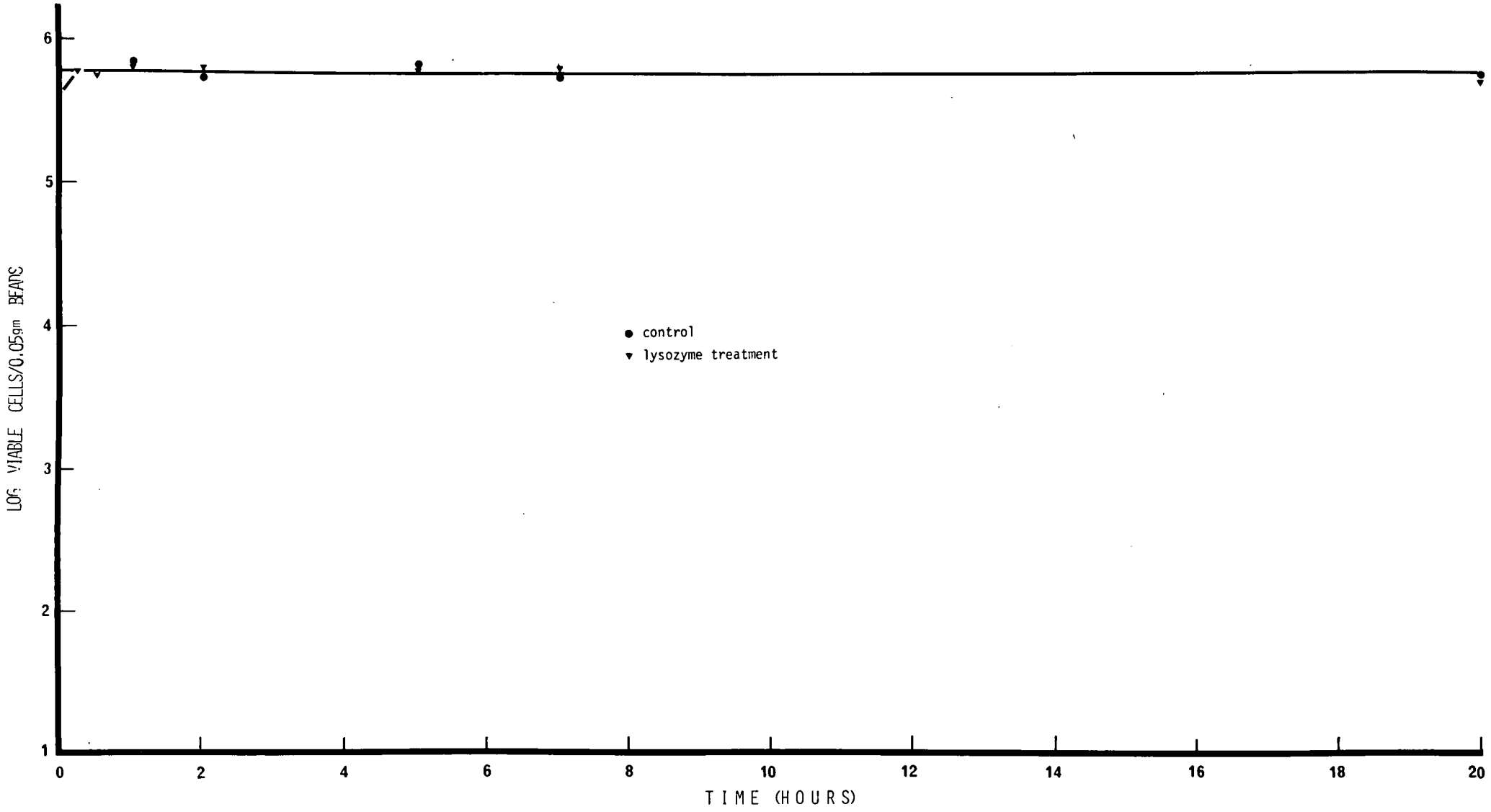

LOG VIABLE CELLS/0.05gm BEADS
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
TIME (HOURS)
control
lysozyme treatment

Table 24

Possible repair by R. japonicum QA372 of desiccation-induced susceptibility to various antibacterial agents.

Time after rehydration (hours)	Log viable count per 0.05 gm. glass beads. Control	Chloramphenicol	Streptomycin	Congo Red	Penicillin	Polymyxin
0	6.99	6.90	6.87	7.00	6.98	6.92
1.0	7.00	6.93	6.93	6.95	6.91	6.86
3.0	6.94	6.93	6.92	6.92	6.86	6.87
6.0	6.89	6.91	6.88	6.91	6.93	6.85
8.0	6.89	6.87	6.87	6.90	6.91	6.92
25.0	6.89	6.91	6.87	6.87	6.86	6.95

Table 25

Repair of desiccation-induced susceptibility to various antibacterial agents by *R.trifolii* SU297/32B.

Time after rehydration (hours)	Log viable count per 0.05 gm. glass beads. Glutamate agar	Yeast-Mannitol Agar	Glutamateagar + rose bengal	Glutamateagar + congo red	Glutamateagar + polymyxin	Glutamateagar + penicillin
0	5.21	4.98	4.17	5.10	4.92	4.76
1.0	5.39	5.04	2.55	5.09	5.04	nd
3.0	5.31	nd	3.31	5.17	5.02	nd
4.0	5.20	5.13	nd	nd	nd	4.82
6.0	5.24	5.30	3.94	5.09	4.89	4.50
9.0	5.22	5.20	3.91	5.01	5.03	4.98
24.0	5.35	nd	3.31	nd	nd	nd

nd = viable count not done.

Hurst et al. (1973) noted alteration of the osmotolerance of heated Staphylococcus aureus. This was not the case with desiccated R.meliloti SU45 (table 25) as the ability of this bacterium to grow on media of low water activity (a_w 0.990) was not affected by dehydration.

The conclusion of this series of experiments was that desiccation to rhizobia did not induce repairable susceptibility to lysozyme. Therefore, damage caused by desiccation did not involve alterations of the integrity of the lipopolysaccharide layer as determined by sensitivity to lysozyme. This is in contrast to the results obtained for Escherichia coli, as desiccation was found to alter the susceptibility of these bacteria to lysozyme.

Further, damage to the lipopolysaccharide layer induced by desiccation (as detected by lysozyme sensitivity) was about the same for both the fast-and the slow-growing rhizobia, i.e. differential susceptibility of the lipoplysaccharide layer to damage by dehydration between the fast-and slow-growing rhizobia, was not an explanation for the difference in survival of the two groups of bacteria when subjected to desiccation.

Table 26

Possible repair by *R.meliloti* SU45 of NaCl susceptibility caused by desiccation.

Time after rehydration (hours)	Log viable count per 0.05gm. glass beads			
	Dried		Not Dried	
	Control	Water Activity =0.990	Control	Water Activity =0.990
0	6.40	6.29	9.00	8.75
1.5	6.29	6.31	9.02	8.78
2.5	6.26	6.22	9.02	9.00
4.0	6.30	6.11	9.20	8.74
6.0	6.35	6.20	9.07	8.82
8.0	6.29	6.16	9.31	8.86
13.5	6.28	6.18	9.19	8.88

G. Use of nuclear magnetic resonance (NMR) spectroscopy to determine the water permeabilities of various microorganisms.

In this study NMR spectroscopy was used to determine the relationship between a variety of cell sizes and the rate at which water passed across the membrane. More specifically, it was the aim of these investigations to find out if differences in the water permeability existed between fast-and slow-growing rhizobia, and if this could be correlated with their differential responses to desiccation.

Dick (1966) obtained a good correlation between cellular water permeabilities and cell surface to volume ratio. Measurements of the dimensions of various bacterial cells and yeast, are presented in table 27, together with calculations of the surface-to-volume (s/v) ratio. The results show that the s/v increased in the order Saccharomyces cerevisiae M6, Bacillus sp. M70 followed by Staphylococcus aureus M3 and gram-negative bacteria including Escherichia coli M13 and various species of Rhizobium.

The results presented in figures 55 and 56 are the spectra obtained using steady-state NMR for Saccharomyces cerevisiae M6 and Bacillus sp. M70 respectively. The significant features of the spectra for Saccharomyces cerevisiae M6 (figure 55) are the lines attributable to extracellular water which have been broadened due to contact with the paramagnetic ions (Mn^{2+}), and the relatively narrow line which was derived from intracellular water (not in contact

Table 27

Calculations of the areas, volumes, surface/volume ratios, and water permeabilities (T2) for various microorganisms.

Organism	Length (μ)	Width (μ)	Volume (μ^3)	Surface area (μ^2)	S/V ratio (μ^2/μ^3)	Line width at half peak height (ppm)	Relative T2 Measurements
Saccharomyces cerevisiae M6	4.72	4.72	5.08	70.02	1.27	2.5	0.127
Bacillus sp. M70	4.55	1.59	9.00	26.67	2.97	7.0	0.0455
Escherichia coli M13	1.54	0.86	0.895	5.31	5.93	-	-
Staphylococcus aureus M3	1.10	1.10	0.70	3.81	5.45	-	-
Rhizobium lupini UT2	2.13	0.60	0.60	4.58	7.63	-	-
R.japonicum QA372	2.04	0.68	0.74	5.08	6.86	-	-
R.lupini UT12	2.66	0.94	1.85	9.24	5.00	-	-
R.trifolii TA1	2.31	0.79	1.16	6.82	5.88	-	-
R.trifolii SU297/31A	2.28	0.92	1.52	7.91	5.20	-	-
R.trifolii SU297/32B	1.96	0.76	0.59	5.58	9.46	-	-
R.trifolii SU298/536D	2.23	0.95	1.61	8.17	5.08	-	-
R.leguminosarum TA101	2.85	1.06	2.51	11.25	4.48	-	-

see Page 250

Table 27 continued

Values for all rhizobia were obtained from K.C.Marshall (pers.comm.).

T_2 estimates were derived from figures 55 and 56.

- indicates that a distinction between the extra-and intracellular water could not be made, therefore, no estimates of T_2 could be obtained.

with Mn^{2+}). The sharp discontinuity between intra-and extracellular water was obvious at all Mn^{2+} concentrations. This enabled estimation of the relative T_2 value, the water permeability, of the yeast by the method described in Methods and Materials. The results are presented in table 27.

Steady state NMR spectra for Bacillus sp. M70 are presented in figure 56. Once again a distinction could be made between the intra-and extracellular water due to the marked discontinuities in the spectra at about 4 and 5 ppm. This enabled calculation of T_2, the results of which are presented in table 27. As can be seen from figure 56, the discontinuity was not as marked at low concentrations (60 mM) of Mn^{2+}, however, it became apparent as the concentrations of these ions was increased. Presumably, at a concentration of 60 mM $MnCl_2.6H_2O$ the concentration of paramagnetic ions was not high enough to induce sufficient line broadening of the signal due to extracellular water to enable differentiation from the signal due to intracellular water. Consequently, merging of the two lines became apparent (figure 56, spectrum A).

A comparison of the spectra in figures 55 and 56 revealed that the line due to intracellular water from Bacillus sp. M70 was broader than the corresponding line for Saccharomyces cerevisiae M6. The line widths at half peak height were 7.0 and 2.5 ppm for Bacillus M70 and S.cerevisiae, respectively. As there is an inverse relationship between line width and T_2, this result demonstrated that the passage of water across the bacterial membrane was faster than across the yeast

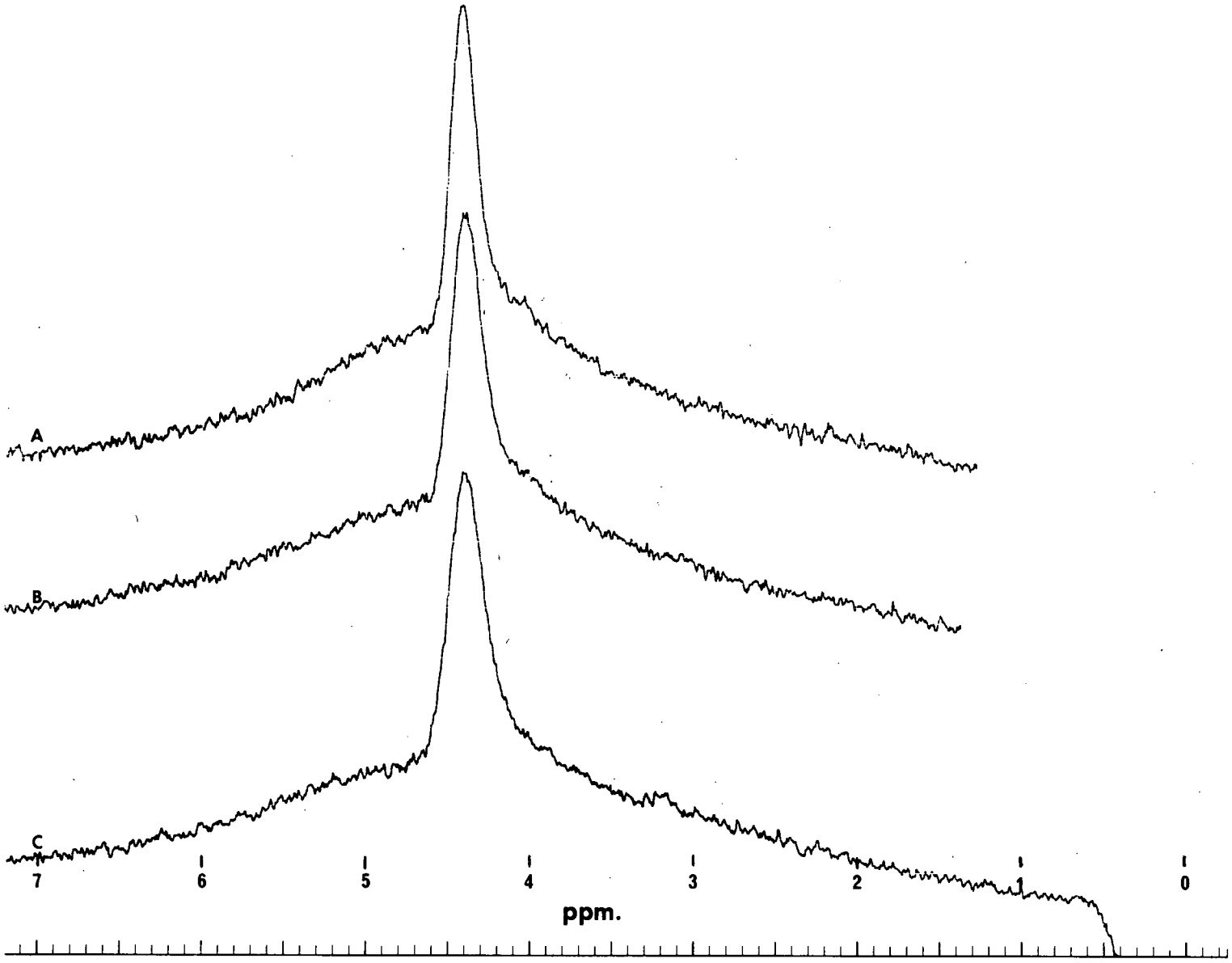
FIGURE 55

Steady-state NMR spectra from studies on water permeability of Saccharomyces cerevisiae M6.

Lines A, B and C demonstrated the effect on the spectra if different $MnCl_2.6H_2O$ concentrations were used.

A = 60 mM Mn^{2+}
B = 72 mM Mn^{2+}
C = 84 mM Mn^{2+}

Note the sharp discontinuity which enabled a distinction to be made between intra-and extracellular water.


A
B
C
7
6
5
4
3
2
1
0
ppm.

74/3/72

FIGURE 56

Steady-state NMR spectra from studies on the water permeability of Bacillus sp. M70 vegetative cells.

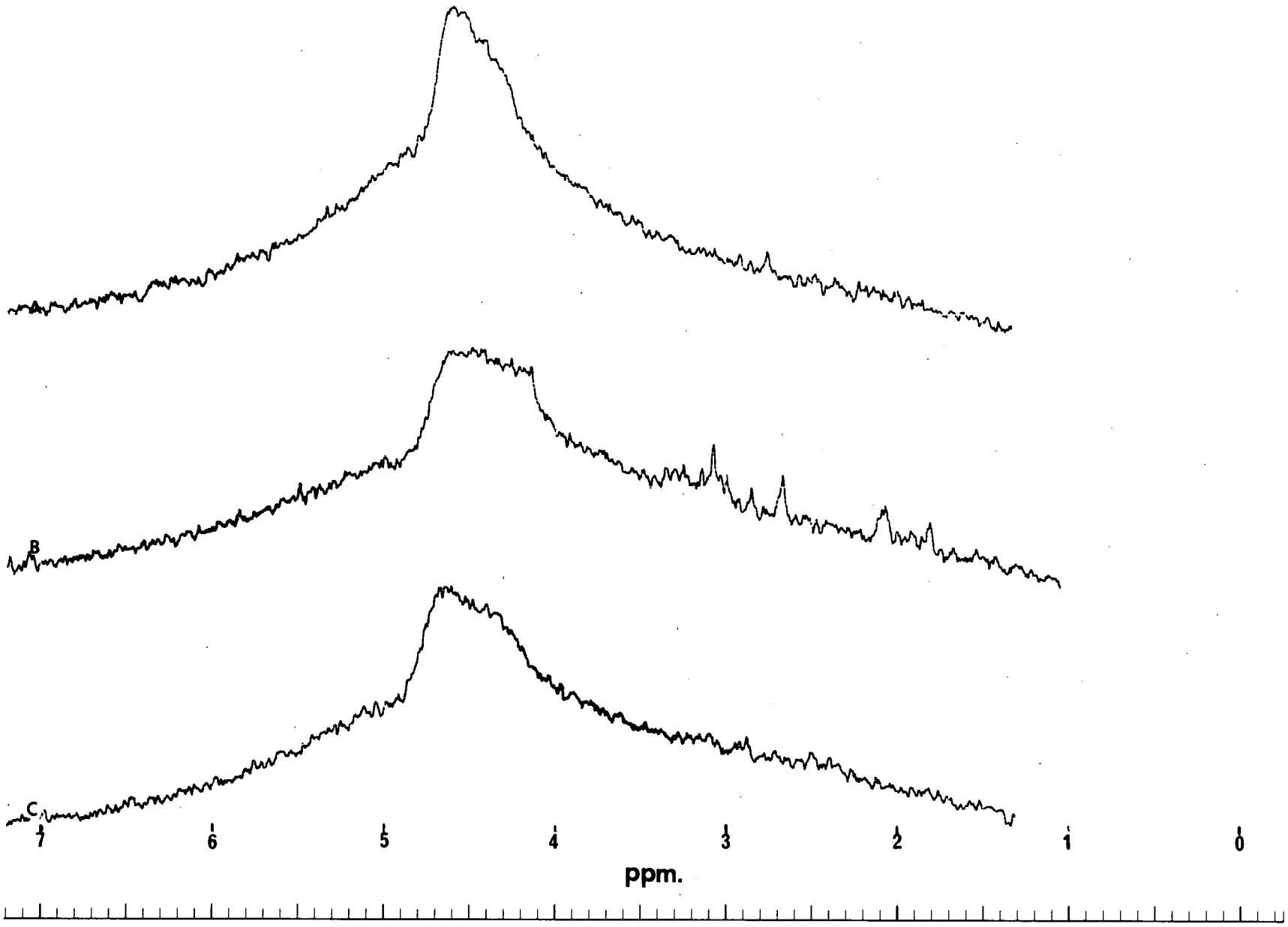
Lines A,B and C demonstrate the effect on the spectra of different concentrations of paramagnetic ions ($MnCl_2 .6H_2O$) were used.

A = 60mM Mn^{2+}

B = 84mM Mn^{2+}

C = 72mM Mn^{2+}

Note the discontinuity which enabled distinction between intra-and extracellular water. This point of inflection was not as pronounced with Bacillus sp.M70 as it was with Saccharomyces cerevisiae M6 (figure 55).


A
B
C
7
6
5
4
3
2
1
0
ppm.

74/3/15

membrane. The ratio of the T_2 values (table 27) for yeast relative to Bacillus sp. M70 was about 3.0, therefore, the rate of passage of water molecules across the membrane of the bacterium was three times greater than for Saccharomyces cerevisiae M6. For the complicated reasons given by Pople et al. (1959) estimates of T_2 obtained by the use of continuous-wave NMR are subject to error, only pulsed techniques giving quantitatively accurate results. Because the spectra in figures 55 and 56 were obtained by the use of continuous-wave NMR, the values of T_2 in table 27 are not accurate and, represent relative differences between the microorganisms. Unfortunately there was no pulsed NMR equipment available at this University.

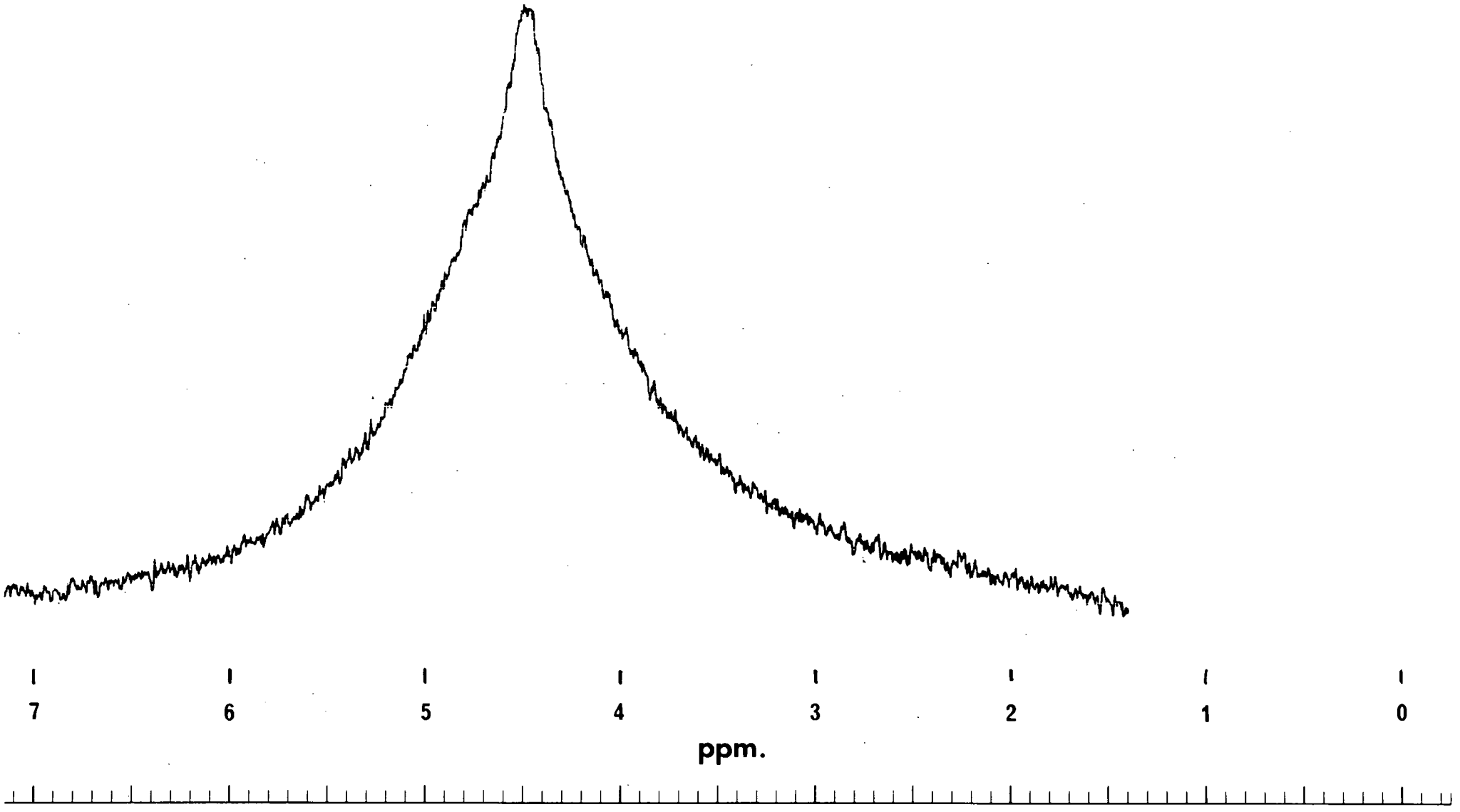
The spectrum presented in figure 57 was typical of those obtained for Escherichia coli M13, and Staphylococcus aureus M3 at all Mn^{2+} concentrations employed. Therefore, only a representative spectrum has been presented. This was obtained using E.coli M13 at a Mn^{2+} concentration of 40mM. Unlike the spectra typical of either Bacillus sp. M70 or Saccharomyces cerevisiae M6, it was not possible to distinguish between intra-and extracellular water when either Escherichia coli M13 or Staphylococcus aureus M3 was used. Consequently, it must be concluded that the passage of water across the membranes of E.coli and S.aureus was too rapid to be determined by this technique. Because of this it was not possible to estimate T2 values for E.coli or S.aureus. Similar results

FIGURE 57

A typical steady-state spectrum obtained for either Escherichia coli M13 or Staphylococcus aureus M3 . This figure is the spectrum obtained for E.coli. Spectra for other cells were not shown because of their similarity to this line.

Concentration of $MnCl_2 \cdot 6H_2O$ = 40 mM.

Note that the distinct point of inflection characteristic of the spectra in figures 55 and 56 is absent. Thus no differentiation could be made between intra-and extra-cellular water.


7
6
5
4
3
2
1
0
ppm.

74/3/74

were obtained for all species of rhizobia and representative spectra are presented in figure 58 for R. japonicum, R. trifolii and R. meliloti. Clearly, these were similar to the spectra of Escherichia coli and Staphylococcus aureus in that it was not possible to distinguish between intra-or extracellular water. Thus estimates of T_2 could not be obtained. In an attempt to overcome this problem, various species of Rhizobium were sent to Dr.R.Outhred at the University of New South Wales, Sydney, who had access to a pulsed NMR spectrometer. However, even with this equipment, it was not possible to distinguish between intra-or extracellular water and T_2 estimates could not be obtained. It was estimated by R.Outhred (pers. comm.) that, at a Mn^{2+} concentration of 50mM, the rate at which water passed across the membrane would be in the order of 0.5 to 1.0msec. Therefore, it appears that for rhizobia, Escherichia coli and Staphylococcus aureus, the membrane offers very little resistance to the movement of water molecules between the cell interior and the external environment.

From the results in table 27, it was possible to obtain T_2 values for Bacillus sp M70 and Saccharomyces cerevisiae M6, but not for other microorganisms. This suggests that estimation of water permeabilities of cells by this technique is limited to microorganisms with a s/v ratio smaller than, or equal to 2.96 (the value for Bacillus sp. M70, table 27).

FIGURE 58

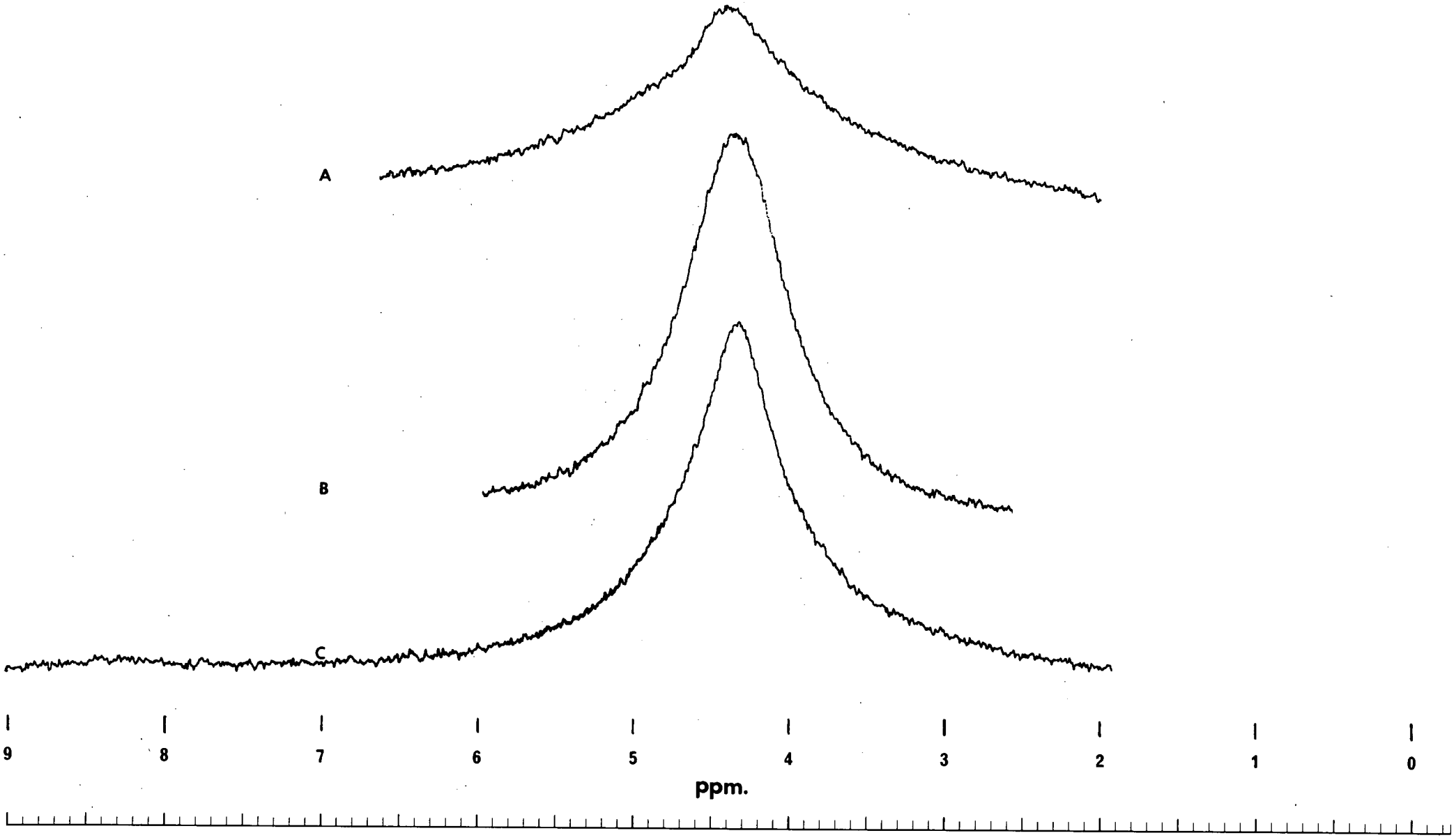
Steady-state NMR spectra obtained for various species of Rhizobium.

A = R.japonicum QA372, packed cell volume was 34% and $MnCl_2.6H_2O$ concentration was 50mM

B= R.trifolii SU297/32B, packed cell volume was 18% and $MnCl_2.6H_2O$ concentration was 40mM.

C= R.meliloti CC131, packed cell volume was 50% and $MnCl_2.6H_2O$ concentration was 60mM.

Note that the distinct points of inflection obvious for yeast and Bacillus sp. M70 (figures 55 and 56) was absent for all species of Rhizobium. No difference could be determined between intra-and extracellular water.


A
B
C
9
8
7
6
5
4
3
2
1
0
ppm.

7/13/73

The results in figures 59 and 60 show the effect of the addition of a suspension of montmorillonite on the steady-state NMR spectra of Saccharomyces cerevisiae M6 and Bacillus sp. M70 in the presence of Mn^{2+}. The very slight broadening of the peak attributable to intracellular water upon the addition of montmorillonite could indicate an affect upon water permeabilities. The effect was slight, however, and could be attributed to the increased viscosity of the montmorillonite-organism mixture. Also, errors resulting from adsorption of Mn^{2+} to montmorillonite particles could be involved. Pulsed NMR techniques would probably be required to determine the true effect of montmorillonite upon the exchange of water molecules between the microorganism and its environment.

The conclusions reached from this series of experiments was that, at any particular dehydration rate, there was no difference between the fast-and slow-growing rhizobia in the rate at which water left the bacteria. Similarly, if rehydration were considered, there would be no significant difference between the effective rates at which water entered any species of rhizobia. Consequently, the reason for the greater ability of the slow-growing rhizobia to survive desiccation relative to the fast-growing species, is not related to any differences in water permeability between the two bacterial groups.

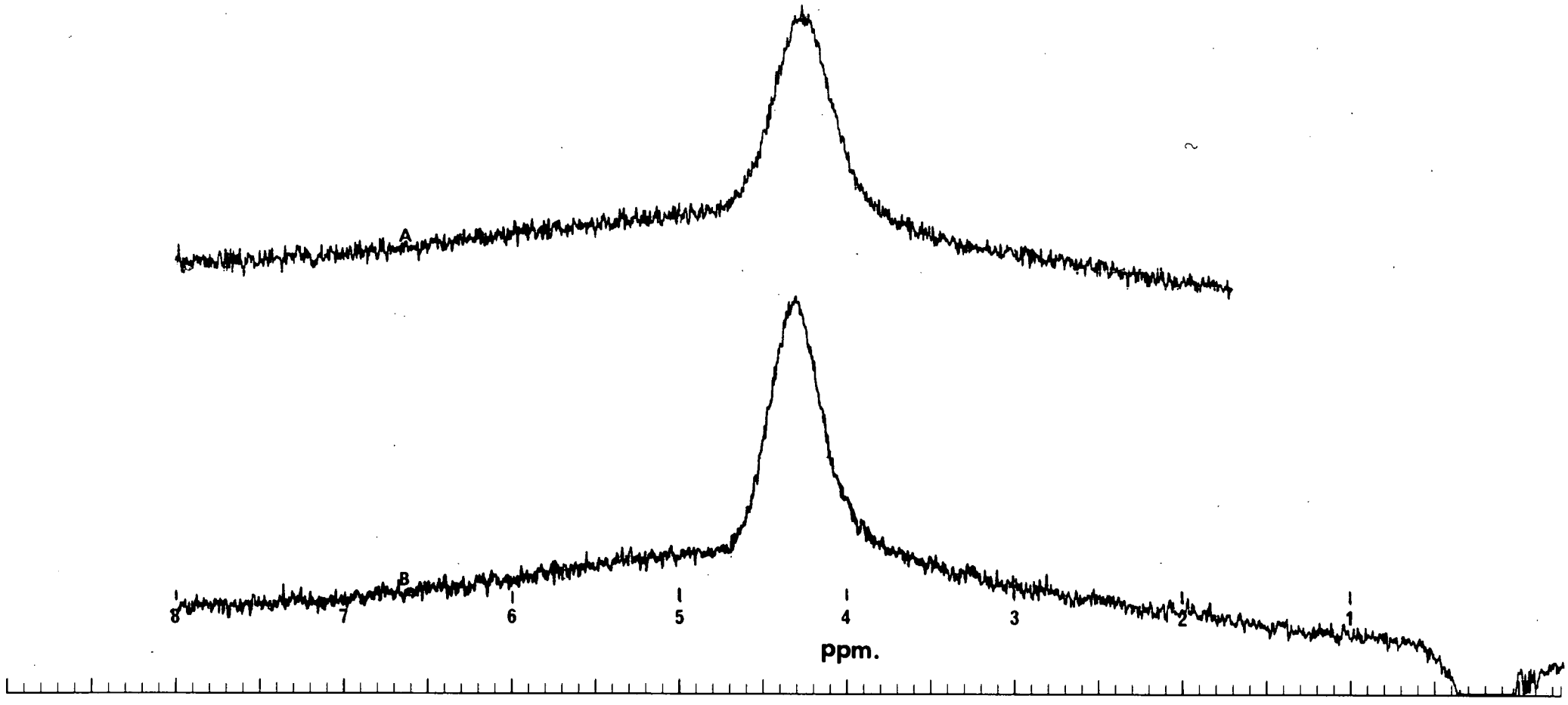
FIGURE 59

The effect of the addition of montmorillonite on the steady-state NMR spectrum obtained for Saccharomyces cerevisiae M6.

$MnCl_2.6H_2O$ concentration = 80 mM for both spectra.

Line A = S.cerevisiae plus montmorillonite at a concentration of 300 µgm./ml.

Line B = S.cerevisiae without montmorillonite.


A
B
8
7
6
5
4
3
2
1
ppm.

7/13/77

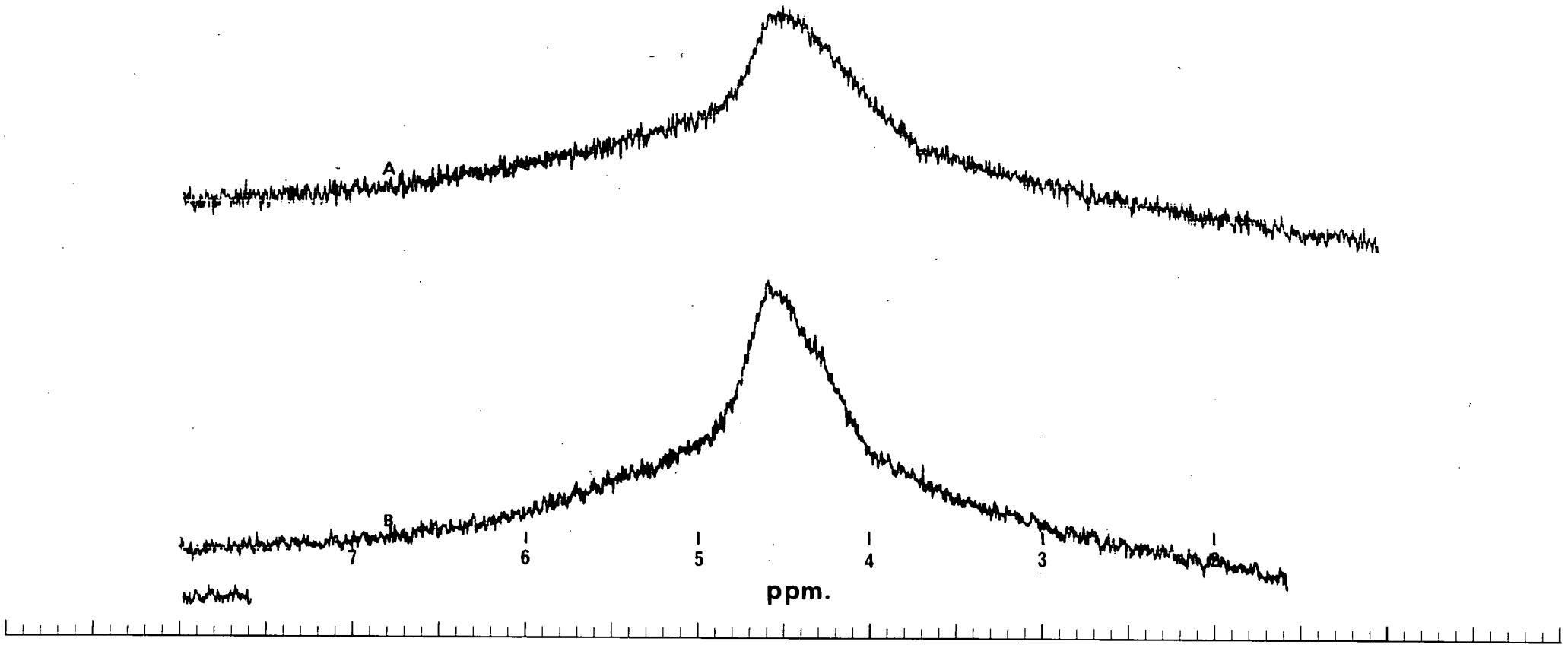
FIGURE 60

The effect of the addition of montmorillonite on the steady-state NMR spectrum obtained for Bacillus sp. M70.

$MnCl_2.6H_2O$ concentration = 60 mM for both spectra.

Line A = Bacillus sp. M70 plus montmorillonite at a concentration of 300 µgm./ml.

Line B = Bacillus sp. M70 without montmorillonite


A
B
7
6
5
4
3
ppm.

7/13/76

H. Internal osmotic pressure of various species of Rhizobium.

Chen and Alexander (1973) suggested that there may be an inverse correlation between the internal osmotic pressure of a microorganism and its ability to withstand dehydration i.e. the lower the osmotic pressure of the internal contents, the greater the ability of bacteria to survive dehydration. Because of the greater ability of slow-growing root-nodule bacteria to survive dehydration relative to the fast-growing rhizobia, it was of interest to determine whether the suggestion of Chen and Alexander (1973) applied to these bacteria.

The results presented in table 28 represent the estimates of the internal solute concentration of various species of Rhizobium, relative to packed cell volume (PCV) measurements. It must be pointed out that all these values would underestimate the true value as no correction has been made for the dilution effect of the suspending medium. To eliminate this error, measurements would have to be made of intra-and extracellular water volumes, enabling expression of the results as per unit intracellular volume. This has not been carried out. Although not correct in an absolute sense, the results obtained gave an indication of the relative differences in internal osmolality between the fast-and slow-growing bacteria and the R.meliloti group.

Table 28

Relative internal osmotic pressures of various species of Rhizobium expressed as per unit packed cell volume (PCV.).

Group	Bacterium		PCV (%)	Osmolality (mOsmols.)	Osmolality (mOsmols/PCV)	Average for group (mOsmols/PCV)
Fast-growing	R.trifolii SU298/534C		18.0	30.5	1.69	
	R.trifolii SU297/32B	(1)	18.0	28.0	1.56	
		(2)	12.0	28.0	2.33	
	R.trifolii SU297/31A	(1)	20.0	20.0	1.00	1.49
		(2)	60.0	40.0	0.67	
	R.leguminosarum TA101	(1)	10.0	15.0	1.50	
		(2)	6.0	12.0	2.00	
	Rhizobium sp. SU343		20.0	22.5	1.13	
Medic rhizobia	R.meliloti SU47A		15.0	32.0	2.13	
	R.meliloti SU47B		20.0	47.0	42.35	
	R.meliloti CC131	(1)	17.0	56.0	3.29	2.36
		(2)	7.5	20.0	2.67	
		(3)	5.0	10.0	2.00	
		(4)	51.0	88.0	1.73	
Slow-growing	R.lupini UT12		10.0	100.0	10.00	
	R.japonicum QA372	(1)	2.0	36.0	18.00	11.69
		(2)	3.5	30.0	8.57	
		(3)	3.0	30.0	10.00	

The results in table 28 showed that the osmolality per unit PCV for the slow-growing rhizobia was about eight times that of the fast-growing rhizobia and about five times that of the R.meliloti group.

As mentioned in Methods and Materials the osmolality also was expressed as per unit $\log_{10}$ of the viable count. These results are presented in table 29. Once again the slow-growing bacteria had consistently higher internal osmotic pressures than the fast-growing root-nodule bacteria.

The results presented in table 30, list the osmolality readings for aliquots of bacterial suspensions disrupted by the use of Triton x-100 and by autoclaving. In every case, autoclaving of the rhizobia resulted in an increase in the osmolality reading. Therefore, the osmotically active ingredients of all groups of rhizobia were not denatured by heat and were probably ionic. The reason for the increase in the observed osmotic pressure is not clear. A possible explanation could be that autoclaving denatured proteins, and it is possible that ions were released by the disruption of salt linkages within the proteins. The ions so released could contribute to the osmotic pressure of the medium after autoclaving.

Included in table 29 are the results of dialysis of rhizobia disrupted by the detergent Triton x-100. The fact that all osmotic activity was removed by dialysis added further credence to the suggestion that the osmotically active

Table 29

Relative osmolality of the internal contents of various species of *Rhizobium*.

Bacterium	Osmolality (mOsmols. per $\log_{10}$ viable count)	Osmolality after dialysis
R.japonicum Q\372	13.16	0.00
	11.84	0.00
	8.99	0.00
R.lupini UT12	5.79	0.00
R.lupini UT2	9.91	0.00
	7.30	0.00
Average for group	9.50	-
R.leguminosarum TA101	4.29	0.00
	4.85	0.00
	3.51	0.00
	2.71	0.00
R.trifolii TA1	2.80	0.00
	2.99	0.00
Rhizobium sp. SU343	2.90	0.00
	2.91	0.00
Average for group	3.37	-

Table 30

Effect of autoclaving and disruption with the detergent Triton x-100 on the measured internal osmotic pressure of various species of Rhizobium.

Group	Bacterium		Osmolality (bacteria disrupted with detergent)	Osmolality[x] (bacteria autoclaved)
Fast-growing	R.trifolii SU297/31A		70.0	165.0
	R.leguminosarum TA101	(1)	104.0	110.0
		(2)	64.0	74.0
		(3)	36.0	41.0
		(4)	40.0	48.0
		(5)	28.5	32.5
		(6)	22.0	28.0
	R.trifolii TA1	(1)	64.0	69.0
		(2)	74.0	81.0
		(3)	105.0	117.0
		(4)	23.0	31.0
	Rhizobium sp. SU343	(1)	83.0	114.0
		(2)	66.0	72.0
		(3)	24.5	27.0
Medic rhizobia	R.meliloti CC131	(1)	93.0	105.0
		(2)	90.0	96.0
Slow-growing	R.japonicum QA372	(1)	395	425
		(2)	174	194
		(3)	190	130
		(4)	105	117
		(5)	87	95
	R.lupini UT12	(1)	170	172
		(2)	50	80
	R.lupini UT2		64	68

This experiment involved direct comparisons of the osmolality of autoclaved and Triton x-100 disrupted rhizobia, neither the PCV nor the log viable counts were estimated for these readings.

constituents were probably ionic. It does not seem possible from the above that osmotically active glycoproteins contributed significantly to the observed osmolality, as these proteins would be denatured by autoclaving and are not dialysable (DeVries et al., 1970).

1. Water adsorption isotherms of montmorillonite and of the fast-and slow-growing root-nodule bacteria.

It was concluded by Scott (1958), Webb (1960b) and Bateman et al. (1962) that the water content of bacteria was dictated by the environmental relative humidity (R.H.). Webb (1960b) and Cox (1968a) suggested that bacterial death by dehydration due to R.H. changes could be via the effect of the environmental vapour pressure on the bacterial water content (vapour pressure = the ratio of the pressure of water present in the atmosphere to the pressure of saturated water vapour at the same temperature. It is equal to relative humidity, divided by 100). Consequently, it was of interest to determine the water sorption characteristics of fast-and slow-growing root-nodule bacteria as the results may suggest reasons for the greater inherent ability of the slow-growing species to survive dehydration.

The results are presented in two subsections:-

(i) the water sorption isotherms

(ii) calculations based on the water adsorption isotherms

I(i) The water sorption isotherms.

The calibration of the silica spring employed in these experiments resulted in the following straight-line relationship between spring extension and added weight:

$$y = 350.96x+115.36$$

where y = the extension of the spring

x = the added weight in grams.

Bovine serum albumin was chosen as the test protein to check that the apparatus was functioning correctly. The results in figure 61 show the typical hysteresis for adsorption and desorption cycles. The average of the adsorption-desorption isotherms for this protein was very close to the average values reported by Bull (1944) for serum albumin (see Appendix Table L).

The results in figures 62 and 63 show adsorption isotherms of representatives of the fast-and slow-growing rhizobia. That hysteresis was involved in an adsorption-desorption cycle of R.japonicum QA372 is shown in figure 62. Apart from this instance, the points plotted in all the graphs were the quantities of water adsorbed by bacteria. Also included in figure 62 is the adsorption isotherm of Ca^{2+} -montmorillonite.

The results in figures 62 and 63 indicate that the fast-growing bacteria adsorbed more water per gram than the slow-growing rhizobia at all R.H.s except perhaps at 90% and above. The isotherms of the various representatives of the

FIGURE 61

Water sorption isotherm for bovine serum albumin. Detailed results are presented in Appendix Table L. Also presented in Appendix Table L, is a comparison of the mean of the experimental data for both sorption and desorption isotherms with the mean figure presented by Bull (1944).

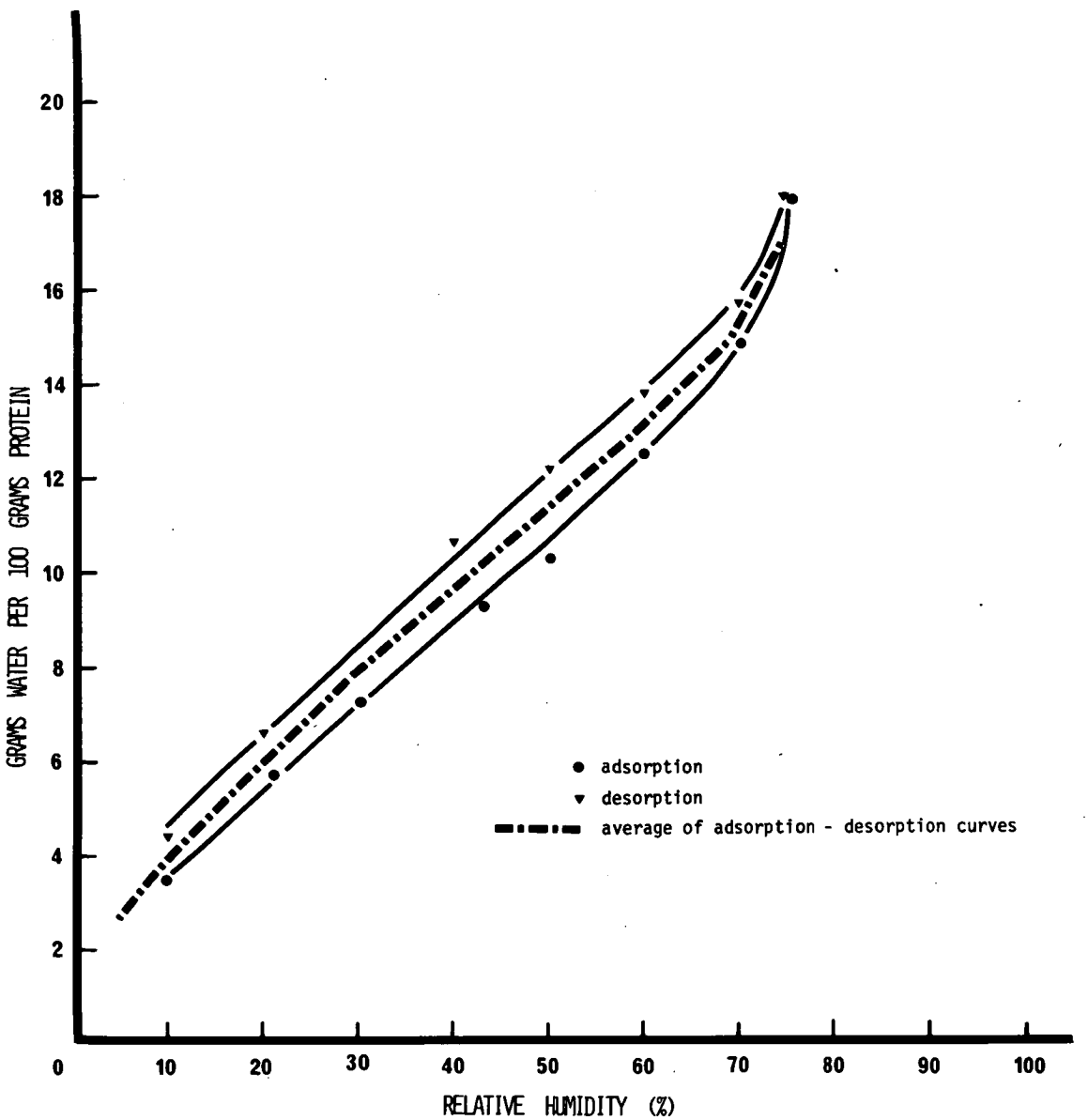

GRAMS WATER PER 100 GRAMS PROTEIN
20
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
90
100
RELATIVE HUMIDITY (%)
adsorption
desorption
average of adsorption - desorption curves

FIGURE 62

Adsorption isotherms for Ca^{2+} - montmorillonite and two species of Rhizobium.

Part of the desorption isotherm as well as the adsorption isotherm for R.japonicum QA372 are shown, demonstrating hysteresis.

The detailed results are presented in Appendix Tables LI,LII and LIII.

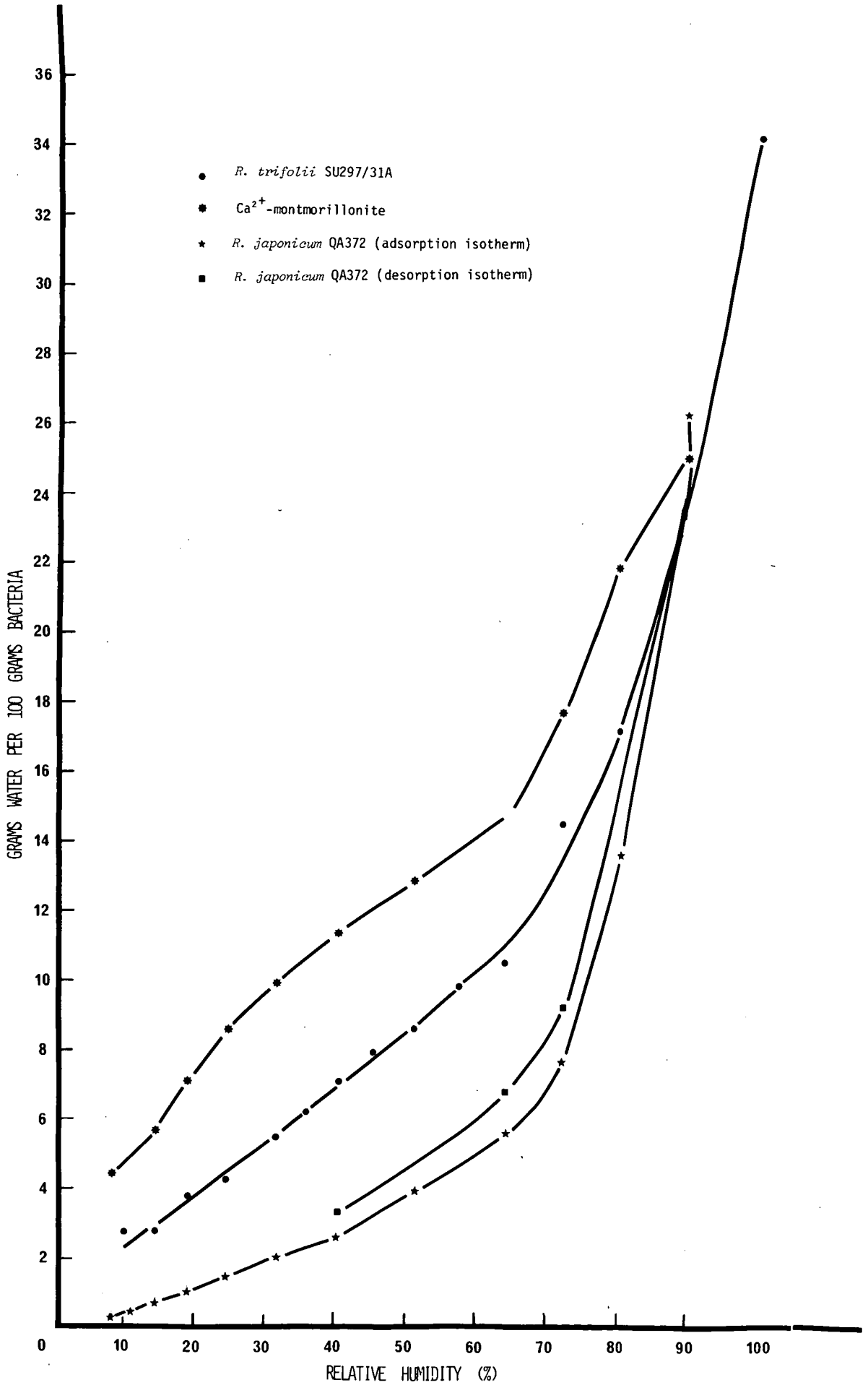
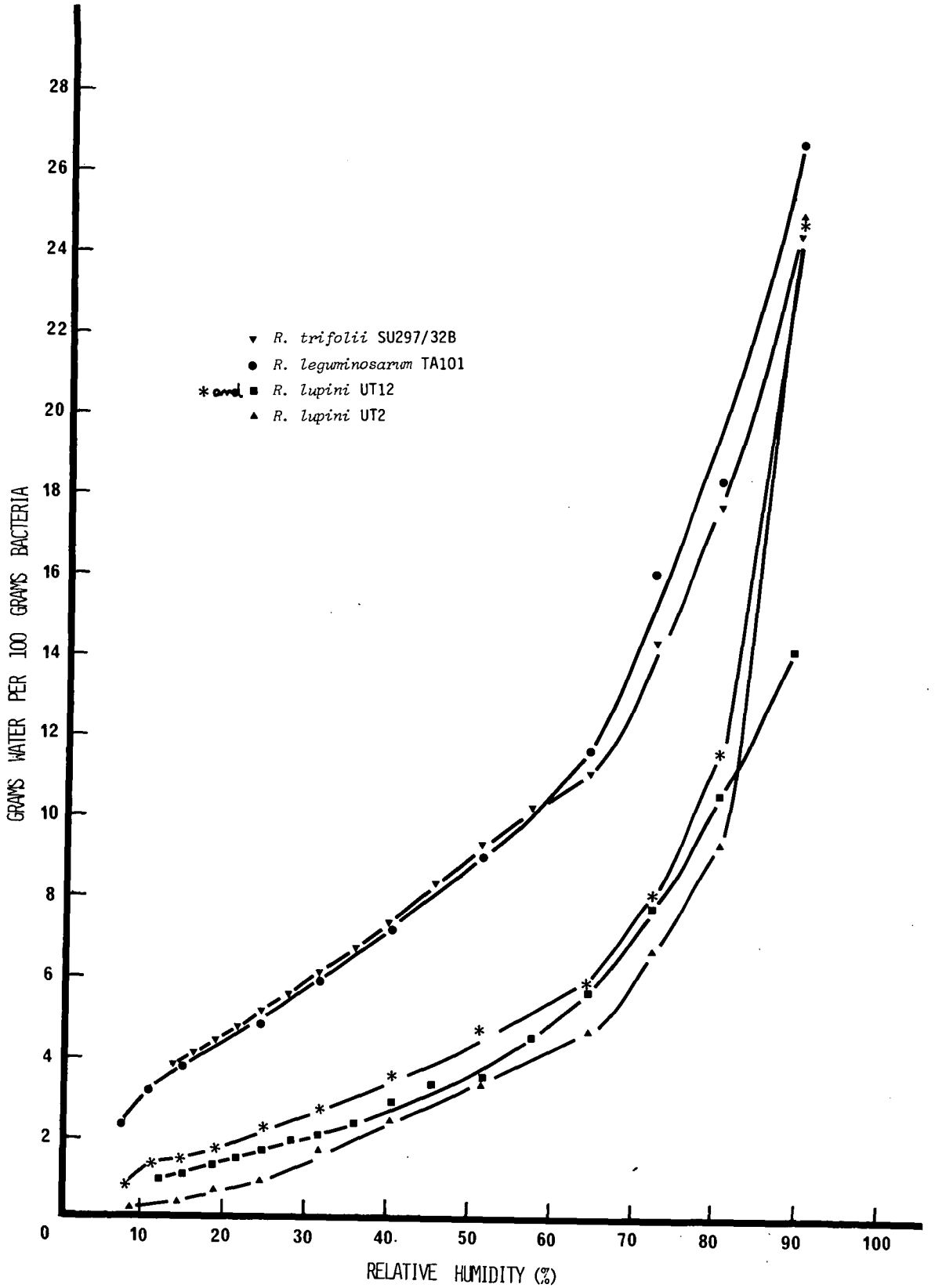

R. trifolii SU297/31A
Ca2+-montmorillonite
R. japonicum QA372 (adsorption isotherm)
R. japonicum QA372 (desorption isotherm)
GRAMS WATER PER 100 GRAMS BACTERIA
RELATIVE HUMIDITY (%)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
10
20
30
40
50
60
70
80
90
100

FIGURE 63

Adsorption isotherms for various species of *Rhizobium*.

The detailed results are presented in the Appendix Tables LIV, LVI, LVII and LVIII.


▼ R. trifolii SU297/32B
● R. leguminosarum TA101
* and ■ R. lupini UT12
▲ R. lupini UT2
GRAMS WATER PER 100 GRAMS BACTERIA
RELATIVE HUMIDITY (%)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
10
20
30
40
50
60
70
80
90
100

fast-growing rhizobia were very similar (figures 62 and 63), but there was more variation between isotherms of the slow-growing species. Most of the variation between isotherms of the latter group of bacteria could be attributed to error in the measurement of the initial dry weight of the cells. Because of the small quantities of water adsorbed at low R.H. by the slow-growing rhizobia, any error of the initial dry weight estimates caused variation in the positioning of the isotherms.

Because of the difficulty in accurately measuring the low quantities of water adsorbed at low R.H., the sigmoid-shaped curve, characteristic of most biological material, was not always obtained. Nevertheless, the curves would belong to the Type II category proposed by Brunauer (1945, cited by Adamson, 1967) which corresponds to monolayer adsorption at low R.H. followed, at higher vapour pressures by multilayer adsorption. The characteristic change in slope at low R.H. values of these sigmoid-shaped curves represents the formation of an adsorbed monolayer. The remainder of the curve represents multilayer adsorption.

The adsorption of water by Ca^{2+}-montmorillonite followed, very closely, the adsorption isotherm reported by Roderick, Senich and Demirel (1969). According to Roderick and Demirel (1966), most of the water adsorbed by montmorillonite at vapour pressures below about 0.20 is restricted to the external surface of the clay platelets. At higher vapour pressures, water molecules penetrate between sheets of montmorillonite resulting

in expansion of the interlayer clay lattice. This results in an increase in the surface area available for adsorption of water vapour.

The results for Ca^{2+}-montmorillonite presented in figure 62 demonstrate that, at all relative humidities studied, the clay adsorbed more water per gram than both the fast-and the slow-growing rhizobia.

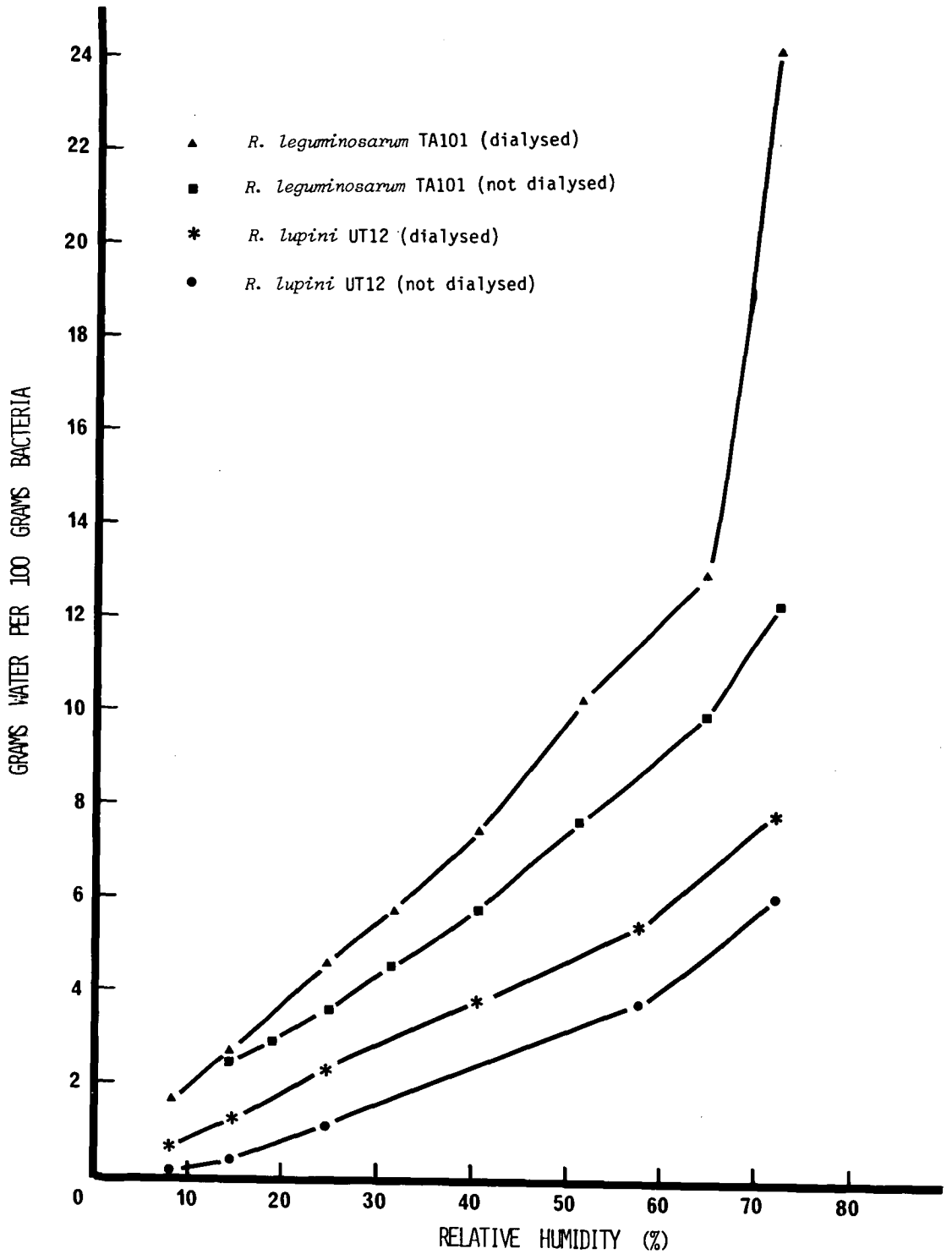
It was reported by Walker et al. (1973) that residual salts bound to myosin B affected the water sorption characteristics of this protein. To test the importance of dialysable salts on the isotherms of rhizobia, bacteria were disrupted with the non-ionic detergent Triton x-100 and the isotherms of both dialysed and undialysed bacterial suspensions were determined. The results for R.leguminosarum TA101 and R.lupini UT12 (figure 64)indicate that water adsorbed by bacteria disrupted by Triton x-100 was slightly less than the quantity of water adsorbed by the untreated bacteria (figure 63). The effect of dialysis of disrupted R.leguminosarum TA101 was to change both the slope and the position of the isotherm. At R.H. values greater than 15%, the disrupted, dialysed R.leguminosarum TA101 adsorbed more water per gram than did the disrupted, undialysed sample. This was especially noticeable at R.H. greater than 65%. The effect of dialysable solutes was to decrease the amount of water adsorbed by macromolecules of this bacterium.

Qualitatively similar results were obtained for R.lupini UT12 (figure 64), although the effect at R.H. values greater than 65% was not as marked as with R.leguminosarum. Therefore,

FIGURE 64

Adsorption of water vapour by R.leguminosarum TA101 and R.lupini UT12. The bacteria used to obtain the isotherms were disrupted with the detergent Triton x-100 and a portion was dialysed to remove the dialysable solutes.

The detailed tabulated results are in Appendix Table LIX and LX.


GRAMS WATER PER 100 GRAMS BACTERIA
RELATIVE HUMIDITY (%)
R. leguminosarum TA101 (dialysed)
R. leguminosarum TA101 (not dialysed)
R. lupini UT12 (dialysed)
R. lupini UT12 (not dialysed)
0
2
4
6
8
10
12
14
16
18
20
22
24
10
20
30
40
50
60
70
80

74/5/132

dialysis of disrupted suspensions of R.lupini UT12 resulted in greater amounts of water adsorbed, relative to the disrupted, undialysed samples.

The results presented in figure 64 suggest that the large differences between the isotherms of the fast-and slow-growing root-nodule bacteria (figures 62 and 63), were not due entirely to the effect of internal solutes on the characteristics of water adsorption by the macromolecules constituting each bacterial group.

I(ii) Calculations based on the water adsorption isotherms.

Application of the B.E.T. equation (as described in Methods and Materials) to the water sorption isotherm data presented in figures 62 and 63 gave the results presented in figures 65 to 67 for the fast-growing R.trifolii SU297/32B and R.leguminosarum TA101, figure 68 for R.japonicum QA372, figures 69 and 70 for R.lupini UT12, and figure 71 for R.lupini UT2. The results in figure 72 represent the application of the B.E.T. equation to water sorption data for Ca^{2+}-montmorillonite. As will be noted in figures 65 to 72, the values of $\frac{P/P_o}{W(1-P/P_o)}$ have not always been plotted up to a vapour pressure of 0.9. These points were omitted because of the large deviations from the straight line. Such deviations are a frequent occurrence when the B.E.T. is applied to sorption of gases on solid surfaces(Bull, 1944; Hoover and Mellon, 1950; Roderick and Demriel, 1966; Ling 1972), and can be attributed to the fact that the B.E.T. equation is largely an empirical

mathematical expression describing water adsorption to "pure" surfaces and not to complex biological systems.

The results in figures 65 and 66 show good agreement between the isotherm data for R.trifolii SU297/32B and the B.E.T. equation, up to a vapour pressure of 0.60. Similarly, there is little deviation from linearity of the points for R.leguminosarum TA101 up to a vapour pressure of 0.70 when plotted by the B.E.T. equation. The greater scatter of the results for the slow-growing rhizobia (figures 68 to 71) is attributed to the difficulty of measuring small amounts of water adsorbed by these bacteria, particularly at low R.H. values.

Values for 1/WmC (the intercept of the line with the y axis) and (C-1)/WmC (the slope of the line) can be determined from figures 65 to 72, and a compilation of these results is presented in table 31. From these parameters, the quantity of water required for monolayer formation (Wm) and the value of C (a constant in the B.E.T. equation related to the average heat of adsorption of a monolayer of water), were calculated for various species of Rhizobium and for Ca^{2+} montmorillonite. The results are presented in table 32. The average Wm value for the fast-growing rhizobia (4.681 gm H_2O/100 gm bacteria) is approximately twice that for the slow-growing bacteria (2.449 gm H_2O/100 gm bacteria) whereas the Wm value for Ca^{2+} montmorillonite is 6.916 gm H_2O/100 gm. clay. This value for Ca^{2+} montmorillonite is in close agreement with the value

FIGURE 65

Application of the B.E.T. equation to the water sorption isotherm data of R.trifolii SU297/32B presented in figure 63.

Derivation of the points plotted are shown in Appendix Table LXI.

The dotted line represents the deviation from linearity at higher vapour pressures.

W = the amount of water adsorbed at vapour pressure, p.

Po = the saturation vapour pressure.

The slope of the line $\frac{(C-1)}{WmC}$ and the intercept $(1/WmC)$ with the y-axis are presented in Table 31.

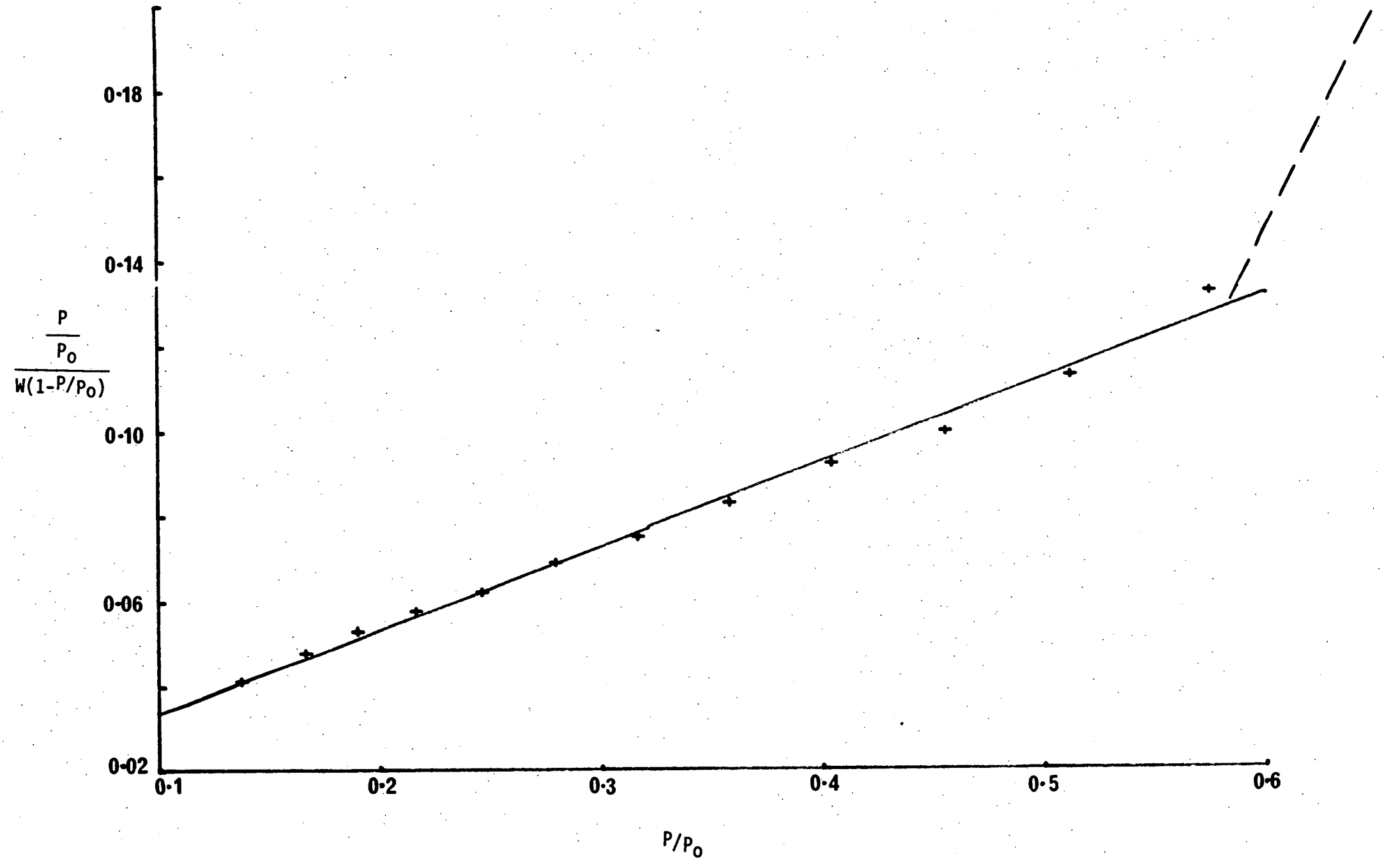

0·18
0·14
0·10
0·06
0·02
$\frac{P}{P_o}$
$W(1-P/P_o)$
0·1
0·2
0·3
0·4
0·5
0·6
P/P_o

FIGURE 66

Application of the B.E.T. equation to the water sorption isotherm data of R.trifolii SU297/32B presented in figure 62.

Derivation of the points plotted are shown in Appendix Table LXII.

Other details are as for figure 65.

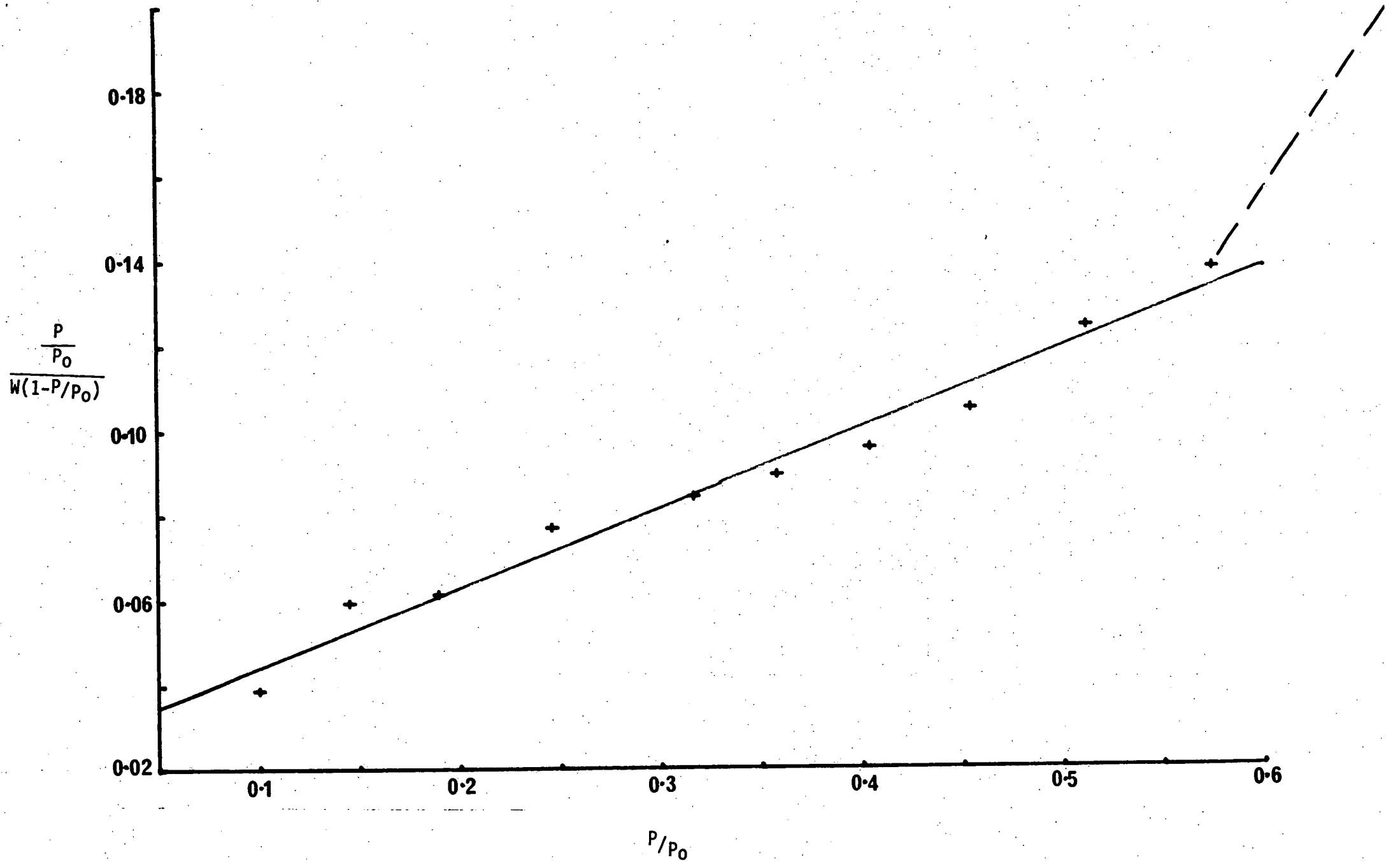

0·18
0·14
0·10
0·06
0·02
$\frac{P}{P_o}$
$W(1-P/P_o)$
0·1
0·2
0·3
0·4
0·5
0·6
P/P_o

FIGURE 67

Application of B.E.T. equation to water sorption isotherm data for R. leguminosarum TA101 presented in figure 63.

Derivation of the points plotted are shown in Appendix Table LXIII.

Other details are as for figure 65.

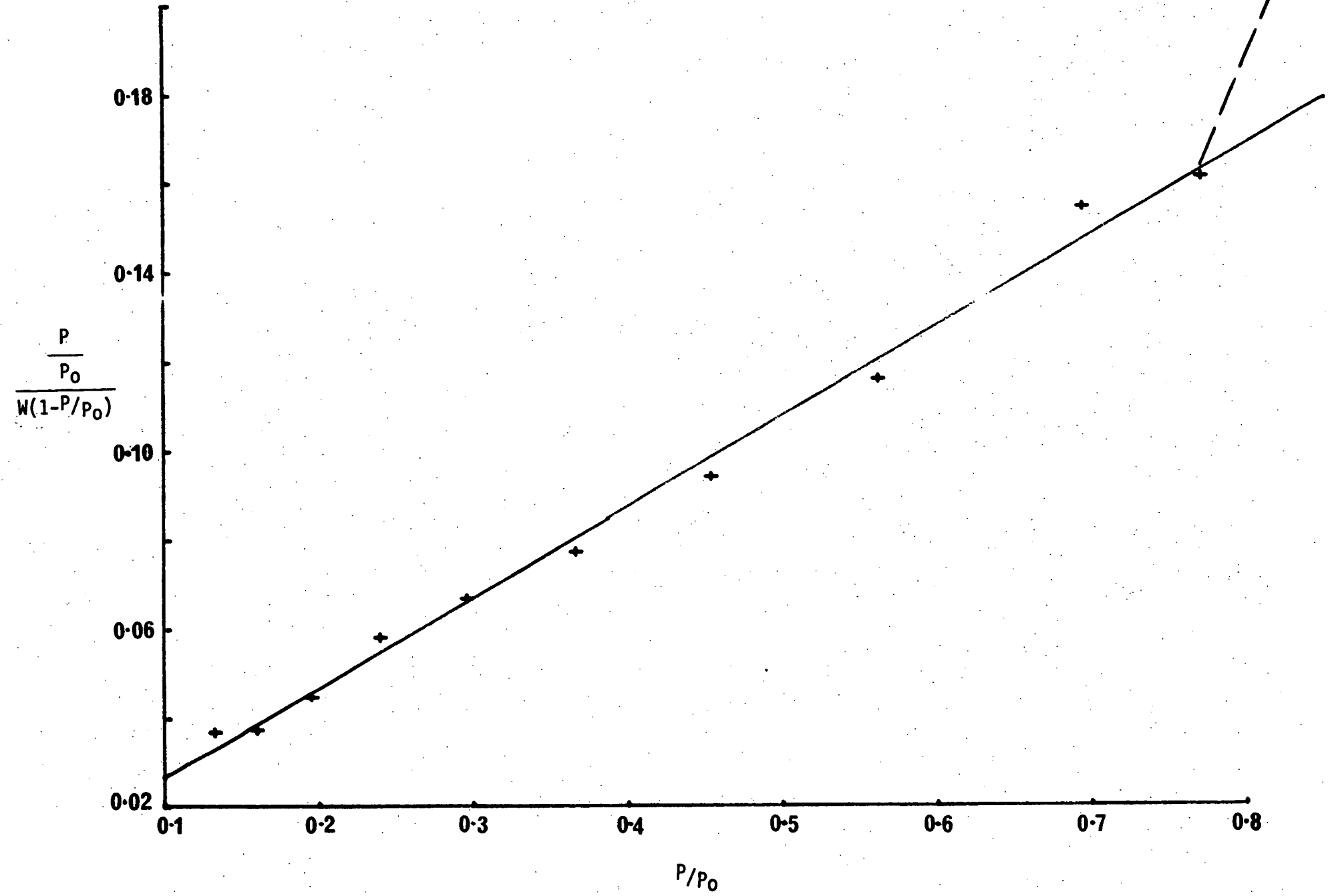

0·18
0·14
0·10
0·06
0·02
P / Po / W(1-P/Po)
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
P/Po

FIGURE 68

Application of B.E.T. equation to the water sorption isotherm data for *R. japonicum* QA372 presented in figure 62.

Derivation of points is shown in Appendix Table LXIV.

Other details are as for figure 65.

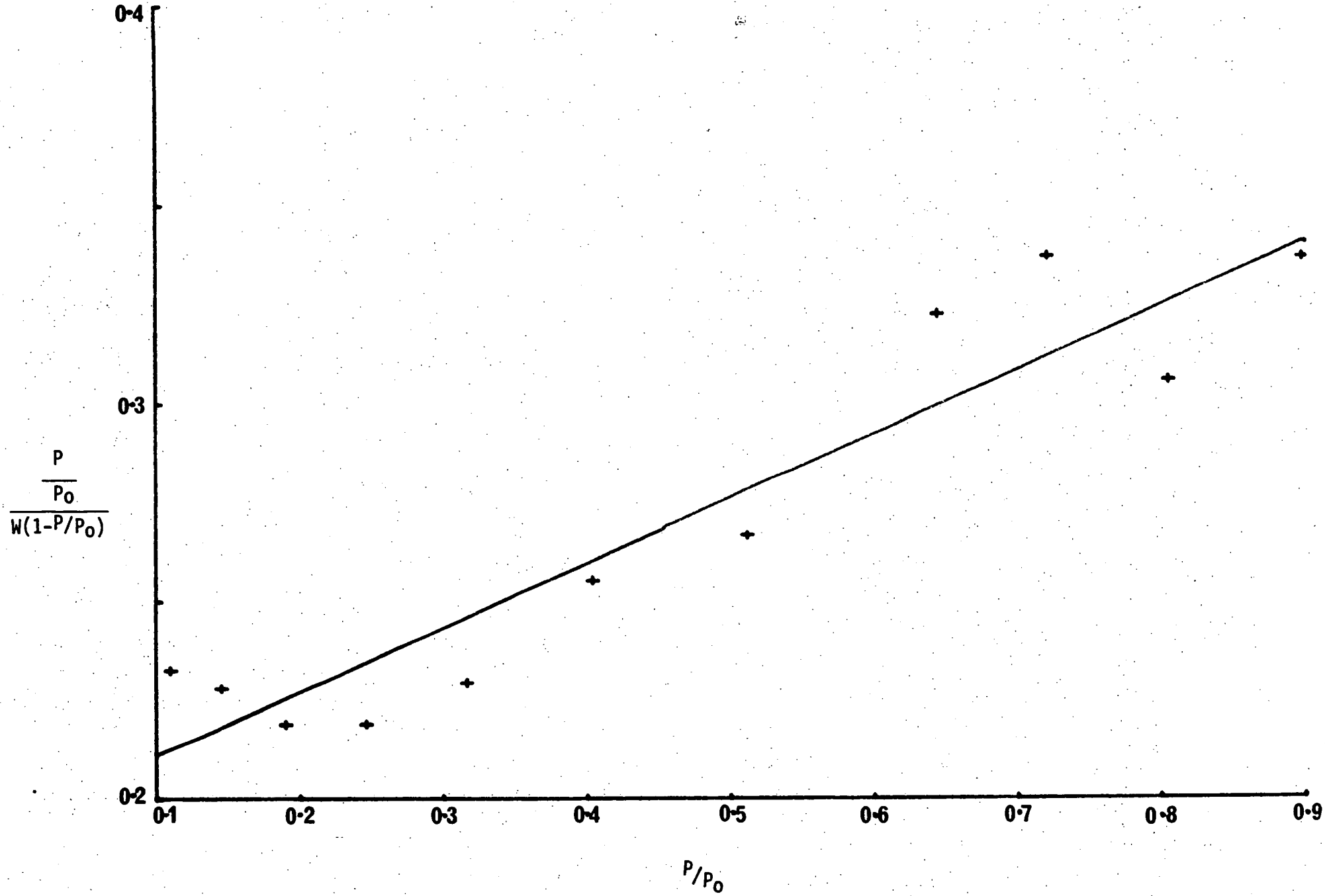

0·4
0·3
0·2
$\frac{P}{P_o}$
$W(1-P/P_o)$
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
P/P_o

FIGURE 69

Application of B.E.T. equation to water sorption isotherm data for R. lupini UT12 presented in figure 63.

Derivation of plotted points is shown in Appendix Table LXV.

Other details are as for figure 65.

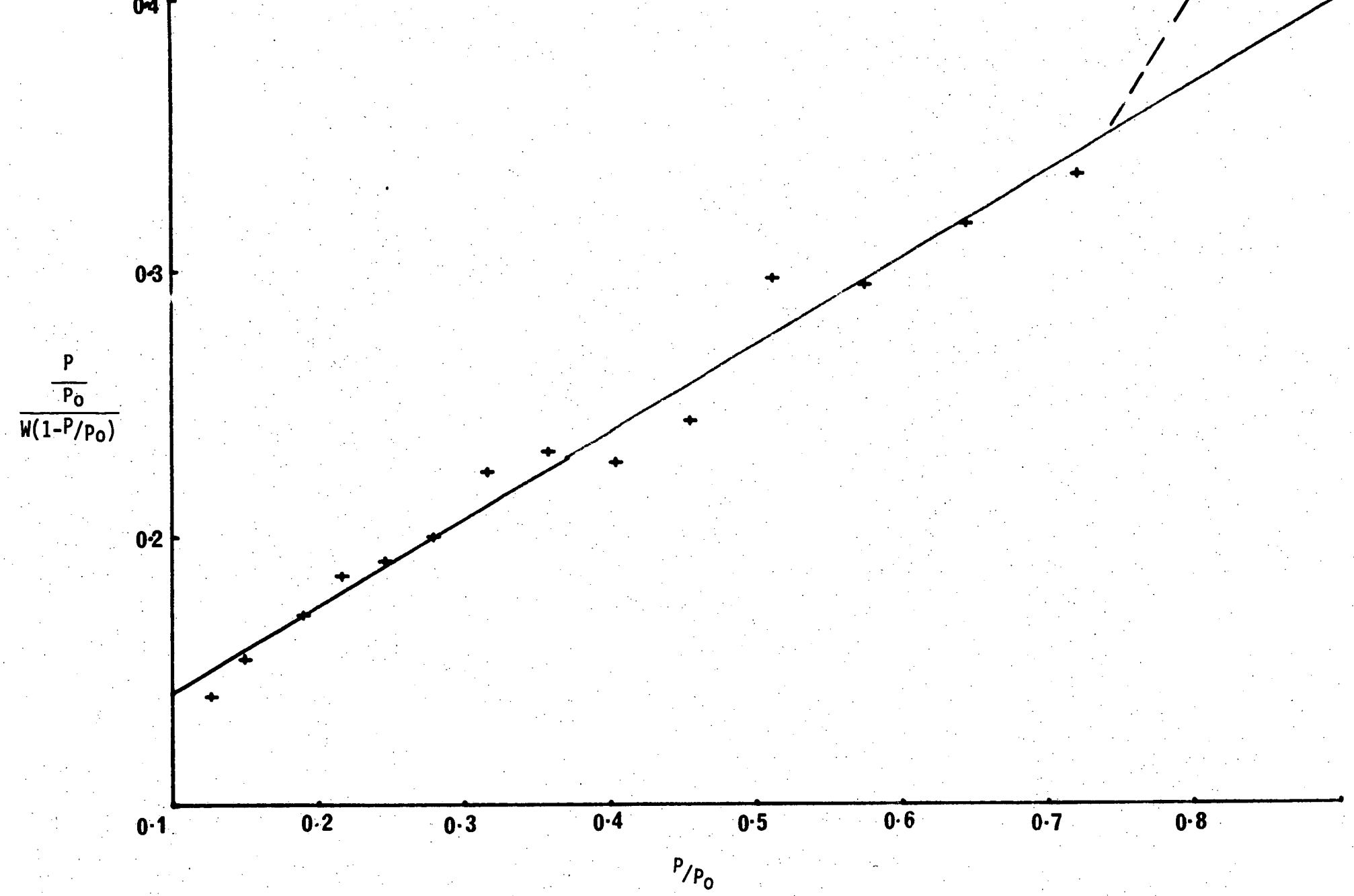

0·4
0·3
0·2
0·1
P / Po / W(1-P/Po)
0·2
0·3
0·4
0·5
0·6
0·7
0·8
P/Po

FIGURE 70

Application of B.E.T. equation to the water sorption isotherm data for R.lupini UT12 presented in figure 63.

Derivation of the points plotted are shown in Appendix Table LXVI.
Other details are as for figure 65.

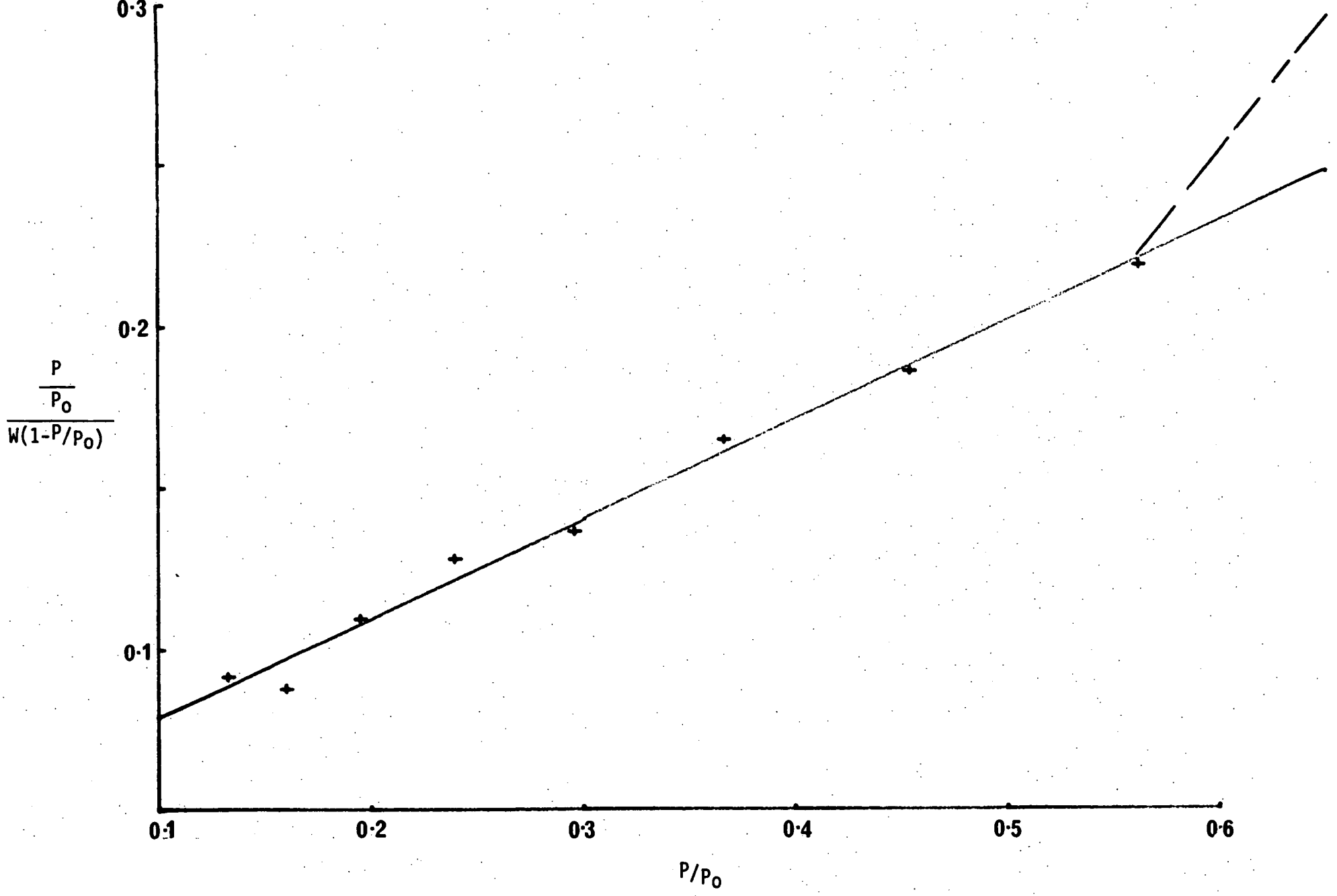

0·3
0·2
0·1
P / Po / W(1-P/Po)
0·1
0·2
0·3
0·4
0·5
0·6
P/Po

FIGURE 71

Application of the B.E.T. isotherm equation to isotherm data for R.lupini UT2 presented in figure 63.

Derivation of the points plotted are presented in Appendix Table LXVII.

Other details are as for figure 65.

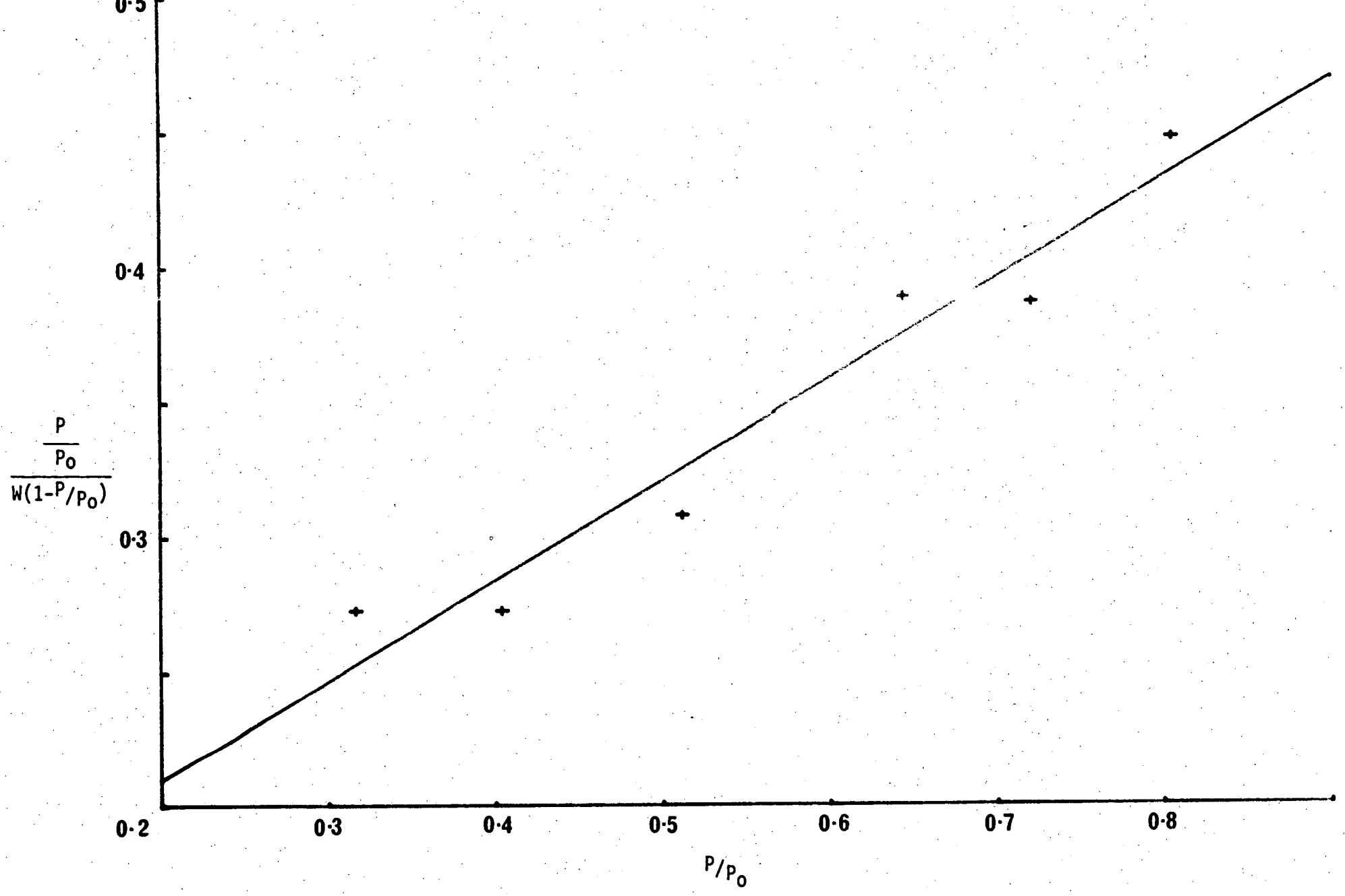

0·5
0·4
0·3
0·2
P / Po / W(1-P/Po)
0·3
0·4
0·5
0·6
0·7
0·8
P/Po

FIGURE 72

Application of the B.E.T. equation to water sorption data of Ca^{2+}-montmorillonite presented in figure 62.

Derivation of the points plotted are presented in Appendix Table LXVIII.

Other details are as for figure 65.

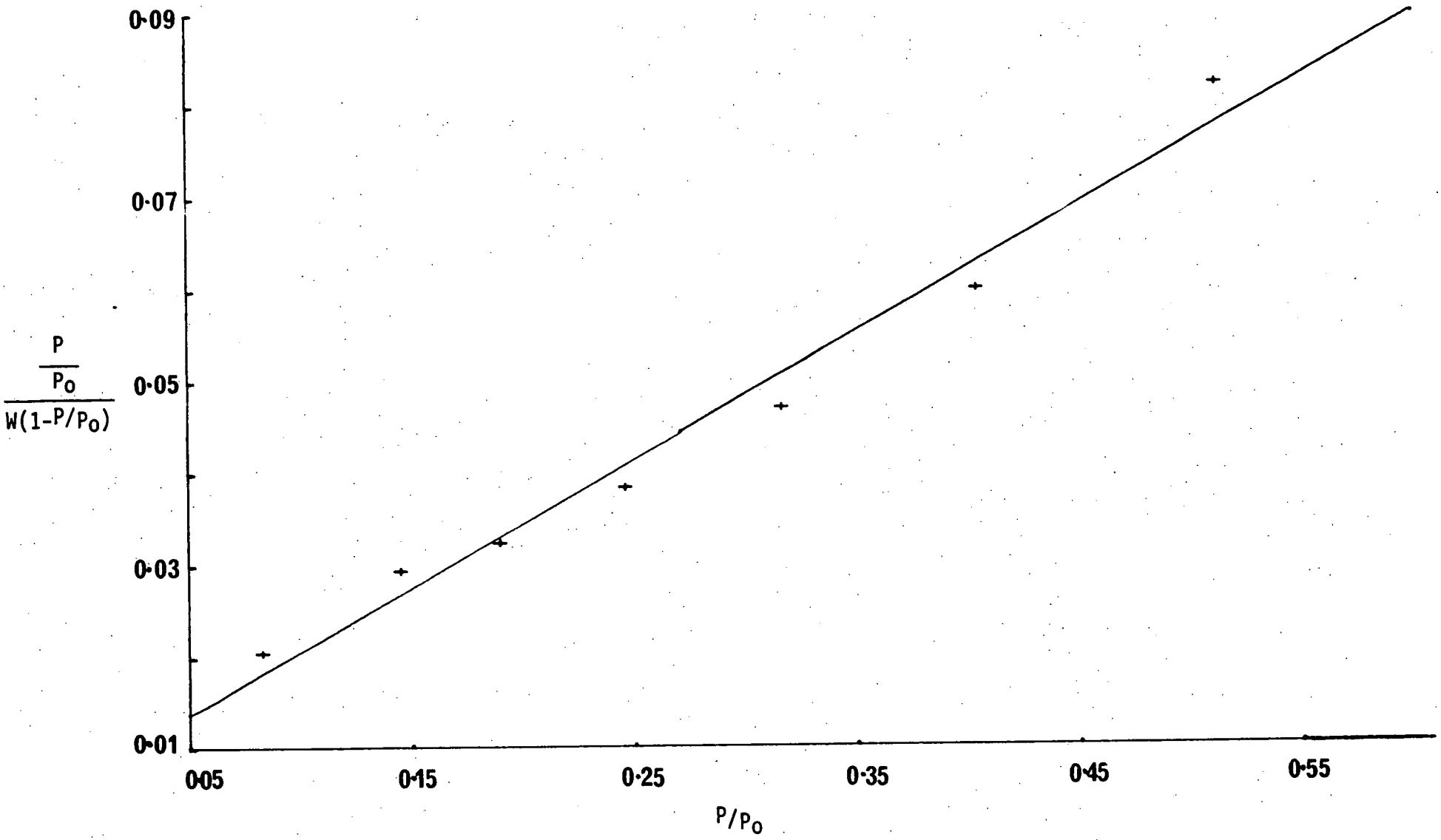

0·09
0·07
0·05
0·03
0·01
P / Po / W(1-P/Po)
0·05
0·15
0·25
0·35
0·45
0·55
P/Po

Table 31

Values derived for the intercepts and slopes of graphs obtained by the application of the B.E.T. equation to the isotherm data presented in Appendix Tables LI to LVIII for various rhizobia and Ca^{2+}- montmorillonite.

Group		Intercept ($1/_{WmC}$)	Slope $\frac{(C-1)}{WmC}$
Fast-growing	R.trifolii SU297/32B	0.0144	0.1953
	R.trifolii SU297/32B	0.0258	0.1865
	R.leguminosarum TA101	0.0166	0.2025
Slow-growing	R.japonicum QA372	0.1951	0.1617
	R.lupini UT12	0.1102	0.3221
	R.lupini UT12	0.0638	0.3056
	R.lupini UT2	0.1358	0.3709
Ca^{2+}-montmorillonite		0.0070	0.1376

Wm=the amount of water required for monolayer formation.

C =a constant in the B.E.T. equation which is related to the heat of adsorption of a monolayer of liquid.

reported by Roderick and Demirel (1966).

If it is assumed that the adsorbed water molecules were in the closest packing arrangement, then the average cross-sectional area for a water molecule would be 10.5 $\mathring{A}^2$ (Roderick and Demirel, 1966) and, from the Wm values, the adsorptive surface areas can be calculated. These are presented in table 32. There are consistent, large differences in the sorptive surface areas between the fast-and slow-growing rhizobia. The average surface area available for water adsorption for the slow-growing species was 86.01 m^2/gm, which is only one half that available in the fast-growing rhizobia. The adsorptive surface area of Ca^{2+}-montmorillonite according to the B.E.T. equation is 242.87 m^2/gm. (table 32).

The results of C (a constant related to the heat of adsorption of a monolayer) presented in table 32, differed for the fast-and slow-growing rhizobia and for Ca^{2+} montmorillonite. This probably reflected the different quantities of water required for monolayer formation because, when C was expressed as per unit weight of water required for monolayer formation (C/Wm), the values for the bacteria and clay were similar, at least within the limits of error (table 32). Although the calculated value of C was only an approximation to the true value for the heat of adsorption, the similarity of the C/Wm quantities in both bacterial groups implied that there was no difference in the energy with which water molecules were held by the different species of rhizobia.

Table 32

Compilation of the constants derived from an application of the B.E.T. equation to water adsorption isotherm data for various rhizobia and Ca^{2+}- montmorillonite. Total free energy changes (Δ F) were obtained by solution of Bangham's free energy equation (see text).

Group	Wm (gm. water sorbed per 100 gm. substance)	C	$\frac{C}{Wm}$	A (m^2/gm)	Δ F (cal./m^2)
Fast-growing					
R.trifolii SU297/32B	4.769	14.563	3.054	167.5	-667.74
R.trifolii SU297/32B	4.710	8.229	1.750	165.4	-619.13
R.leguminosarum TA101	4.564	13.199	2.892	160.3	-711.86
Group Means	4.681	11.997	2.565	164.9	-666.24
Slow-growing					
R.lupini UT12	2.707	5.790	2.139	95.06	-613.27
R.lupini UT12	2.313	3.920	1.696	81.23	-604.05
R.lupini UT2	1.974	3.731	1.891	69.32	-547.94
R.japonicum QA372	2.803	1.829	0.653	98.43	-490.54
Group Means	2.449	3.818	1.595	86.01	-563.95
Ca^{2+}-montmorillonite	6.916	20.657	2.987	242.87	-668.22

Wm = quantity of water required to form a monolayer.
C=a constant related to the heat of adsorption of the monolayer.
A=specific surface area of the adsorbing material.

A more accurate estimate of the energy involved in wetting of bacteria and montmorillonite may be obtained by application of Bangham's (1937) free energy equation to the isotherm data. The equation can be expressed as:-

$$\Delta F = \frac{RT}{MA} \int_0^1 W/P/Po \; d \; (P/Po)$$

R=the gas constant

T=the absolute temperature

M=the molecular weight of water

A=the specific surface area of the adsorbing material

W=the weight of water adsorbed at a vapour pressure of P

Po=the saturation vapour pressure

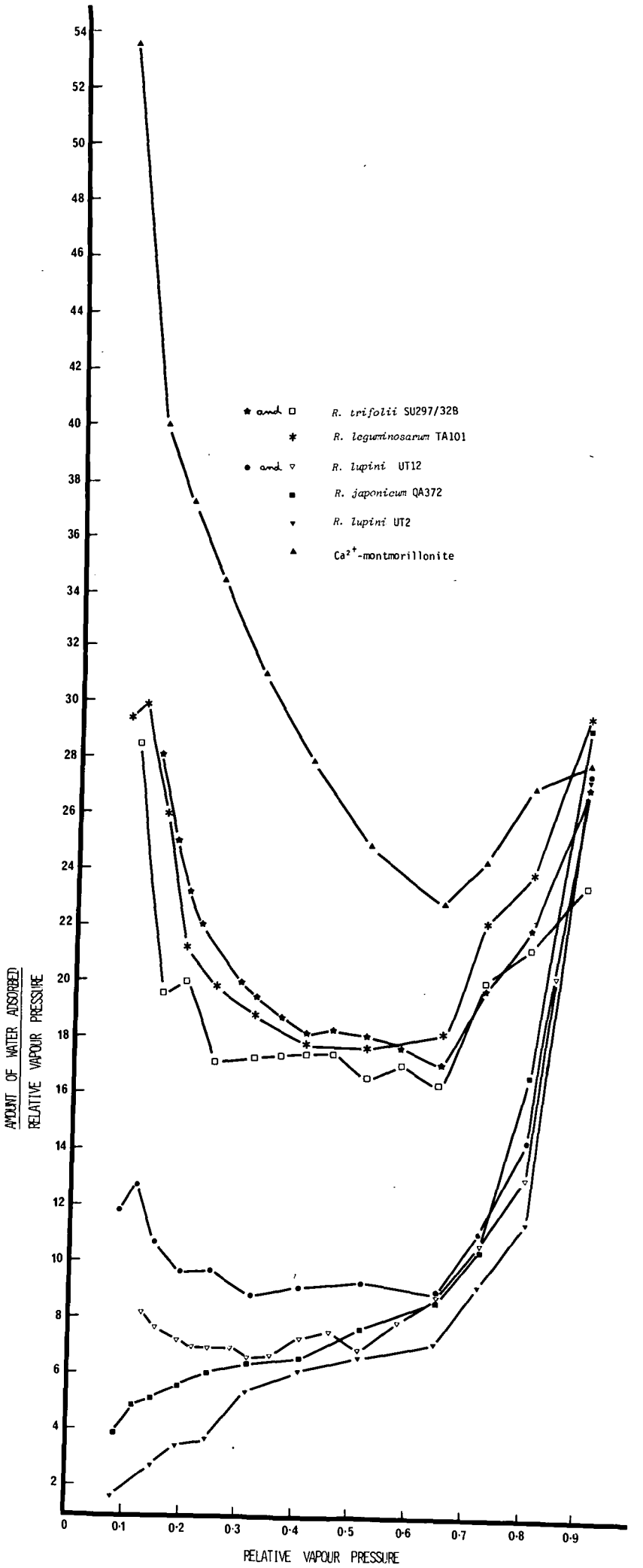
Δ F=the change in free energy associated with wetting.

Evaluation of ΔF was obtained by graphical integration of plots of W/P/Po versus P/Po shown in figure 73. The areas under the curves were obtained by the use of a planimeter (A.OTT,Kempten) and are presented in appendix table LXXI as AΔ F values for each bacterium and the Ca^{2+}-montmorillonite. By the use of estimates of the adsorptive surface areas obtained from the B.E.T. data, the values of Δ F were obtained and are presented in table 32. The Δ F values determined for the rhizobia fall in the range of values reported by Bull (1944) for

FIGURE 73

Plots for the integration of Bangham's free energy equation for water adsorption to Ca^{2+}-montmorillonite and various fast- and slow-growing root-nodule bacteria.

For details of values plotted, see Appendix Tables LXIX and LXX.


AMOUNT OF WATER ADSORBED
RELATIVE VAPOUR PRESSURE
54
52
50
48
46
44
42
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
RELATIVE VAPOUR PRESSURE
★ and □ R. trifolii SU297/32B
✱ R. leguminosarum TA101
● and ▽ R. lupini UT12
■ R. japonicum QA372
▼ R. lupini UT2
▲ Ca²⁺-montmorillonite

various proteins. Generally, the total ΔF values for the fast-growing rhizobia were greater than those for the slow-growing species although a range of values is evident for both groups of bacteria i.e. the average total free energy change per unit area associated with the adsorption of water vapour up to a R.H. of 90% is greater for the fast-growing rhizobia than for the slow-growing species. As ΔF is a measure of the affinity of the adsorbing material for water (Bull, 1944) it would seem that the fast-growing root-nodule bacteria have a slightly higher affinity for water than the slow-growing species, in addition to a greater adsorptive surface area. There is little further comment to be made on the total free energy changes on hydration because they are probably the summation of a number of complicated factors.

If Bangham's free energy equation is evaluated at various P/Po values up to 0.90, an indication of the change in ΔF due to adsorption of water can be obtained. Bull (1944) and Sharma et al. (1969) evaluated the integral:-

$$\frac{-RT}{M}\int_{0}^{P/Po} W/P/Po \; d(P/Po)$$

to give values of $A\Delta F$ for porous solids. The values obtained, $A\Delta F$, are the free energy changes per gram of solid. The $A\Delta F$ values were plotted because of uncertainties about the validity of the B.E.T. equation when dealing with adsorption of polar gases to porous solids over a wide range of vapour pressures. Because of this uncertainty the B.E.T. surface areas may not apply at all vapour pressures and be subject to

errors. The results presented in figure 74 show the relationship between the free energy change/gm of solid and the relative vapour pressure. At all vapour pressures, the free energy changes per gram or the surface energy (Sharma et al. 1969) for the fast-growing rhizobia are greater than the values for the slow-growing species. The surface energies per gram of Ca^{2+}-montmorillonite are very much greater than the values of either the fast-or the slow-growing rhizobia at most vapour pressures. Bull (1944) studied the free energy changes of various proteins as a function of water content and noted that the free energy change per gram of water adsorbed was a constant from zero to between 1 and 4% water depending on the protein. This was followed by a steep upswing in the curve i.e. there was a gain in the affinity of proteins for water as water was adsorbed. The results presented for the fast-and slow-growing rhizobia and for Ca^{2+}-montmorillonite, are in qualitative agreement with the trends reported for proteins by Bull (1944).

The results presented in figure 75 were obtained by dividing the surface energies at different vapour pressures (see figure 74) by the surface area for each solid as derived by the B.E.T. equation (see table 32). As stated above, there is some doubt as to the validity of the surface area estimates of porous solids as determined by the B.E.T. theory when polar liquids (such as water) are used. Therefore, the values plotted in figure 75 may be subject to error. The results in figure 75 still show the trend demonstrated in figure 74, as the free

FIGURE 74

Plots of the change in surface energy ($A\Delta F$) per gram for both fast-and slow-growing rhizobia and for Ca^{2+}-montmorillonite.

The values plotted are presented in Appendix Table LXI.

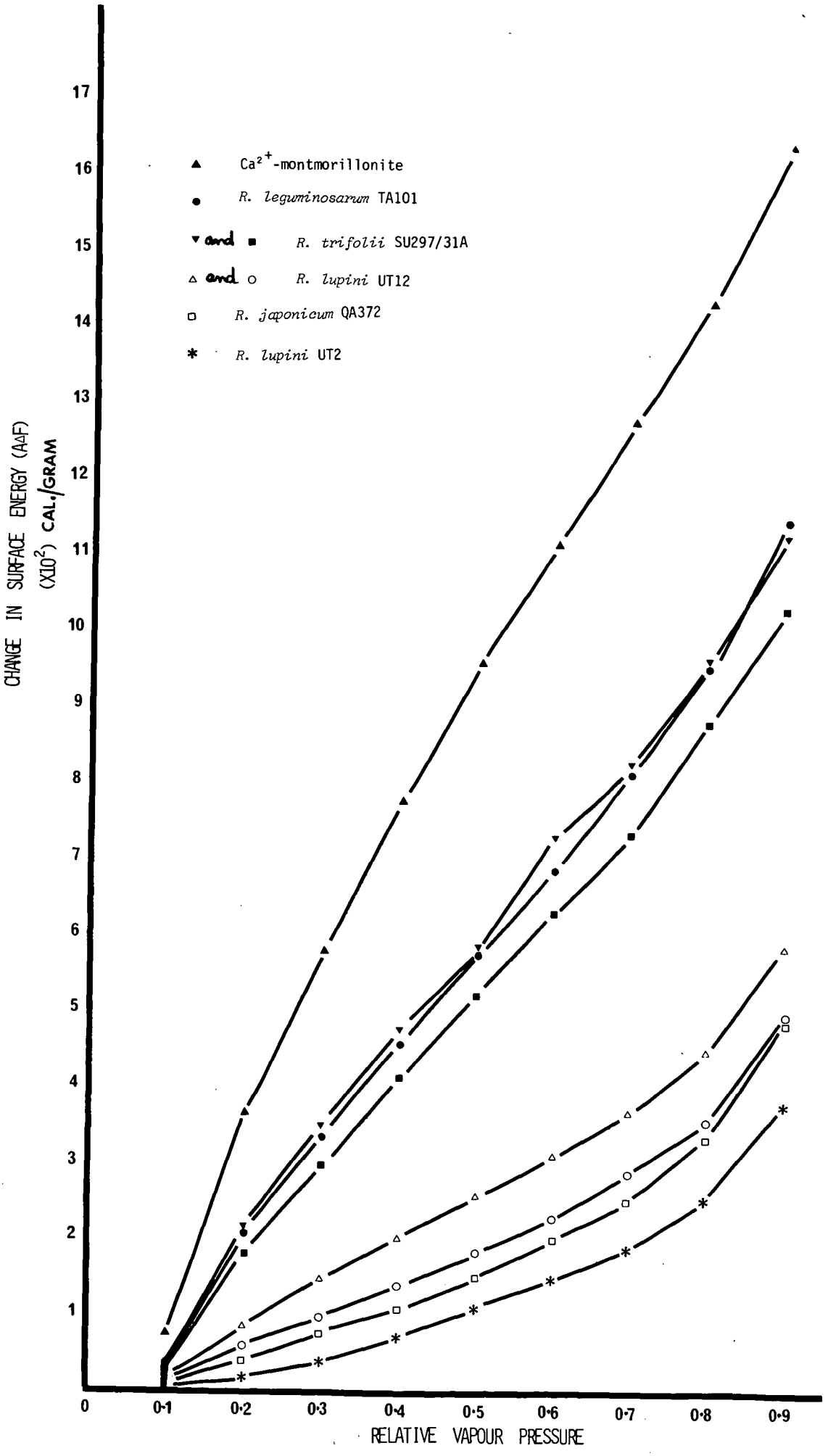
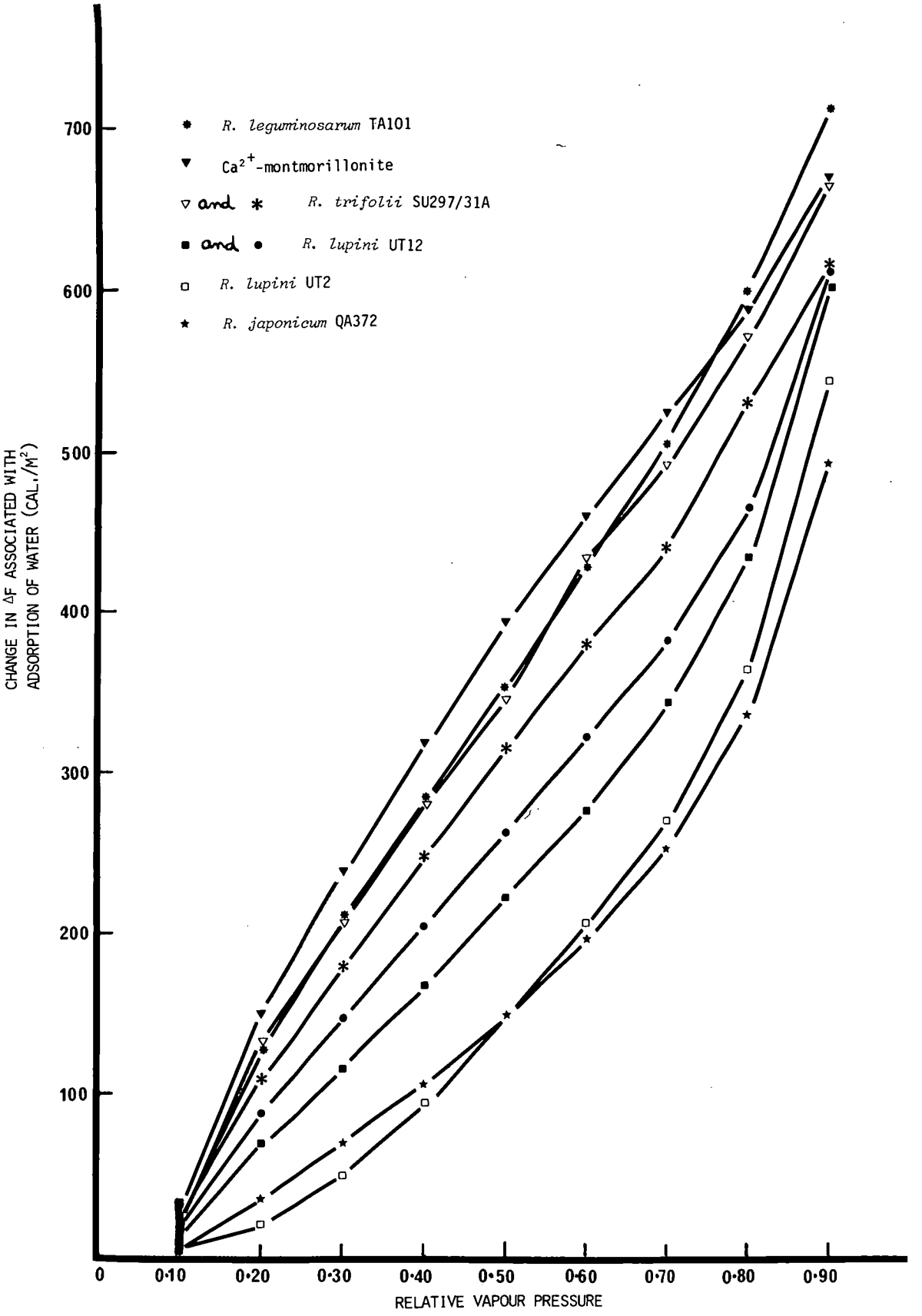

▲ Ca^{2+}-montmorillonite
● *R. leguminosarum* TA101
▼ and ■ *R. trifolii* SU297/31A
△ and ○ *R. lupini* UT12
□ *R. japonicum* QA372
* *R. lupini* UT2
CHANGE IN SURFACE ENERGY (AΔF) (X10²) CAL./GRAM
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
RELATIVE VAPOUR PRESSURE

FIGURE 75

Plots of the change in free energy per m^2 associated with the adsorption of water vapour by fast-and slow-growing rhizobia, as well as for Ca^{2+}-montmorillonite.

The values plotted are presented in Appendix Table LXII and were obtained by dividing the surface energies at different vapour pressures (see figure 74) by the surface area for each solid as derived by the B.E.T. equation (see table 32).


R. leguminosarum TA101
Ca2+-montmorillonite
R. trifolii SU297/31A
and
R. lupini UT12
and
R. lupini UT2
R. japonicum QA372
CHANGE IN ΔF ASSOCIATED WITH ADSORPTION OF WATER (CAL./M2)
700
600
500
400
300
200
100
0
0·10
0·20
0·30
0·40
0·50
0·60
0·70
0·80
0·90
RELATIVE VAPOUR PRESSURE

energies/m^2 for the fast-growing rhizobia are greater than the values for the slow-growing species. Similarly, the free energy changes/m^2 for the Ca^{2+}-montmorillonite are greater than both the fast-and the slow-growing rhizobia at all vapour pressures except 0.90.

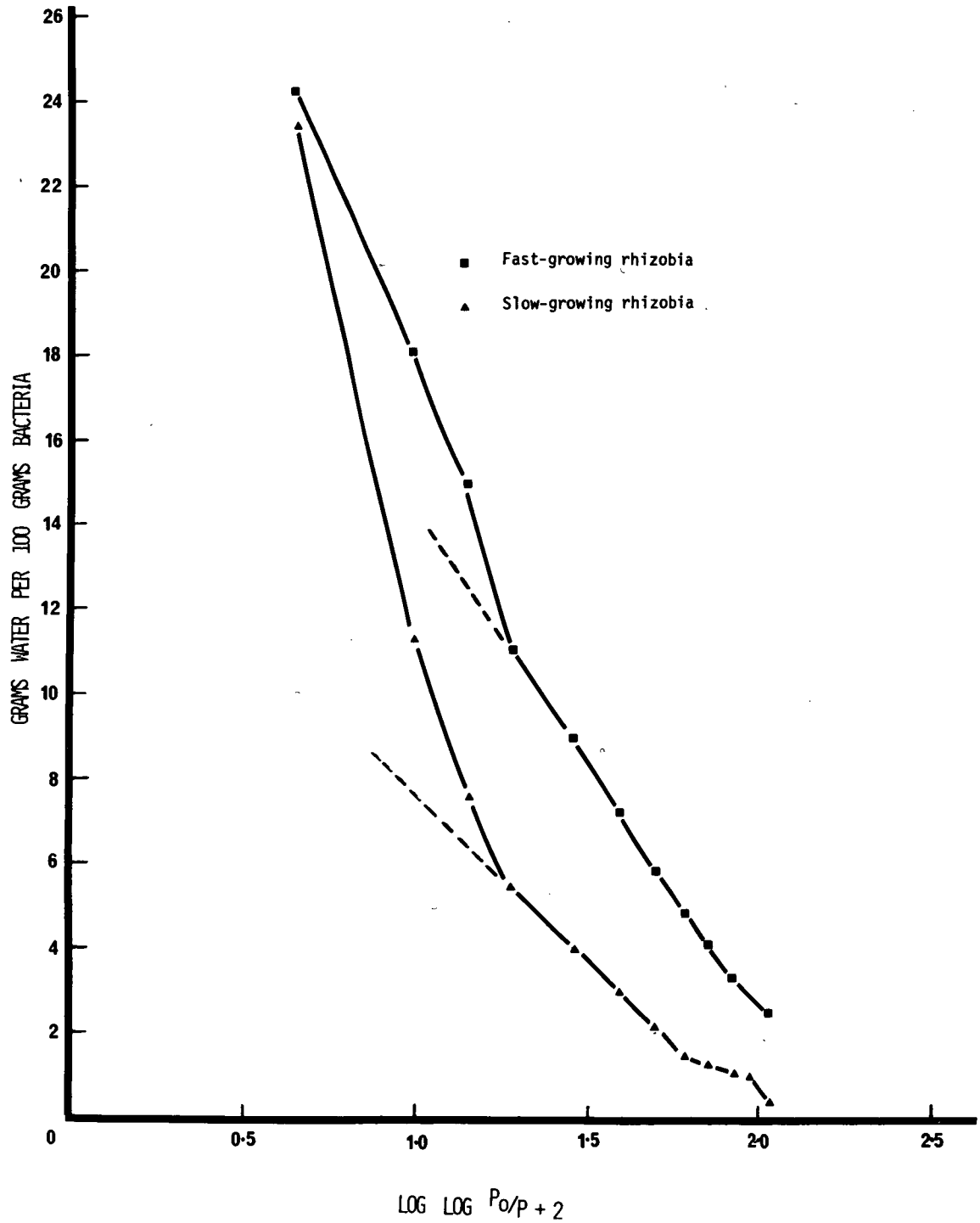
To test the closeness-of-fit of the isotherm data to the Bradley equation, average values for the fast-growing rhizobia and for the slow-growing species were used. The values of log log Po/P versus W (the amount of water adsorbed at any vapour pressure) are plotted in figure 76. A constant of +2 was added to the log log Po/P term in the graphical representation of the Bradley equation so that all the plotted points were of a positive sign. As stated in the Methods and Materials, this plot should give a straight line. As is obvious from figure 76, the lines for both the fast-and slow-growing rhizobia began to diverge from linearity at about the same point. This point corresponded to a vapour pressure of about 0.65. Deviation from linearity also occurred at low relative humidities. This was expected because the function log log Po/P approaches infinity as P approaches zero.

The purpose in testing the goodness-of-fit of the isotherm data to the Bradley equation for both groups of rhizobia, was to compare the accuracy of the predictions of this equation with those of the B.E.T. theory. Ling (1965,1972) has used the closeness-of-fit of isotherm data to the Bradley equation as circumstantial evidence in support of his contention

FIGURE 76

Plots of the moisture adsorbed by an average fast-and an average slow-growing Rhizobium in terms of the Bradley isotherm equation.

Details of the calculations for each point plotted are presented in Appendix Table LXXIII.


GRAMS WATER PER 100 GRAMS BACTERIA
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0·5
1·0
1·5
2·0
2·5
Fast-growing rhizobia
Slow-growing rhizobia
LOG LOG Po/P + 2

that water in cells exists as multiple polarized layers on charged polymers such as proteins. Bradley derived his equation describing isotherm data by taking into account the possible interaction between the permanent dipolar nature of water molecules and a charged adsorbing surface. Thus, monolayer adsorption of dipolar water molecules on active sites of proteins takes place at low R.H. Subsequent multilayer adsorption is by induced polarization (or alignment) of the adsorbing water molecules by the active surface. The theory of Bradley, therefore, introduced a mechanism for the propagation of dipolar forces throughout the dipolar water system. In the B.E.T. theory, multiple layers of water were assumed to be held by the usual vander Waals forces present in liquid water.

As can be seen from an application of the B.E.T. equation (figures 65 to 71) and the Bradley theory (figure 76) both equations generally fail to predict the amount of water adsorbed above a relative humidity of 65%. Consequently, little can be said about the suitability of one equation over the other when describing the adsorption of water by the rhizobia.

5. DISCUSSION

The stability and proper functioning of macromolecules, structures and, ultimately whole bacteria, after dehydration stress must depend on the degree and type of interactions between constituent molecules during treatment. Complete removal of water must involve large electrostatic interactions as charged sites on polymers are transformed from a medium of high dielectric constant to an essentially anhydrous medium of low dielectric constant. Thus, there would be many opportunities for extensive intra-and intermolecular reactions to occur under these conditions. Associated with the removal of water from bacteria is the inactivation of biological molecules by oxygen at low R.H., as well as concentration of intracellular solutes, some of which may reach toxic concentrations. These are all factors which must contribute to the death of dried microorganisms.

The results reported in this thesis indicated that the slow-growing root-nodule bacteria had a greater inherent ability to survive dehydration than the fast-growing species. Survival of the medic rhizobia was usually in between these two groups. By desiccation of the fast-growing bacteria in some montmorillonite-amended soil samples, substantial increases in the survival were obtained. These results are in agreement with those published by Marshall (1964) who demonstrated the capability of montmorillonite to protect R. trifolii from desiccation.

As an approximation, desiccation of the slow-growing rhizobia reduced the viable count from 10^8 to about 10^6 bacteria per 0.05 gm soil. The effect upon the fast-growing species was to reduce the viable numbers from 10^8 to about 10^4 per 0.05 gm. unamended soil, and to about 10^6 bacteria per 0.05 gm. in montmorillonite-amended soil samples. Therefore, desiccation caused a very large percentage decline in the viable population of both the fast-and the slow-growing rhizobia and, on a percentage basis, the effect of montmorillonite on the survival of fast-growing rhizobia was small. In terms of the field situation, however, the increase of approximately 100 fold of the viable population of fast-growing rhizobia when dried in association with montmorillonite, would represent a significant increase in the availability of bacteria for root colonization and nodulation of the legume host.

Measurements of the relative amounts of water associated with desiccated fast-and slow-growing rhizobia have been made by determination of their water sorption isotherms. There was a good inverse correlation between the water content of rhizobia at various vapour pressures and dehydration resistance. More water was associated with the dehydration susceptible fast-growing species than was retained by the resistant ones at all vapour pressures except 0.90 and above. This must reflect differences in the water sorption characteristics of the macromolecules which constitute each group of rhizobia. An analysis of the surface energy changes associated with the

adsorption of water revealed that, at all the vapour pressures tested, the surface energies of the fast-growing rhizobia are greater than those for the slow-growing species. This indicated that the affinity for water in the fast-growing bacteria was greater than that in the slow-growing group. This reinforces the conclusion outlined above, that the differences in the water sorption isotherms between the two groups of rhizobia is a reflection of variation in the number (and possibly the type) of water-accessible adsorption sites on the macromolecules from the fast-and slow-growing root-nodule bacteria. This conclusion is also suggested by the greater adsorptive surface area of the former bacteria relative to that of theslow-growing species.

The hydration of macromolecules, especially proteins, has been the subject of much study and the literature has been reviewed by Ling (1972). The author concluded that the primary sites for water sorption to globular proteins at all vapour pressures, are the polar side chains. For the fibrous proteins, polar side chains are the main sites of hydration only at low R.H., but at high vapour pressures the pepide bond is important. For this reason, the fibrous proteins characteristically adsorb much larger quantities of water than the globular macromolecules (Ling, 1972). Although the polymers in the systems described by Ling (1972) were simple when compared to the complexity of the intracellular environment of

bacteria, the principles established are probably still applicable. Therefore, it can be postulated that the disimilarity between the water sorption isotherms of the fast-and slow-growing rhizobia may be due to differences in the number of water-accessible polar sites on the macromolecules. Perhaps a greater proportion of the peptide backbone of proteins in the fast-growing rhizobia are available for water adsorption than in the slow-growing species. Variations of this type between the rhizobia could account for the differences of the isotherms. Vincent and Humphrey (1970) reported differences in the internal antigenic constituents between the fast-and slow-growing rhizobia, and De Ley (1965) found variation of the G:C ratio between these two bacterial groups. Therefore, it is reasonable to postulate that differences in the water sorption isotherms are due to qualitative variations in the variety of macromolecules between the two groups of bacteria.

It has been reported by many authors (eg. Bull and Breese, 1970; Walker et al., 1973) that residual salt drastically effects the characteristics of the water isotherms of proteins. Removal of intracellular solutes from the fast-and slow-growing rhizobia altered their water adsorption properties. For both groups of rhizobia, the effect was to increase the amount of water adsorbed. The tendency for these bacteria to adsorb water, therefore, is reduced by the internal ionic environment. It was expected that the effect of dialysis on water uptake by

slow-growing rhizobia would be very significant because of their higher internal osmotic pressure relative to the fast-growing species. This was not the case, however, as the effect of dialysis on the uptake of water by R.leguminosarum was more pronounced than the effect on the slow-growing R.lupini. Consequently, the importance of the differences in internal osmotic pressure between the fast-and slow-growing rhizobia in determining the characteristics of the isotherms of these bacteria, is probably minimal.

The good correlation between the amount of water associated with a bacterium and its susceptibility to desiccation, suggested a reason for the variation in response to this stress. If the larger amount of water retained by the fast-growing rhizobia was sufficient to allow slow, but significant, metabolic activity to proceed at low moisture contents, lethal physiological upsets could result. Because the slow-growing rhizobia retained small quantities of water at the same R.H.s, the postulated residual metabolic activity may not occur. The drier proteins would be "frozen" in both configuration and activity. In support of this hypothesis, some enzymes such as lecithinase and urease (Todd, 1972) are active with only a monolayer of water adsorbed, and the activity of lipase (Acker, 1969) and urease (Skujins and Mclaren, 1967) closely paralleled the water sorption isotherm of the protein.

This capacity for some enzymes to operate at extreme states of dehydration could cause dislocation of metabolic activities which might contribute to death in desiccated bacteria. Residual metabolic activity in dehydrated, fast-growing rhizobia may account for their greater susceptibility to desiccation than the slow-growing species.

Barker (1933 cited by Bull and Breese, 1968) demonstrated that the temperature at which egg albumin was denatured increased significantly as the relative humidity decreased i.e. the lower the amount of water associated with the protein the greater was its ability to survive heating. At any vapour pressure, the slow-growing root-nodule bacteria are drier than the fast-growing species, therefore, it can be postulated that the former bacteria should survive dry heat stress better than the latter group. Marshall (1964) has shown that this is so, as the slow-growing R. lupini can survive exposure to dry heat better than R. trifolii.

The results of the isotherm data for the root-nodule bacteria suggested that bacterial susceptibility to desiccation may be related to the amount of water retained during desiccation. The rates of water exchange (permeabilities) for the rhizobia have also been measured and the results show that the rate at which water passes across the bacterial membrane is probably the same for both groups of rhizobia. In fact, the results tended to fit the postulate of Dick (1966), that there was an inverse

relationship between the cell surface-to-volume ratio and water permeability. Therefore, differences in permeability between the fast-and slow-growing rhizobia are almost certainly not a reason for the greater ability of the latter group to survive desiccation. Conversely, there probably are no differences between groups of rhizobia in the rate at which water entered the cell upon rehydration. It follows that, upon rehydration of desiccated root-nodule bacteria, differential rates of water uptake could not explain the divergence of the two groups in their response to desiccation.

Despite this similarity in permeability properties, the groups of rhizobia do respond differently to changes in the water activity of the rehydration medium. Rehydration of desiccated rhizobia in media of a_w less than 1.0 would only allow partial rehydration. In all cases, regardless of the solute used to adjust the a_w of the medium, the slow-growing rhizobia survived better than the fast-growing species. Both groups of rhizobia exhibited a susceptibility to high salt concentrations, as the decrease in the viable count was greater when NaCl or KCl was used to adjust the a_w of the medium than if either sucrose or glycerol was the solute. Frequently, the decrease in the viable count of either the fast-or slow-growing rhizobia rehydrated in media of the same water activity was greater when the solute was sucrose than when glycerol was used. As the water availability in both cases was the same, the degree of rehydration would have been similar.

This differential effect of the solute (at the same a_w) in the rehydration medium has been reported by Ray *et al.* (1971a) and Leach and Scott (1959). Generally, these authors reported lower survival in glycerol adjusted media than in media containing sucrose. A possible explanation for this could be the relative permeabilities of rehydrated bacteria to glycerol and sucrose. Zimmerman (1962) reported that sucrose was only minimally penetrable to aerosolized bacteria and Hambleton (1970) stated that *Escherichia coli* was freely permeable to glycerol. If the rhizobia exhibited this differential permeability to sucrose and glycerol then, upon rehydration in media adjusted to the same water availability with glycerol and sucrose, the *total* intracellular water content of bacteria in glycerol adjusted media would probably be greater than in the sucrose adjusted media. As the relative penetrabilities of the rhizobia to glycerol and sucrose are not known, this reason for the effect of either solute on the survival of rehydrated bacteria, is speculative. However the results of these experiments do indicate that the solute used to adjust the a_w of the rehydration medium was as important in influencing bacterial survival as the effect of the water activity as such.

The greater susceptibility of the fast-growing rhizobia to partial rehydration may have important consequences in the field situation when dry soils are partially rewet by sporadic rainfall. As complete rehydration of desiccated root-nodule

bacteria gave optimum survival, it is possible that, in the field, partial rehydration of desiccated fast-growing rhizobia could further decrease the viable populations.

It has been demonstrated that the rhizobia vary in their ability to grow at water activities below a_w 0.999 (obtained by the use of NaCl). Generally, the fast-growing species did not grow below a_w 0.999, whereas growth of the slow-growing rhizobia was possible at a_w 0.995 and growth of strains of R.meliloti was apparent at a_w 0.980. These results demonstrating the variation of growth between rhizobia in NaCl enriched media may imply that the medic rhizobia have an ecological advantage over the fast-and slow-growing species in partially desiccated soils. Undoubtedly, the availability of water for use by bacteria in partially dried soils would constitute a selective stress, and bacteria able to grow in high salt or low water activity would possess a competitive advantage under such circumstances.

A brief review of the literature dealing with the effect of water potential on the activity of soil microorganisms was published by Griffin (1972). The author demonstrated that within natural soil, some fungi were more efficient than others at colonizing available substrates at low water contents, however, very little was reported about differences between bacteria in their ability to colonize soils at low water availabilities. Ethiraj (1972) did not find inhibition of growth of rhizobia isolated from lucerne, berseem or Sesbania aculeata L. when

grown in media adjusted with NaCl. Salts such as Na_2SO_4, KCl and K_2SO_4 were inhibiting to berseem isolates but not to lucerne or Sesbania aculeata rhizobia. These results suggest that further work with the medic rhizobia is warranted, especially monitoring their growth responses in soil at different relative humidities.

Many attempts were made to select desiccation resistant species of Rhizobium by growth at low activities. However, these experiments were not successful as it was not possible to increase the ability of rhizobia to survive desiccation irrespective of the a_w of the growth medium. Chen and Alexander (1973) grew an unidentified species of Rhizobium at low water activities and reported enhanced survival when subjected to desiccation. Their conclusions were not equivocal because some dehydration-tolerant bacteria could only grow at high water activities, whereas some dehydration-susceptible bacteria were able to grow at low a_w values. Chen and Alexander (1973) concluded that the ability to grow in salt-rich media was "not the sole factor protecting cells from drought inactivation". Boylen (1973) was unable to select progeny of Arthrobacter for increased resistance to desiccation. If the ability to multiply in salt enriched media was a major factor determining bacterial desiccation resistance, then the medic rhizobia should survive drying better than either the fast-or the slow-growing rhizobia. This was not found to be the case and, therefore, it is concluded

that the role of the a_w of the growth medium in determining desiccation resistance of rhizobia, is relatively small. Similarly, Griffin (1972) was unable to relate ability of Aspergillus and Penicillium to grow at low water potentials with the prevalence of these fungi in desert soils, and concluded that this was not an important factor determining the distribution of these fungi in the soil.

Although the cell wall of gram-negative bacteria contains peptidoglycan, the substrate for lysozyme, these organisms are usually resistant to attack by the enzyme. This resistance is probably due to the lipopolysaccharide (LPS) component which overlays the peptidoglycan and prevents access of the enzyme to the substrate (Salton, 1957; Weidel et al., 1960). Sensitivity of gram-negative bacteria to lysozyme can be induced by certain treatments which alter the permeability of the LPS layer and expose the substrate to the enzyme (Salton, 1957). Consequently, induced sensitivity to lysozyme can be used to estimate the extent of disruption of the outer LPS layer caused by a particular treatment. The results presented in this thesis for Escherichia coli M13 demonstrated that air-drying these bacteria rendered some of them susceptible to the bactericidal action of lysozyme. Therefore, desiccation caused changes in the integrity of the outer LPS layer of E.coli M13. This sensitivity to lysozyme was reversible, i.e. damage to the LPS layer induced by desiccation was repaired by the bacteria. These results were similar to those reported for

aerosolized E.coli by Hambleton (1970, 1971).

The removal of water from either the fast-or slow-growing rhizobia did not drastically alter their permeability to lysozyme. This lack of appreciable induced susceptibility to lysozyme in those rhizobia surviving desiccation suggested that the outer cell wall LPS layers of both fast-and slow-growing root-nodule bacteria remained relatively unaltered when the bacteria were desiccated. Therefore, the similar tolerance of this LPS layer to damage by dehydration between surviving fast-and slow-growing species of Rhizobium does not provide an explanation for the differential responses of these two groups of bacteria to desiccation. This is in contrast to the findings of Hambleton (1970), who showed that strains of Escherichia coli that were sensitive to aerosolization exhibited induced susceptibility to lysozyme, but strains that were stable in the aerosolized state remained resistant to the enzyme.

Studies on the interaction between the fluorescent probe ANS and undried fast-and slow-growing rhizobia indicate quantitative differences in the ANS-binding sites between the two bacterial groups. ANS fluoresces when bound at charged polar/non-polar regions of biological structures (Radda and Vanderkooi, 1972), therefore, the differences in the relative fluorescence between undried fast-and slow-growing rhizobia indicates differences in the number of ANS-accessible polar/non-

polar sites between groups of rhizobia. Because Radda and Vanderkooi (1972) suggested the involvement of the charge of polar/non-polar groups in determining the interaction of ANS with macromolecules, it is possible that the difference in fluorescence intensity between the fast-and slow-growing rhizobia were related to their different surface charge characteristics. Marshall (1967) demonstrated that the surface charge of R.trifolii TA1 was similar to that of the slow-growing root-nodule bacteria. These species contained only negatively charged carboxyl ionogenic groups, whereas such species as R.leguminosarum and strains of R.trifolii (other than strain TA1), had more complex amino-carboxyl ionogenic surfaces. The fact that R.trifolii TA1 fluoresced as intensely with ANS as the other fast-growing rhizobia, suggested that the different surface charge characteristics was not an explanation for the differential response of the fast-and slow-growing rhizobia to ANS. The concept of surface charge as used by Marshall (1967) was obviously different than that implied (but not defined) by Radda and Vanderkooi (1972). Consequently, the differences of ANS fluorescence between the fast-and slow-growing rhizobia are probably related to true differences in the polar/non-polar nature of their surfaces. This may reflect a qualitative difference between molecules constituting the cell wall of fast- and slow-growing root-nodule bacteria and could be a reason for the different responses of these bacteria to desiccation.

Results have been presented which suggest that sub-lethal damage to desiccated rhizobia did not involve drastic alterations to the LPS-layer. However, studies on the interaction of the fluorescent probe ANS with dried bacteria indicated substantial alterations of the surface features of most of the bacterial population. Prior to desiccation, the fast-growing rhizobia fluoresced strongly when mixed with ANS, whereas the slow-growing and medic rhizobia showed only minimal reaction. The increased fluorescence resulting from desiccation of slow-growing and medic rhizobia could be due to the exposure of ANS-reactive sites at the bacterial surface which were not accessible to ANS before dehydration. Alternatively, dehydration may have altered the permeability of the cytoplasmic membrane such that the internal contents were released and covered the bacterial surface. Because of the polar/non-polar nature of the intracellular material, enhanced fluorescence would result. It is noteworthy that the water used to rehydrate the desiccated rhizobia fluoresced after removal of bacteria by centrifugation. This suggests cytoplasmic membrane damage and leakage of intracellular material and, in support of this Todd (1972) indicated that the maintenance of the structure of the cytoplasmic membrane was of prime importance in desiccation resistance.

The proposal by Marshall (1964) that montmorillonite has the ability to protect fast-growing rhizobia from desiccation has been confirmed. However, the precise relationship between the additive and bacteria is very important as, under some

circumstances, montmorillonite did not protect desiccated fast-growing rhizobia. It was not possible to demonstrate protection of the slow-growing rhizobia by this additive.

The working hypothesis used throughout this thesis was that the mechanism by which montmorillonite protects bacteria from desiccation was via an effect on the final bacterial water content after dehydration. By either increasing or decreasing the final intracellular water content of desiccated bacteria, montmorillonite could have enabled a greater proportion of fast-growing rhizobia to survive drying. It has been demonstrated that montmorillonite-amended soil retains more water than unamended samples. However, a comparison between the water contents of unamended soil and montmorillonite-amended samples at the point at which bacteria undergoing desiccation began to die, revealed that more water could be lost from the unamended soil before bacterial death occurred than from the amended samples. This was taken as circumstantial evidence that the extra water retained by amended soil samples was not necessarily available to bacteria undergoing desiccation.

The water adsorption isotherms indicated that at all R.H. values less than 90% Ca^{2+}-montmorillonite adsorbed more water than either the fast-or the slow-growing rhizobia. Determinations of whether this is available to bacteria must involve comparisons of the relative affinities for water between montmorillonite and

each species of Rhizobium. The equilibrium distribution of water at any vapour pressure between closely associated montmorillonite particles and bacteria will depend upon the relative affinities of the two particles for water. If the additive has a greater affinity for water than bacteria, then the bulk movement of water would be from the bacterium to the additive. The mass flow of water would be in the reverse direction if bacteria had a higher affinity for water than the additive.

Bull (1944) has stated that the magnitude of the surface energy changes associated with adsorption of water vapour is a measure of the affinity of the surface for the vapour. Measurements of the surface energies per gram of Ca^{2+}-montmorillonite and various species of rhizobia have been presented and indicate that, at all vapour pressures, Ca^{2+}-montmorillonite has a greater affinity for water than any species of Rhizobium. Consequently, at any relative humidity, an equilibrium distribution of water between Ca^{2+}-montmorillonite and rhizobia would be reached by the movement of water from rhizobia to montmorillonite particles and not in the opposite direction. It is postulated that the mechanism by which montmorillonite protects fast-growing rhizobia from desiccation is by decreasing the intracellular water content below the equilibrium value reached when the bacteria are associated with other soil particles such as sand grains. This is not in agreement with the suggestion put forward by Marshall (1968a),

that protection of desiccated rhizobia by montmorillonite involved altered rates of water movement between bacteria and their environment during drying and rewetting. Also the very rapid rate at which water moves across the bacterial membrane would tend to argue against the suggestion that montmorillonite protected bacteria by increasing the rate of evaporation of water from bacteria enveloped by this clay.

Although the affinity of the slow-growing rhizobia for water is less than that of Ca^{2+}-montmorillonite, this additive does not protect these bacteria. It is quite possible that montmorillonite does reduce the hydration state of the slow-growing rhizobia at any vapour pressure, however, these bacteria have such a low affinity for water that protection from desiccation would not involve the further removal of water. Perhaps the effect of montmorillonite on the hydration state of the fast-growing rhizobia at any vapour pressure is to reduce the quantity of water retained to about the value held by the slow-growing species. It is interesting that the viable numbers of fast-growing rhizobia surviving desiccation in montmorillonite-amended soils are of the same order of magnitude as the numbers of slow-growing bacteria surviving desiccation in unamended soil samples. The hypothesis outlined above does imply that there is a certain (as yet undefinable) quantity of water which must be removed from bacteria undergoing desiccation in order to maximize survival. Once below this value, further decreases in the water content have no effect upon the ability of microorganisms to survive and protection, by sugars for example, must be achieved by other mechanisms. If this postulate is correct, then the quantity of water retained by a fast-growing rhizobia is probably above the

"critical level", whereas the amount of water adsorbed by the slow-growing rhizobia is less than this value. In support of the hypothesis of a "critical moisture level", Monk and McCaffrey (1957) showed that the maximum death rate of Serratia marcescens occurred at water contents between 15% and 70%. Below 15% water, the death rate was negligible. Similarly, Zimmerman (1962) suggested that the minimally penetrable solutes protected S.marcescens from aerosolization by removal of intracellular water to levels below an undefined critical level. Unprotected cells probably died because their intracellular water content remained too high for too long.

It has been demonstrated that not all fast-growing rhizobia/montmorillonite associations increased the ability of these bacteria to survive desiccation. For instance, when aqueous suspensions of montmorillonite and bacteria were mixed prior to desiccation, no protection was obtained. Similarly, the protective ability of montmorillonite was removed when rhizobia were added to moist montmorillonite-amended soil samples. An explanation of these seemingly contradictory results could be the finding that clay films prepared by the evaporation of water from aqueous suspensions of fully hydrated montmorillonite retained more water than the partially hydrated clay in the amended soil. This was demonstrated by the results of experiments using a pressure membrane apparatus to measure the amount of water retained soil samples. The explanation for this phenomenon given by Barrer and MacLeod (1954) was that montmorillonite platelets in suspension were sufficiently lubricated to orient themselves into roughly parallel configurations so that water was

held between these parallel plates as well as within the clay lattice. It is possible in the experiments involving survival of rhizobia associated with different forms of montmorillonite, that when sufficiently lubricated, some clay particles were drawn by surface tension forces into a structure surrounding one or a group of bacteria. This regular array of platelets probably retained more water at any vapour pressure than a purely random array. This water could be available to bacteria and increase their final equilibrium hydration state when desiccated. As discussed above, this appears to be detrimental to the survival of bacteria in the dried state.

These results highlight the importance of the precise clay-bacterium association as not all interactions were able to protect living cells. It has been demonstrated by Marshall (1968b) that, when in aqueous suspensions, platelets of montmorillonite are adsorbed to the surface of fast-growing rhizobia in edge-to-face and face-to-face orientations. Marshall (1968a) suggested that this association between R.trifolii and montmorillonite particles was sufficient to give protection from desiccation. The results presented here do not agree with this, as protection of fast-growing rhizobia was not obtained when aqueous montmorillonite/bacteria suspensions were dried. Enhanced survival was only obtained when fast-growing rhizobia were added to dry montmorillonite-amended soil samples. It is likely that the bacterium/clay association under such circumstances would differ greatly from that suggested by Marshall (1968b; 1969a).

It is worthy of note here that many of the lime-pelleting treatments used to inoculate temperate legume seeds with bacteria were not successful when applied to tropical or subtropical seeds. Norris (1972) and Date (1968) rightly attributed this to the detrimental effects of increased pH on the cowpea type rhizobia, and to toxic factors in the seed coats of some seeds. In view of the above discussion, however, it is possible that some pelleting techniques applied to the cowpea rhizobia were detrimental because they resulted in increased retention of intracellular water.

Of all the additives studied during this investigation, the effect of montmorillonite upon the response of all groups of rhizobia was of prime interest. The reason for determining the effects of other additives was to help explain the effect of clay and if possible to make generalizations as to which substances gave protection to desiccated rhizobia in the system used.

The results obtained with the hydrophilic aerosil were somewhat surprising. Because of its colloidal size (average primary particle size =12mμ; obtained from literature supplied by Degusa), hydrophilic nature, and extracellular positioning it was expected that similar results to the effect of montmorillonite would be obtained. However, in all cases the hydrophilic aerosil decreased the ability of both fast-and slow-growing bacteria to survive desiccation whereas the hydrophobic aerosil had no significant effect. An

explanation for this behaviour of the hydrophilic aerosil is not obvious, but it may be that the surfaces of these particles are able to interact with bacteria in such a way as to render them susceptible to dehydration.

A comparison of the protective ability of other additives reveals that only the sugars (maltose, sucrose and glucose) and PVP were able to protect both fast-and slow-growing rhizobia from desiccation. The effect of PEG 6000 was similar to montmorillonite in that it protected the fast-growing species but not the slow-growing rhizobia. Glycerol and the lower molecular weight polymers of PEG decreased the ability of both fast-and slow-growing root-nodule bacteria to survive desiccation. It is suggested that glycerol, PEG 400 and PEG 1500 were able to penetrate to the cell interior and, because of their hydrophilic nature, may have increased the retention of intracellular water by desiccated bacteria. As stated above, this could be detrimental to microorganisms in the dried state. In support of this, Cox (1967) and Hambleton (1970) reported that glycerol was freely permeable to Escherichia coli. The results reported by Cox (1967) demonstrated that glycerol protected E.coli aerosolized into environments of high relative humidity, but at low vapour pressures glycerol was toxic to dried bacteria. At low relative humidities, an impenetrable agent such as raffinose was required to protect aerosolized E.coli (Cox,1967). The results reported in this investigation demonstrated that

glycerol did not protect rhizobia at low water contents in the system used.

The penetrabilities of sucrose, maltose and glucose for rhizobia have not been determined, but Zimmerman (1962) described Serratia marcescens as being freely permeable to glucose but minimally permeable to sucrose and maltose. If the same relationship holds for the rhizobia then the mechanism of protection of airdried root-nodule bacteria by these sugars cannot be a function of penetrability. Vincent et al. (1962) were unable to explain the superiority of maltose as a protective agent over all other sugars tested by its penetrability, osmotic effect, molecular size or solubility. Therefore, the mechanism of action of these sugars remains unknown. It is interesting to note that Mazur (1970) reported that protection by sucrose of red blood cells desiccated by freezing did not require penetration of the sugar into the cell. Similarly, red blood cells were protected by glycerol when its penetration into the cell was blocked. These results suggested that the ability of a cell to survive dehydration by freezing may depend on protection of the cell surface rather than of the cell interior. It is suggested that maltose, sucrose and glucose are able to protect rhizobia by stabilizing structures external to the cell interior, this could include the cytoplasmic membrane.

Because of the size of the PVP molecule, it is highly unlikely that it would penetrate to the cytoplasm of the rhizobia. Mazur (1970) reported that red blood cells were impermeable to this molecule and yet it was able to protect these cells from damage due to freezing. Therefore, the necessity for permeation of the additive to obtain protection is in doubt. As reported by Klotz (1964), PVP has a structuring effect on water and Mazur (1970) reported its anomalously high freezing point depression. When mixed with bacteria, this extracellular polymer could preferentially bind water molecules and, therefore, reduce the amount of intracellular water available to the dried bacteria. However, a more direct mechanism may be involved because PVP may penetrate the cell wall to the external surface of the cytoplasmic membrane and by interaction with this structure, enhance its stability to desiccation. Klotz (1964) also reported that tetrabutylammonium bromide was more efficient than PVP in structuring water. Consequently, the dramatic lowering of the ability of rhizobia to survive desiccation when mixed with this compound was surprising. The only suggestion for this behaviour could be that tetrabutylammonium bromide, in contrast to PVP 40,000, was able to penetrate the cell interior with consequent adverse effects due to increased retention of intracellular water at low relative humidities.

The results presented for the effect of various additives on the storage of dried R.leguminosarum QA372 indicated that maltose was the only additive that protected the bacterium during storage. Although PVP increased the survival of this slow-growing bacterium, its action was limited to the actual period during which water was being removed from the microorganism. After this was completed, the death rate during storage in the dried condition was not significantly different from the control as indicated by the similarity of the regression coefficients. The same applied to other additives such as montmorillonite and tetrabutylammonium bromide.

Conclusions

1. The fast-growing rhizobia are more susceptible to desiccation than the slow-growing species.
2. The different response of the two bacterial groups to desiccation is probably related to the different amounts of water retained (isotherms) by these bacteria at any vapour pressure rather than differences in the rate of movement of water between the various rhizobia and the environment (determined by NMR spectroscopy). The surface area available for the adsorption of water by the fast-growing rhizobia was greater than that for the slow-growing species. Similarly, the fast-growing bacteria had a higher surface energy at any vapour pressure than the slow-growing bacteria.

3. Very little difference was detected between the fast- and slow-growing root-nodule bacteria in the degree of desiccation-induced susceptibility to lysozyme. It is concluded, therefore, that sub-lethal damage probably does not involve alterations of the integrity of the lipopolysaccharide layer of the survivors as determined by induced lysozyme sensitivity.

4. Desiccation did cause drastic changes in the surface features of rhizobia as determined by fluorescence of the bacteria in the presence of ANS. It is suggested that this could be due to alteration of the permeability of the cytoplasmic membrane in killed bacteria. This would allow leakage of intracellular macromolecules which adsorbed to the external surface of bacteria and enhanced their fluorescence when mixed with ANS.

5. Montmorillonite protects only the fast-growing and not the slow-growing rhizobia from the effects of desiccation, but protection of the former bacteria is dependent upon the precise clay / bacterium association, as not all combinations resulted in increased ability of these bacteria to survive dehydration. It is suggested that the mechanism of action of montmorillonite is to decrease the intracellular water content of desiccated fast-growing rhizobia below an undefined critical level. As the water content of desiccated slow-growing rhizobia is normally below this value, further

removal of intracellular water has no effect upon survival.

6. Of all the other additives investigated only maltose, sucrose, glucose and PVP protected both fast-and slow-growing rhizobia. The effect of PEG 6000 was similar to the effect of montmorillonite, as the polymer only protected the fast-growing rhizobia and not the slow-growing species. All other additives tested either had no effect upon survival or were detrimental to desiccated bacteria. The mechanism of protection of sugars and PVP is unknown, but it has been suggested that the site of action of these hydrophilic substances may be external to the cell and may involve stabilization of the cytoplasmic membrane to desiccation.

6. BIBLIOGRAPHY

ACKER, L.W., (1969). Water activity and enzyme activity. Food Technol., 23, 27.

ADAMSON, A.W., (1967). "Physical chemistry of surfaces". John Wiley & Sons, Interscience Publ., New York.

AMARGER, N., JACQUEMETTON, M. and BLOND, G. (1972). Influence of the age of the culture on the survival of Rhizobium meliloti after freeze drying and storage. Arch. Mikrobiol., 81, 361.

ANDERSON, J.D., (1966). Biochemical studies of lethal processes in aerosols of Escherichia coli. J. Gen. Microbiol., 45, 303-313.

ANDERSON, J.D. and COX, C.S. (1967). Microbial survival. Symp. Soc. Gen. Microbiol., 17, 203-226.

ANDERSON, J.D. and DARK, F.A. (1967). Studies on the effects of aerosolization on the rates of efflux of ions from populations of Escherichia coli strain B.J. Gen. Microbiol., 46, 95.

ANDERSON, J.D., DARK, F.A. and PETO, S. (1968). The effect of aerosolization upon survival and potassium retention by various bacteria. J. Gen. Microbiol., 52, 99.

ANTHEUNISSE, J. (1973). Viability of lyophilized microorganisms after storage. Antonie van Leeuwenhoek, 39, 243-248.

BANGHAM, D.H. (1937). The Gibbs adsorption equation and adsorption on solids. Trans. Farad. Soc., 33, 805-811.

BARRER, R.M. and MACLEOD, D.M. (1954). Intercalation and sorption by montmorillonite. Trans. Farad. Soc., 50, 980-989.

BATEMAN, J.B., STEVENS, C.L., MERCER, W.B. and CARSTENSEN, E.L. (1962). Relative humidity and the killing of bacteria: the variation of cellular water content with external relative humidity or osmolality. J. Gen. Microbial., 29, 207-219.

BENBOUGH, J.E. (1967). Death mechanisms in airborne Escherichia coli. J. Gen. Microbiol., 47, 325-333.

BENBOUGH, J.E. (1969). Factors affecting the toxicity of oxygen towards airborne coliform bacteria. J. Gen. Microbiol., 56, 241.

BENBOUGH, J.E., HAMBLETON, P., MARTIN, K.L. and STRANGE, R.E. (1972). Effect ofaerosolization on the transport of α-methyl glucoside and galactosides into Escherichia coli. J. Gen. Microbiol., 72, 511.

BENEDICT, R.G., SHARPE, E.S., CORMAN, J., MEYERS, G.B., BAER, E.F., HALL, H.H., AND JACKSON, R.W. (1961). Preservation of microorganisms by freeze-drying.
11. The destructive action of oxygen. Additional stabilizers for Serratia marcescens. Experiments with other microorganisms. Appl. Microbiol., 9, 256.

BERGERSEN, F.J., BROCKWELL, J. and THOMPSON, J.A. (1958). Claver seed pelleted with bentonite and organic material as an aid to inoculation with nodule bacteria. J. Aust. Inst. Agric. Sci., 24, 158-160.

BISSETT, K.A. (1952). Complete and reduced life cycles in Rhizobium. J. Gen. Microbiol., 7, 233.

BOWEN, G.D. and KENNEDY, M. (1959). Effect of high soil temperatures on Rhizobium sp. Qd. J. Agric. Sci., 16, 177-197.

BOYLEN, C.W. (1973). Survival of Arthrobacter crystallopoites during prolonged periods of extreme desiccation. J. Bacteriol, 113, 33.

BREWER, R.(1962). Techniques used in fabric and mineral analysis of soil materials. CSIRO, Division of soils. Divisional report 9/62.

BREWLEY, J.D. (1972). The conservation of polyribosomes in the moss Tortula ruralis during total desiccation. J.Exp. Bot., 23, 692.

BREWLEY, J.D. (1973) Polyribosomes conserved during desiccation of the moss Tortula ruralis are active. Pl. Physiol., 51, 285-288.

BROCKWELL, J. (1962). Studies on seed pelleting as an aid to legume seed inoculation.

1. Coating materials, adhesives and methods of inoculation. Aust. J. Agric. Res., 13, 638-649.

BROCKWELL, J. and PHILLIPS, L.J. (1970). Studies on seed pelleting as an aid to legume seed inoculation.

3 Survival of Rhizobium applied to seed sown into hot, dry soil. Aust. J. Expt. Agric. Anim. Husb., 10, 739.

BROCKWELL, J. and WHALLEY, R.D.B. (1962). Incorporation of peat inoculant in seed pellets for inoculation of Medicago tribuloides Desr. sown into dry soil. Aust. J. Sci., 24, 458-459.

BROCKWELL, J. and WHALLEY, R.D.B. (1970). Studies on seed pelleting as an aid to legume seed inoculation.

2. Survival of Rhizobium meliloti applied to medic seed sown into dry soil. Aust. J. Exp. Agric. Anim. Husb., 10, 455.

BRUNAUER, S., EMMETT, P.H. and TELLER, E. (1938). Adsorption of gases in multimolecular layers. J. Amer. Chem. Soc., 60, 309-319.

BULL, H.B. (1944). Adsorption of water vapour by proteins. J. American Chem. Soc., 66, 1499.

BULL, H.B. and BREESE, K. (1968). Protein hydration. 1. Binding sites. Arch. Biochem. Biophys., 128, 488.

BULL, H.B. and BREESE, K. (1970). Water and solute binding by proteins. 1. Electrolytes. Arch. Biochem. Biophys., 137, 299.

CHATEL, D.L. and GREENWOOD, R.M. (1973a). The colonization of host-root and soil by rhizobia. 11. Strain differences in the species of Rhizobium trifolii. Soil Biol. Biochem., 5, 433.

CHATEL, D.L. and GREENWOOD, R.M. (1973b). Differences between strains of Rhizobium trifolii in ability to colonize soil and plant roots in the absence of their specific host plants. Soil Biol. Biochem., 5, 809.

CHATEL, D.L. and PARKER, C.A. (1973a). Survival of field-grown rhizobia over the dry summer period in Western Australia. Soil Biol. Biochem., 5, 415.

CHATEL, D.L. and PARKER, C.A. (1973b). The colonization of host-root and soil by rhizobia. 1. Species and strain differences in the field. Soil Biol. Biochem., 5, 425.

CHATEL, D.L.,GREENWOOD, R.M. and PARKER, C.A. (1968). Saprophytic competence as an important character in the selection of Rhizobium for inoculation. 9th Intern. Congr. Sci. Trans., Vol. 11, 65-73.

CHEN, M. and ALEXANDER, M. (1973). Survival of soil bacteria during prolonged desiccation. Soil Biol. Biochem., 5, 213-221.

CHOATE, R.V. and ALEXANDER, M.T. (1967). The effect of the rehydration temperature and rehydration medium on the viability of freeze-dried Spirillum atlanticum. Cryobiol., 3, 419-422.

CHOWDHURY, M.S. (1965). Growth and persistence of Rhizobium in soil. PhD Thesis, University of Western Australia.

CONLON, T. and OUTHRED, R. (1972). Water diffusion permeability of erythrocytes using an NMR technique. Biochem. Biophys. Acta., 288, 354-361.

COX, C.S. (1966a). The survival of Escherichia coli sprayed into air and into nitrogen from distilled water and from solutions of protecting agents, as a function of relative humidity. J. Gen. Microbiol., 43, 383.

COX, C.S. (1966b). The survival of Escherichia coli in nitrogen atmospheres under changing conditions of relative humidity. J. Gen. Microbiol., 45, 283.

COX, C.S. (1967). The aerosol survival of Escherichia coli JEPP sprayed from protecting agents into nitrogen atmospheres under changing relative humidity conditions. J. Gen. Microbiol., 49, 109.

COX, C.S. (1968a). The aerosol survival of Escherichia coli B in nitrogen, argon and helium atmospheres and the influence of relative humidity. J. Gen. Microbiol., 50, 139.

COX, C.S. (1968b). The aerosol survival and cause of death of Escherichia coli K12. J. Gen. Microbiol., 54, 169.

COX, C.S. (1969). The cause of loss of viability of airborne Escherichia coli K12. J. Gen. Microbiol., 57, 77.

COX, C.S. (1970). Aerosol survival of Escherichia coli B disseminated from the dried state. Appl. Microbiol., 19,604.

COX, C.S. (1971). Aerosol survival of Pasturella tularensis disseminated from the wet and dry states. Appl. Microbiol., 21, 482.

COX, C.S. (1972). Aerosol survival of Pasturella tularensis and the influence of relative humidity. Appl. Microbiol., 23,1.

COX, C.S. and BALDWIN, F. (1966). The use of phage to study the causes of loss of viability of Escherichia coli in aerosols. J. Gen. Microbiol., 44, 15.

COX, C.S. and BALDWIN, F. (1967). The toxic effect of oxygen upon the aerosol survival of Escherichia coli B. J.Gen. Micro., 49, 115.

COX, C.S. and HECKLY, R.J. (1973). The effects of oxygen upon freeze-dried and freeze thawed bacteria: viability and free-radical studies. Can. J. Microbiol., 19, 189-194.

COX, C.S. BAXTER, J. and MAIDMENT, B.J. (1973). A mathematical expression for oxygen-induced death in dehydrated bacteria. J. Gen. Microbiol., 75, 179.

COX, C.S., BONDURANT, M.C. and HATCH, M.T. (1971). Effects of oxygen on aerosol survival of radiation sensitive and resistant strains of Escherichia coli B. J. Hyg., 69, 661.

CRAFTS, A.S. (1968). Water structure and water in the plant body. In "Water deficits and plant growth" volume 1, pp23-47. Edited by T.T. Kozlowski. Academic Press, New York.

DARBYSHIRE, B. (1973). The function of the carbohydrate units of glucose oxidase in its desiccation resistance. 14th Annual General Meeting, Aust. Soc. Plant Physiol., Canberra, Paper No. 59.

DARBYSHIRE, B. and STEER, B.T. (1973). Dehydration of macromolecules 1. Effect of dehydration-rehydration on indoleacetic acid oxidase, ribonuclease, ribulosediphosphate carboxylase, and ketose-1-phosphate aldolase. Aust. J. Biol. Sci., 26, 591-604.

DART, P.J., ROUGHLEY, R.J. and CHANDLER, M.R. (1969). Peat culture of Rhizobium trifolii: an examination by electron microscopy. J. Appl. Bacteriol., 32, 352.

DATE, R.A. (1968). Rhizobial survival on the inoculated legume seed. 9th Congr. Soil Sci. Trans., Vol. 11, 75-83.

DATE, R.A. (1973). Nitrogen, a major limitation in the productivity of natural communities, crops and pastures in the pacific area. Soil Biol. Biochem., 5, 5-18.

DATE, R.A., BATTHYANY, C. and JAURECHE, C. (1965). Survival of rhizobia in inoculated and pelleted seed. Proc. 9th Int. Grassld. Cong., Sao Paulo. pp.263-269.

DAVIS, M.S. and BATEMAN, J.B. (1960a). Relative humidity and the killing of bacteria. I Observations on Escherichia coli and Micrococcus lysodeikticus. J. Bact., 80, 577.

DAVIS, M.S., and BATEMAN, J.B. (1960b). Relative humidity and the killing of bacteria II. Selective changes in oxidative activity associated with death. J. Bact., 80, 580.

DeLEY, J. (1965). DNA composition, flagellation and taxonomy of the genus Rhizobium. J. Gen. Microbiol., 41, 85.

DeVRIES, A.L. KOMATSU, S.K. and FEENEY, R.E. (1970). Chemical and physical properties of freezing point-depressing glycoproteins from Antarctic fish. J. Biol. Chem., 245, 2901-2908.

DEWALD, R.R. (1966a). Preservation of Serratia marcescens by high-vacuum lyophilization. Appl. Microbiol., 14, 561.

DEWALD, R.R. (1966b). Kinetic studies on the destructive action of oxygen on lyophilized Serratia marcescens. Appl. Microbiol., 14, 568.

DICK, D.A.T. (1966). "Cell water". Washington Butterworths Inc.

DIMMICK, R.L., HECKLY, R.J. and HOLLIS, D.P. (1961). Free-radical formation during storage of freeze-dried Serratia marcescens. Nature, 192, 776.

DUDMAN, W.F. (1968). Capsulation in Rhizobium species. J. Bacteriol., 95, 1200.

ESTERMAN, E.F. and McLAREN, A.D. (1959). Stimulation of bacterial protedysis by adsorbents. J. Soil Sci, 10, 64.

ETHIRAJ, S., SHARMA, H.R. AND VYAS, S.R. (1972). Studies on the salt tolerance of rhizobia. Indian J. Microbiol., 12, 87-91.

FALK, M., HARTMAN, K.A. and LORD, R.C. (1963). Hydration of deoxyribonucleic acid. 11. An infrared study. 111. A spectroscopic study of the effect of hydration on the structure of deoxyribonucleic acid. J. Am. Chem. Soc., 85, 387.

FOULDS, W. (1971). Effect of drought on three species of Rhizobium. Pl. Soil, 35, 665.

FRITZ, O.G. Jr. and SWIFT, T.J. (1967). The state of water in polarized and depolarized frog nerves, A proton Magnetic resonance study. Biophys. J., 7, 675.

GRAHAM, P.H. (1964). The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes. J.Gen. Microbiol., 35, 511-517.

GRAHAM, P.H., PARKER,C.A., OAKLEY, A., LANGE, R.T. and SANDERSON, I,J.V. (1963). Spore formation and heat resistance in Rhizobium. J. Bact., 86, 1353-1354.

GRIFFIN, D.M. (1972). "Ecology of soil fungi." Chapman and Hall, London.

HAMBLETON, P. (1970). The sensitivity of gram-negative bacteria, recovered from aerosols, to lysozyme and other hydrolytic enzymes. J. Gen. Microbiol., 61, 197-204.

HAMBLETON, P. (1971). Repair of wall damage in Escherichia coli recovered from an aerosol. J. Gen. Microbiol., 69, 81.

HATTORI, T. (1973). "Microbiol life in the soil an introduction." Marcel Dekker, Inc., New York.

HECKLY, R.J. and DIMMICK, R.L. (1967). Electron paramagnetic resonance in frozen and dried biological materials. Nature, 216, 1003.

HECKLY, R.J. and DIMMICK, R.L. (1968). Correlations between free radical production and viability of lyophilized bacteria. Appl. Microbiol., 16, 1081.

HECKLY, R.J., DIMMICK, R.L. and WINDLE, J.J. (1963). Free radical formation and survival of lyophilized microorganisms. J. Bact., 85, 961-966.

HELY, F.W. and BROCKWELL, J. (1962). An exploratory survey of the ecology of Rhizobium meliloti in inland New South Wales and Queensland. Aust. J. Agric. Res., 13, 864-879.

HESS, G.E. (1965). Effects of oxygen on aerosolized Serratia marcescens. Appl. Microbiol., 13, 781.

HOOVER, S.R. and MELLON, E.F. (1950). Application of polarization theory to sorption of water vapour by high polymers. J. Amer. Chem. Soc., 72, 2562.

HURST, A., HUGHES, A., BEARE-ROGERS, J.L. and COLLINS-THOMPSON, D.L. (1973). Physiological studies on the recovery of salt tolerance by Staphylococcus aureus after sublethal heating. J. Bacteriol., 116, 901.

JANSSEN, D.W. and BUSTA, F.F. (1973). Influence of milk components on the injury, repair of injury, and death of Salmonella anatum cells subjected to freezing and thawing. Appl. Microbiol., 26, 725.

JENSEN, H.L. (1961). Survival of Rhizobium meliloti in soil culture. Nature, 192, 682-683.

KLOTZ, I.M. (1965). Role of water structure in macromolecules. Fed. Proc., 15, 5-24.

LAHAV, N. (1962). Adsorption of sodium bentonite particles on Bacillus subtilis. Pl. Soil, 17, 191.

LEACH, R.H. and SCOTT, W.J. (1959). The influence of rehydration on the viability of dried microorganisms. J. Gen. Microbiol., 21, 295-307.

LION, M.B. (1963). Quantitative aspects of the protection of freeze dried Escherichia coli against the toxic effect of oxygen. J. Gen. Microbiol., 32, 321.

LION, M.B. and AVI-DOR, Y. (1963). Oxygen induced inactivation of NADH oxidase in lyophilized cells of Escherichia coli. Israel J. Chem., 1, 374.

LION, M.B. and BERGMANN, E.D. (1961a). The effect of oxygen on freeze-dried Escherichia coli. J. Gen. Microbiol, 24, 191-200.

LION, M.B. and BERGMANN, E.D. (1961b). Substances which protect lyophilized Escherichia coli against the lethal effect of oxygen. J. Gen. Microbiol., 25, 291-296.

LION, M.B., KIRBY-SMITH, J.S. and RANDOLPH, M.L. (1961). Electron-spin resonance signals from lyophilized bacterial cells exposed to oxygen. Nature, 192, 34.

LING, N.G. (1965). The physical state of water in living cell and model systems. Ann. New York. Acad. Sci., 125, 401-417.

LING, G.N. (1972). Hydration of macromolecules. In "Water and aqueous solutions, structure, thermodynamics and transport processes." pp663-700. Edited by R.A. Horne. Wiley - Interscience, New York.

LOVEDAY, J. (1957). The soils of the Sorell-Carlton-Copping area, South-east Tasmania, with special reference to the soils formed on basalt. C.S.I.R.O., Soil Publication No. 8.

LUSCOMBE, B.M. and GRAY, T.R.G. (1973). Effects of growth conditions on the physiology of a bacterium. Am. Soc. Microbiol., 73rd Annual Meeting, No. G19, p.29.

McLAREN, A.D. and SKUJINS, J. (1968). The physical environment of micro-organisms in soil. In "The Ecology of Soil Bacteria". Edited by T.R.G. Gray and D. Parkinson. Liverpool University Press.

McLEOD, R.W. and ROUGHLEY, R.J. (1961). Freeze-dried cultures as commercial legume inoculants. Aust. J. Exp. Agric. Anim. Husb., 1, 29-33.

MACLEOD, R.A., SMITH, L.D.H. and SELINAS, R. (1966). Metabolic injury to bacteria. 1. Effect of freezing and storage on the requirements of Aerobacter aerogenes and Escherichia coli for growth. Can. J. Microbiol., 12, 61.

MALTMAN, J.R. and WEBB, S.J. (1971). The action of hydrolytic enzymes and vapour rehydration on semidried cells of Klebsiella pneumoniae. Can. J. Microbiol., 17, 1443.

t'MANNETJE, L. (1967). A re-examination of the taxonomy of the genus Rhizobium and related genera using numerical analysis. Antonie van Leeuwenhoek, 33, 477-491.

MARSHALL, K.C. (1964). Survival of root-nodule bacteria in dry soils exposed to high temperatures. Aust. J. Agric. Res., 15, 273-281.

MARSHALL, K.C. (1967). Electrophoretic properties of fast-and slow-growing species of Rhizobium. Aust. J. Biol. Sci., 20, 429-438.

MARSHALL, K.C. (1968a). The nature of bacterium-clay interactions and its significance in survival of Rhizobium under arid conditions. 9th Int. Congr. Soil Sci. Trans., 3, 275-280.

MARSHALL, K.C. (1968b). Interaction between colloidal montmorillonite and cells of Rhizobium species with different ionogenic surfaces. Biochem. Biophys. Acta. 156, 179-186.

MARSHALL, K.C. (1969a). Studies by microelectrophoretic and microscopic techniques of the sorption of illite and montmorillonite to rhizobia. J. Gen. Microbiol., 56, 301-306.

MARSHALL, K.C. (1969b). Orientation of clay particles sorbed on bacteria possessing different ionogenic surfaces. Biochem. Biophys. Acta, 193, 472-474.

MARSHALL, K.C. (1971). Sorptive interactions between soil particles and microorganisms. In "Soil Biochemistry" volume 2 pp409-445. Edited by A.D. McLaren and J.J. Skujins. Marcel Dekker Inc., N.Y.

MARSHALL, K.C., MULCAHY, M.J. and CHOWDHURY, M.S. (1963). Second-year clover mortality in Western Australia - a microbiological problem. J. Aust. Inst. Agric. Sci., 29, 160-164.

MARSHALL, K.C. and ROBERTS, F.J. (1963). Influence of fine particle material on survival of Rhizobium trifolii in sandy soil. Nature. 198, 410-411.

MAZUR, P. (1970). Cryobiology: The freezing of biological systems. Science, 168, 939.

MILES, A.A. and MISRA, S.S. and IRWIN, J.O. (1938). Estimation of the bactericidal power of blood. J. Hyg. Camb., 38, 732-749.

MONK, G.W. and McCAFFREY, P.A. (1957). Effect of sorbed water on the death rate of washed Serratia marcescens. J. Bacteriol., 73, 85.

MORTLAND, M.M. and KEMPER, W.D. (1965). Specific surface. In "Methods of Soil Analysis Part I Physical and Mineralogical Properties, Including Statistics of measurement and sampling." (C.A. Black, D.D. Evans, J.L. White, L.E. Ensminger, F.E. Clark, Eds.). American Soc. Agron., Inc., Madison, Wisconsin, U.S.A.

NOLLER, E.C. and HARTSELL, S.E. (1961). Bacteriolysis of Enterabacteriaceae. I. Lysis of four lytic systems using lysozyme. J. Bacteriol, 81, 482.

NORRIS, D.O. (1972). Leguminous plants in tropical pastures. Tropical Grasslands, 6, 159-170.

NOVICK, O., ISRAELI, E. and KOHN, A. (1972). Nucleic acid and protein synthesis in reconstituted lyophilized Escherichia coli exposed to air. J. Appl. Bact., 35, 185-191.

PAGANELLI, C.V. and SOLOMON, A.K. (1957). The rate of exchange of tritiated water across the human red cell membrane. J. Gen. Physiol., 41, 259.

PARKER, J. (1972). Protoplasmic resistance to water deficits. In "Water deficits and plant growth" volume 3. pp125-176. Edited by T.T. Kozlowski. Academic Press, New York.

PARKINSON, D. GRAY, T.R.G. and WILLIAMS, S.T. (1971). "Methods for studying the ecology of soil micro-organisms." Blackwell Scientific Publications, Oxford.

PEELE, T.C. (1936). Adsorption of bacteria by soils. Cornell Univ. Agr. Expr. Stat. Memo., 197, 1-18.

POON, C.P.C. (1966). Studies on the instantaneous death of airborne Escherichia coli. Amer. J. Epidemiol., 84, 1-9.

POPLE, J.A., SCHNEIDER, W.G. and BERNSTEIN, H.J. (1959). "High-resolution nuclear magnetic resonance". McGraw-Hill Co. Inc., New York.

RADCLIFFE, J.C., McGUIRE, W.S. and DAWSON, M.D. (1967). Survival of rhizobia on pelleted seeds of Trifolium subterraneum L. Agron. J., 59, 56-58.

RADDA, G.K. and VANDERKOOI, J. (1972). Can fluorescent probes tell us anything about membranes? Biochem. Biophys. Acta, 265,509.

RAY, B., JANSSEN, D.W. and BUSTA, F.F. (1972). Characteristation of the repair of injury induced by freezing Salmonella anatum. Appl. Microbiol., 23, 803-809.

RAY, B. and SPECK, M.L. (1972). Repair of injury induced by freezing Escherichia coli as influenced by the recovery medium. Appl. Microbiol., 24, 258.

RAY, B., JEZESKI, J.J. and BUSTA, F.F. (1971a). Effect of rehydration on recovery, repair, and growth of injured freeze-dried Salmonella anatum. Appl. Microbiol., 22, 184-189.

RAY, B., JEZESKI, J.J. and BUSTA, F.F. (1971b). Repair of injury in freeze-dried Salmonella anatum. Appl. Microbiol., 22, 401-407.

RICHARDS, L.A. (1949). Methods of measuring soil moisture tension. Soil Science, 68, 95.

RODERICK, G.L. and DEMIREL, T. (1966). Water vapour-sodium montmorillonite interaction. Highway Res. Record, 128, 45-67.

RODERICK, G.L., SENICH, D. and DEMIREL, T. (1969). X-ray diffraction and adsorption isotherm studies of the montmorillonite-water system. Proc. Int. Clay Conf., Vol.1, 659.

ROUGHLEY, R.J. and VINCENT, J.M. (1967). Growth and survival of Rhizobium sp. in peat culture. J. Appl. Bact., 30, 362-376.

SALTON, M.R.J. (1957). The properties of lysozyme and its action on micro-organisms. Bact. Rev., 21, 82.

SANDERSON, I.J.V. (1962). A study of some aspects of the heat-resistance of Rhizobium. Hons. Thesis. University of Western Australia.

SCHWARTZ, H.M. (1970). Effect of oxygen on freezing damage. 1. Effect on survival of Escherichia coli B/r and Escherichia coli B_{s-1}. Cryobiol., 6, 546.

SCHWARTZ, H.M. (1971). Effect of oxygen on freezing damage: 11. physical-chemical effects. Cryobiol., 8, 255-264.

SCOTT, W.J. (1958). The effect of residual water on the survival of dried bacteria during storage. J. Gen. Microbiol., 19, 624-633.

SHARMA, M.L., UEHARA, G. and MANN, J.A. (1969). Thermodynamic properties of water adsorbed on dry soil surfaces. Soil Science, 107, 86.

SHERWOOD, M.T. (1972). Inhibition of Rhizobium trifolii by yeast extracts or glycine is prevented by calcium. J. Gen. Microbiol., 71, 351.

SKUJINS, J.J. and McLAREN, A.D. (1967). Enzyme rates at limited water activities. Science, 158, 1569.

STEELE, G.D. and TORRIE, J.H. (1960). Principles and procedures of statistics. McGraw-Hill Book Co., Inc., New York.

STEER, B.T. (1973). Dehydration of macromolecules. 11. Protective effects of certain anions on ribulosediphosphate carboxylase subjected to low water potentials in vitro. Aust. J. Biol. Sci., 26, 1435-1442.

STOTZKY, G. (1968). Relevance of soil microbiology to search for life on other planets. Adv. Appl. Microbiol., 10, 17-45.

THOMPSON, J.A. (1964). Studies on the survival of root-nodule bacteria on seed and in soil. Ph.D. Thesis, Univ.Sydney.

TODD, G.W. (1972). Water deficits and enzymatic activity. In "Water deficits and plant growth". Volume 3, pp177-216. Edited by T.T. Kozlowski. Academic Press, New York.

VIEIRA, F.L., SHAFOET, R.I. and SOLOMON, H.K. (1970). The state of water in human and dog red cell membranes. J. Gen. Physiol., 55, 451.

VINCENT, J.M. (1958). Survival of root-nodule bacteria. Univ. Nottingham, 5th Easter School Agric. Sci., Symp. "Nutrition of the Legumes" Ed. E.G. Hallsworth. (Butterworth Sci. Publ.: London). pp108-123.

VINCENT, J.M. (1970). "A manual for the practical study of the root-nodule bacteria." Blackwell Scientific Publications, Oxford and Edinburgh.

VINCENT, J.M. (1972). Nitrogen from microbes. J. Aust. Inst. Agric. Sci., 38, 236.

VINCENT, J.M. and COLBURN, J.R. (1961). Cytological abnormalities in Rhizobium trifolii due to a deficiency of calcium or magnesium. Aust. J. Sci., 23, 269-270.

VINCENT, J.M. and HUMPHREY, B. (1970). Taxonomically significant group antigens in Rhizobium. J. Gen. Microbiol., 63, 379.

VINCENT, J.M., THOMPSON, J.A. and DONOVAN, K.O. (1962). Death of root-nodule bacteria on drying. Aust. J. Agric. Res., 13, 258.

VOSS, J.G. (1967). Effects of organic cations on the gram-negative cell wall and their bactericidal activity with EDTA and surface active agents. J. Gen. Microbiol., 48, 391.

WALKER, J.E., WOLF, M. and KAPSALIS, J.G. (1973). Adsorption of water vapour on myosin A and myosin B. J. Agr. Food Chem., 21, 878.

WALTER, J.A. and HOPE, A.B. (1971). Nuclear magnetic resonance and the state of water in cells. Progr. Biophys. and Mol. Biol., 23, 1.

WANG, J.H. (1954). Theory of the self-diffusion of water in protein solutions. A new method for studying the hydration and shape of protein molecules. J. Am. Chem. Soc., 76, 4755.

WEBB, S.J. (1960a). Factors affecting the viability of airborne bacteria.II. The effect of chemical additives on the behaviour of airborne cells. Can. J. Microbiol., 6, 71.

WEBB, S.J. (1960b). Factors affecting the viability of airborne bacteria. III. The role of bound water and protein structure in the death of airborne cells. Can. J. Microbiol., 6, 89.

WEBB, S.J. (1965). "Bound water in biological integrity". Charles C. Thomas, Springfield, Illinois, USA.

WEBB, S.J. (1967a). Mutation of bacterial cells by controlled desiccation. Nature, 213, 1137.

WEBB, S.J. (1967b). The influence of oxygen and inositol on the survival of semidried microorganisms. Can. J. Microbiol., 13, 733.

WEBB, S.J. (1969). The effects of oxygen on the possible repair of dehydration damage by Escherichia coli. J. Gen. Microbiol, 58, 317-326.

WEBB, S.J. and DUMASIA, M.D. (1967). The induction of lambda prophage by controlled desiccation. Can. J. Microbiol., 13, 33.

WEBB, S.J., DUMASIA, M.D. and BHORJEE, S.J. (1965). Bound water, inositol, and the biosynthesis of temperate and virulent bacteriophages by air-dried Escherichia coli. Can. J. Microbiol., 11, 141.

WEBB, S.J. and WALKER, J.L. (1968a). The effects of mutation and nucleic acid base analogues on the sensitivity of Escherichia coli to partial dehydration. Can. J. Microbiol., 14, 557.

WEBB, S.J. and WALKER, J.L. (1968b). The influence of cell water content on the inactivation of DNA by partial desiccation and ultraviolet light. Can. J. Microbiol., 14, 565.

WEIDEL, W., FRANK, H. and MARTIN, H.H. (1960). The rigid layer of the cell wall of Escherichia coli strain B.J. Gen. Microbiol, 22, 158.

WILKINS, J. (1967). The effects of high temperature on certain root-nodule bacteria. Aust. J. Agric. Res., 18, 299-304.

WILKINSON, S.G. (1968). Studies on the cell walls of pseudomonas species resistant to EDTA. J. Gen. Microbiol., 54, 195.

WON, W.D. and ROSS, H. (1969). Reaction of airborne Rhizobium meliloti to some environmental factors. Appl. Microbiol., 18, 555.

ZIMMERMAN, L. (1962). Survival of Serratia marcescens after freeze drying or aerosolization at unfavourable humidity. I. Effect of sugars. J. Bacteriol., 84, 1297-1302.

ZVYAGINTSEV, D.G. (1962). Adsorption of microorganisms by soil particles. Soviet Soil Science (English transl.)' 140.

7. APPENDIX

APPENDIX TABLE I

Response of various species of rhizobium to desiccation in soil.

Bacteria	Log viable count per 0.05 gm soil. Replication Number 1	2	3	Means
R.trifolii SU297/31A	4.37	4.28	4.23	4.30
R.trifolii SU297/32B	3.52	3.28	3.21	3.34
R.leguminosarum TA101	3.10	3.11	3.25	3.17
R.meliloti SU47	6.08	6.11	6.16	6.12
R.lupini UT12	7.21	7.41	7.44	7.37

Log initial viable count per 0.05 gm. soil were:

R.trifolii SU297/31A	7.34
R.trifolii SU297/32B	7.39
R.leguminosarum TA101	7.03
R.meliloti SU47	8.22
R.lupini UT12	8.57

Analysis of variance (based on log data.)

Source of Variation	DF	Sum of Squares	Mean Square	F
Replication	2	0.001	0.0005	
Bacteria	4	40.049	10.012	720.3 xxx
Error	8	0.111	0.0139	
Total	14	40.161		

APPENDIX TABLE 1

continued

Duncan's Multiple Range Test

Bacterium	Mean of log viable count per 0.05 gm. soil	
R. leguminosarum TA101	3.17	
R. trifolii SU297/32B	3.34	
R. trifolii SU297/31A	4.30	
R. meliloti SU47	6.12	
R. lupini UT12	7.37	

APPENDIX TABLE 11

Response of various species of rhizobia to desiccation in soil.

Bacteria	Log viable count per 0.05gm. soil Replication Number 1	2	Means
Rhizobium sp. 3C1f1	6.80	6.29	6.61
Rhizobium sp. 3I1b117	6.48	6.40	6.44
Rhizobium sp. 3I1b125	6.59	6.48	6.54
Rhizobium sp. CB756	6.56	6.62	6.59
Rhizobium sp. CB421	6.28	6.30	6.30
Rhizobium sp. UT53	6.60	6.71	6.67
Rhizobium sp. SU343	4.11	4.12	4.13
R.meliloti CB112	5.54	4.98	5.35
Rhizobium sp. 3G4b10	6.47	6.28	6.38

Log initial viable counts per 0.05gm soil were:

Rhizobium sp. 3C1f1	8.79
Rhizobium sp. 3I1b117	8.57
Rhizobium sp. 3I1b125	8.47
Rhizobium sp. CB756	8.39
Rhizobium sp. CB421	8.17
Rhizobium sp. UT53	8.74
Rhizobium sp. SU343	8.33
R. meliloti CB112	7.98
Rhizobium sp. 3G4b10	8.29

APPENDIX TABLE 11

continued

Analysis of variance (based on log data)

Source of Variation	DF	Sum of Squares	Mean Square	F
Replication	1	0.087	0.087	2.96 NS
Bacteria	8	11.615	1.452	49.39 xxx
Error	8	0.235	0.0294	
Total	17	11.937		

Duncan's Multiple Range Test

Bacterium	Mean of log viable count per 0.05gm. soil
Rhizobium sp. SU343	4.13
R.meliloti CB112	5.35
Rhizobium sp. CB421	6.30
Rhizobium sp. 3G4b10	6.38
Rhizobium sp. 3I1b117	6.44
Rhizobium sp. 3I1b125	6.54
Rhizobium sp. 3C1f1	6.61
Rhizobium sp. CB756	6.59
Rhizobium sp. UT53	6.67

APPENDIX TABLE 111

Response of various rhizobia to desiccation in soil.

Bacteria	Log viable count per 0.05gm. soil Replication Number 1	2	Means
R.meliloti SU45	4.68	4.68	4.68
R.meliloti SU47A	4.40	4.04	4.27
R.trifolii SU297/31A	5.47	5.44	5.46
R.trifolii SU297/32B	4.35	4.50	4.46
R.trifolii SU298/531B	5.09	4.78	4.98
R.trifolii SU298/534C	5.58	5.53	5.64

Initial viable counts per 0.05gm. soil were:

R.meliloti	SU45	8.27
R.meliloti	SU47 A	8.93
R.trifolii	SU297/31A	8.25
R.trifolii	SU297/32B	8.05
R.trifolii	SU298/531B	8.52
R.trifolii	SU298/534C	8.17

APPENDIX TABLE 111

continued

Analysis of variance (based on log data)

Source of variation	DF	Sum of Squares	Mean Square	F
Replication	1	0.03	0.03	1.56
Bacteria	5	2.943	0.5886	30.66xxx
Error	5	0.096	0.0192	
Total	11	3.069		

Duncan's Multiple Range Test

Bacterium	Mean of log viable count per 0.05gm. soil
R.meliloti SU47A	4.27
R.trifolii SU297/32B	4.46
R,meliloti SU45	4.68
R. trifolii SU298/531B	4.98
R.trifolii SU297/31A	5.46
R.trifolii SU298/534C	5.64

APPENDIX TABLE IV

Effect of alternate desiccation - rehydration cycles on the survival of two species of Rhizobium in soil.

		Log viable count per 0.05 gm. soil.			
		R. trifolii SU297/32B		R. japonicum QA372	
Treatment Number	Replication Number		Mean		Mean
1	1	4.71		7.37	
	2	4.66	4.72	7.29	7.34
	3	4.73		7.34	
2	1	2.47		6.29	
	2	2.20	2.30	6.32	6.33
	3	2.10		6.35	
3	1	0.00		5.24	
	2	0.00	0.00	5.16	5.16
	3	0.00		5.07	
4	1	0.00		4.37	
	2	0.00	0.00	4.53	4.47
	3	0.00		4.47	
5	1	0.00		3.81	
	2	0.00	0.00	3.83	3.82
	3	0.00		3.83	
6	1	0.00		1.76	
	2	0.00	0.00	1.53	1.82
	3	0.00		2.16	

Initial viable counts per 0.05 gm. soil were:

R. trifolii	SU297/32B	8.46
R. japonicum	QA372	8.54

APPENDIX TABLE IV

continued

Analysis of variance (based on log data from treatments 1 and 2 only).

Source of Variation	DF	Sum of Squares	Mean Square	F
Replication	2	0.02	0. 01	
Bacteria	1	33.634	33.634	336.0xxx
Treatment	1	8.962	8.962	896 xxx
Bacteria x Treatment	1	1.533	1.533	153 xxx
Error	6	0.061	0.0102	
Total	11			

APPENDIX TABLE V

Effect of various concentrations of natural montmorillonite on the survival of desiccated R.trifolii TA1 in soil.

	Log viable count per 0.05 gm soil							
Replication	Montmorillonite concentration (% w/w)							
Number	Control	1	2	3	4	5	10	20
1	4.50	4.77	4.73	4.94	4.76	5.43	5.62	6.07
2	4.75	4.66	4.83	4.87	4.96	4.84	5.70	5.89
3	4.59	4.62	5.03	4.86	5.29	4.96	5.36	5.85
Means	4.64	4.69	4.88	4.90	5.06	5.16	5.59	5.95

Log initial viable count per 0.05 gm. soil was 8.34

Analysis of variance (based on log data)

Source of Variation	DF	Sum of Squares	Mean Square	F
Replication	2	0.008	0.004	
Additive	7	4.285	0.612	16.63 xxx
Error	14	0.515	0.0368	
Total	23	4.808		

Duncan's Multiple Range Test

Montmorillonite concentration (%)	Control	1	2	3	4	5	10	20
Means of log viable counts per 0.05 gm. soil.	4.64	4.69	4.88	4.90	5.06	5.16	5.59	5.95

APPENDIX TABLE V1

Effect of PVP, maltose and various concentrations of natural montmorillonite on the survival of desiccated R.trifolii SU297/31A in soil.

Additive concentration (% w/w)	Log viable count per 0.05 gm. soil Replication Number 1	2	Mean
0	5.56	5.71	5.64
3	5.89	5.96	5.93
6	6.01	5.83	5.93
9	6.18	6.21	6.19
12	6.25	6.18	6.27
15	6.34	6.26	6.31
20	6.16	6.34	6.32
30	6.38	6.46	6.43
Maltose	6.34	6.50	6.43
PVP	6.18	6.26	6.22

Log initial viable count per 0.05 gm. soil = 7.75

Analysis of variance (based on log data)

Source of variation	DF	Sum of Squares	Mean Square	F
Replication	1	0.013	0.013	1.63
Additive	9	1.139	0.1266	15.83 xxx
Error	9	0.078	0.008	
Total	19	1.23		

APPENDIX TABLE V1

continued

Duncan's Multiple Range Test

Additive Concentration (% w/w)	Mean log viable count per 0.05 gm. soil
Control	5.64
6	5.93
3	5.93
9	6.19
12	6.22
PVP	6.22
15	6.31
20	6.32
30	6.43
Maltose	6.43

APPENDIX TABLE V11

Effect of Maltose, PVP and various concentrations of natural montmorillonite on the survival of desiccated R.trifolii SU297/32B in soil.

	Log viable count per 0.05 gm. soil		
Additive Concentration (% w/w)	Replication Number 1	2	Mean
0	3.32	3.15	3.25
3	3.71	3.68	3.70
6	3.79	3.83	3.81
9	3.83	3.86	3.84
12	3.91	3.92	3.92
15	3.94	3.92	3.93
20	3.78	3.96	3.88
30	3.69	3.75	3.72
Maltose	4.78	4.78	4.78
PVP	4.18	4.14	4.16

Log initial viable count per 0.05 gm. soil was 6.02

Analysis of variance (based on log data)

Source of variation	DF	Sum of Squares	Mean Square	F
Replication	1	0.001	0.001	
Additive	9	2.744	0.305	80.3 xxx
Error	9	0.034	0.0038	
Total	19	2.779		

APPENDIX TABLE V11

continued

Duncan's Multiple Range Test

Additive Concentration (% w/w)	Mean Log viable count per 0.05 gm. soil	
Control	3.25	
3	3.70	
30	3.72	
6	3.81	
9	3.84	
20	3.88	
12	3.92	
15	3.93	
PVP	4.16	
Maltose	4.78	

APPENDIX TABLE VIII

Effect of various additives on the survival of desiccated Rhizobium sp. SU343 in soil.

Log viable count per 0.05 gm. soil.

Replication	Control	Montmorillonite concentration (% w/w) 1	2	Maltose	PVP
1	4.89	4.95	6.63	5.85	4.36
2	4.52	4.69	6.34	5.99	4.59
Mean	4.71	4.82	6.35	5.92	4.50

Log initial count per 0.05 gm. soil was 8.18

Analysis of variance (based on log data)

Source of variation	DF	Sums of Squares	Mean Square	F
Replication	1	0.008	0.008	
Additive	4	5.483	1.371	52.33 xxx
Error	5	0.131	0.0262	
Total	10	5.622		

APPENDIX TABLE V111

continued

Duncan's Multiple Range Test

Additive Concentration (%)	Mean log viable count per 0.05 gm soil	
PVP	4.48	|
Control	4.71	|
1% montmorillonite	4.82	|
Maltose	5.92	|
20% montmorillonite	6.35	|

APPENDIX TABLE IX

Effect of various concentrations of natural montmorillonite on the survival of desiccated R.leguminosarum TA101 in soil.

Montmorillonite Concentration % (w/w)	Log viable count per 0.05gm. soil Replication Number 1	2	3	Mean
0	5.99	5.98	5.96	5.98
1	5.97	6.27	5.73	6.04
2	6.24	6.18	6.41	6.30
3	6.33	6.27	6.03	6.23
4	6.57	6.31	6.17	6.38
5	6.55	6.43	6.44	6.48
10	6.74	6.67	6.71	6.71

Log initial viable count per 0.05 gm soil was 8.32.

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.066	0.033	
Additive	6	1.216	0.203	9.62xxx
Error	12	0.253	0.0211	
Total	20	1.535		

Duncan's Multiple Range Test

Montmorillonite Concentration (%w/w)	0	1	3	2	4	5	10
Mean log viable count per 0.05 gm. soil	5.98	6.04	6.23	6.30	6.38	6.48	6.71

APPENDIX TABLE X

Effect of various concentrations of natural montmorillonite on the survival of desiccated R.leguminosarum TA101 in soil.

Replication No.	Log viable count per 0.05 gm. soil Montmorillonite concentration (% w/w)										
	0	1	2	3	4	5	10	15	20	25	30
1	5.30	5.92	6.03	5.99	6.47	6.53	7.02	7.00	7.08	7.02	7.24
2	5.60	5.98	5.94	5.99	6.49	6.54	6.87	6.93	7.13	6.95	7.26
3	5.46	5.90	6.04	6.05	6.46	6.55	6.94	7.14	7.12	7.04	7.17
Mean	5.48	5.94	6.01	6.02	6.48	6.56	6.95	7.03	7.11	7.01	7.23

Log initial viable count per 0.05 gm soil = 8.38

Analysis of variance (based on log data)

Source of Variation	D.F.	Sum of Squares	Mean Square	F
Replication	2	0.003	0.0015	
Additive	10	10.56	1.06	265xxx
Error	20	0.08	0.004	
Total	32	10.67		

Duncan's Multiple Range Test

Montmorillonite Concentration (%)	Log viable count per 0.05gm soil
0	5.48
1	5.94
2	6.01
3	6.02
4	6.48
5	6.56
10	6.95
25	7.01
15	7.02
20	7.11
30	7.23

APPENDIX TABLE X1

Effect of various additives on the survival of desiccated *R.trifolii* SU297/31A in soil.

Replication Number	Log viable count per 0.05 gm soil: Additive					
	control	goethite	haema-tite	kaol-inite	illite	montmor-illonite
1	3.87	3.51	3.48	3.52	4.37	5.06
2	3.83	3.42	3.58	3.37	4.28	4.77
3	3.78	3.42	3.61	3.13	4.38	5.07
Mean	3.83	3.46	3.56	3.37	4.35	4.89

Log initial viable count per 0.05 gm soil = 7.36

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.029	0.0145	
Additive	5	5.918	1.1836	90.35 xxx
Error	10	0.131	0.0131	
Total	17	6.078		

Duncan's Multiple Range Test

Additive	kaol-inite	goethite	haema-tite	control	illite	montmor-illonite
Mean log viable count per 0.05 gm soil	3.37	3.46	3.56	3.83	4.35	4.89

APPENDIX TABLE X11

Effect of Maltose, PVP and various concentrations of montmorillonite on the survival of desiccated R.meliloti CC131 in soil.

Additive Concentration (% w/w)	Log viable count per 0.05 gm soil Replication Number 1	2	Mean
0	6.28	6.05	6.20
1	6.77	6.53	6.67
2	6.65	6.44	6.57
3	6.44	6.45	6.45
4	6.40	6.32	6.37
5	6.08	6.15	6.15
10	5.97	6.03	5.88
20	6.83	6.88	6.86
Maltose	6.77	7.06	6.95
PVP	6.48	6.47	6.48

Log initial viable count per 0.05 gm soil = 8.36

Analysis of variance (based on log data)

Source of Variation	D.F	Sums of Squares	Mean Square	F
Replication	1	0.004	0.004	
Additive	9	1.668	0.1853	13.24 xxx
Error	9	0.124	0.014	
Total	19	1.796		

APPENDIX TABLE X11

continued

Duncan's Multiple Range Test

Additive Concentration (% w/w)	Mean log viable count per 0.05 gm soil
10	5.88
5	6.15
0	6.20
4	6.37
3	6.45
PVP	6.48
2	6.57
1	6.67
20	6.86
Maltose	6.95

APPENDIX TABLE X111

Effect of various concentrations of natural montmorillonite on the survival of desiccated R. japonicum QA372 in soil.

Montmorillonite Concentration (% w/w)	Log viable count per 0.05gm soil Replication Number 1	2	3	Mean
0	7.02	6.93	6.99	6.98
1	6.62	6.79	6.76	6.73
2	6.45	6.38	6.53	6.47
3	6.64	6.28	6.30	6.44
4	6.34	6.42	6.53	6.44
5	6.10	6.43	6.35	6.31
10	6.32	6.27	6.32	6.31
15	5.93	6.26	6.01	6.05
20	5.64	5.82	5.79	5.76
25	5.74	5.96	6.06	5.94
30	5.96	5.93	5.97	5.95

Log initial viable count per 0.05 gm soil was 8.18

Analysis of variance (based on log data)

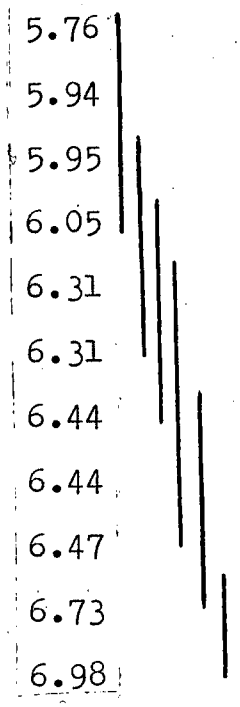
Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.038	0.016	
Additive	10	3.945	0.3945	20.33 xxx
Error	20	0.387	0.0194	
Total	32	4.27		

APPENDIX TABLE X111

continued

Duncan's Multiple Range Test

Montmorillonite Concentration (%)	Mean log viable count per 0.05 gm soil
20	5.76
25	5.94
30	5.95
15	6.05
5	6.31
10	6.31
3	6.44
4	6.44
2	6.47
1	6.73
0	6.98



This Duncan's multiple range test was done at $p < 0.01$

APPENDIX TABLE XIV

Effect of Maltose, PVP and various concentrations of natural montmorillonite on the survival of desiccated R.lupini UT12 in soil.

Additive Concentration (% w/w)	Log viable count per 0.05gm soil Replication Number 1	2	3	Mean
0	4.53	4.93	4.45	4.70
1	4.59	4.99	4.70	4.80
2	4.70	4.63	4.72	4.69
3	4.89	4.46	4.71	4.72
4	4.69	4.71	4.70	4.70
5	4.23	4.71	4.70	4.60
10	5.06	4.78	4.64	4.87
15	4. 68	4.96	4.70	4.81
PVP	6.03	6.06	6.18	6.10
Maltose	6.95	6.98	7.05	7.00

Log initial viable count per 0.05 gm soil = 7.45

Analysis of variance (based on log data)

Source of Variation	D.F	Sums of Squares	Mean Square	F
Replication	2	0.041	0.021	
Additive	9	17.623	1.958	60.43 xxx
Error	18	0.583	0.0324	
Total	29	18.247		

APPENDIX TABLE XIV

continued

Duncan's Multiple Range Test

Additive Concentration (% w/w)	Mean log viable count per 0.05 gm soil
5	4.60
Control	4.70
2	4.69
3	4.72
4	4.70
1	4.80
15	4.81
10	4.87
PVP	6.10
Maltose	7.00

APPENDIX TABLE XV (a)

Effect of removal of water from both unamended and montmorillonite-amended soil samples on the survival of R.lupini UT12.

(a) Unamended soil

Weight of water lost from soil (% w/w)	Log viable count per 0.05 gm soil		
	Replication Number		
	1	2	Mean
26.8	8.29	8.39	8.35
74.7	8.29	8.34	8.32
83.7	8.28	8.23	8.26
88.6	8.30	8.49	8.41
92.0	8.31	8.17	8.26
94.5	8.09	8.17	8.15
97.0	8.26	8.21	8.23
98.7	8.34	7.56	8.10
99.8	7.48	7.65	7.58
99.8	6.90	6.92	6.91
100 (24 hrs. drying)	6.61	6.02	6.42

Log initial viable count per 0.05 gm soil = 8.52

APPENDIX TABLE XV (a)

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.046	0.046	
Water loss	10	9.238	0.9238	18.97 xxx
Error	10	0.487	0.0487	
Total	21	9.771		

Duncan's Multiple Range Test

Water lost (% w/w)	Mean Log Numbers per 0.05 gm soil
100	6.42
99.8	6.91
99.8	7.58
98.7	8.10
94.5	8.15
92.0	8.26
97.0	8.23
83.7	8.26
74.7	8.32
26.8	8.35
88.6	8.41

APPENDIX TABLE XV (b)

Effect of removal of water from both unamended and montmorillonite amended soil samples on the survival of R.lupini UT12.

(b) Soil amended with 5% montmorillonite

Weight of water lost from amended soil (% w/w)	Log viable count per 0.05 gm soil. Replication Number 1	2	Mean
35.2	8.38	8.21	8.33
82.2	8.18	8.29	8.24
85.7	8.02	8.02	8.02
89.6	8.12	8.07	8.10
92.9	7.74	7.80	7.77
94.3	7.76	7.97	7.88
96.1	7.77	7.55	7.68
97.5	7.54	7.13	7.38
97.9	7.34	6.83	7.16
98.0	7.02	6.89	6.79
98.8 (24 hrs. drying)	5.66	5.67	5.67

Log initial viable count per 0.05 gm soil = 8.52

APPENDIX TABLE XV (b)
continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.055	0.055	2.33
Water loss	10	11.742	1.174	49.746 xxx
Error	10	0.236	0.0236	
Total	21	12.033		

Duncan's Multiple Range Test

Water lost (% w/w)	Mean Log Numbers per 0.05 gm soil
98.8	5.67
98.0	6.79
97.9	7.16
97.5	7.38
96.1	7.68
92.9	7.77
94.3	7.88
85.7	8.02
89.6	7.77
82.2	8.24
35.2	8.33

APPENDIX TABLE XVI

Effect of various concentrations of montmorillonite added as a powder and as a suspension, on the survival of desiccated R.lupini UT12 in soil.

	Log viable count per 0.05 gm soil							
	Montmorillonite concentration: as a suspension -ugm/ml--					% powder - in soil-		
Replication	control	17.5	175	1750	17500	5	10	20
1	5.51	5.74	5.57	5.42	5.10	5.44	5.65	5.49
2	5.68	5.71	5.76	5.52	5.15	5.37	5.59	5.55
3	5.84	5.85	5.62	5.60	4.98	5.62	5.57	5.73
Mean	5.70	5.77	5.71	5.52	5.10	5.50	5.61	5.60

Log initial viable count per 0.05 gm. soil = 7.80

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.05	0.0025	
Additive	7	0.92	0.131	14.6 xxx
Error	14	0.13	0.009	
Total	23	1.1		

Duncan's Multiple Range Test

Montmorillonite Concentrations	17500	5%	1750	20%	10%	175	control	17.5
Mean log viable count per 0.05 gm soil	5.10	5.50	5.52	5.60	5.61	5.71	5.70	5.77

APPENDIX TABLE XV11

Effect of various concentration of montmorillonite added as a suspension, on the survival of desiccated R.leguminosarum TA101 in soil.

	Log viable count per 0.05 gm soil. Montmorillonite concentration in suspension (ugm/ml)							
Replication Number	control	17.5	175	400	500	1000	1750	2000
1	4.91	4.75	4.79	4.89	4.89	4.69	5.02	4.75
2	4.79	5.20	4.84	4.88	4.58	4.68	4.90	4.54
3	4.98	4.71	5.05	4.95	4.97	4.95	4.91	4.57
Mean	4.90	4.95	4.91	4.91	4.84	4.80	4.94	4.63

Log initial viable count per 0.05 gm soil = 8.06

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.03	0.015	
Additive	7	0.24	0.034	1.1 NS
Error	14	0.44	0.031	
Total	23	0.71		

APPENDIX TABLE XVIII

Effect of various concentrations of montmorillonite added as a powder and as a suspension, on the survival of desiccated R.leguminosarum TA101 in soil.

Log viable count per 0.05 gm soil.

Replication Number	Montmorillonite concentration -ugm/ml- control	70	140	1400	-soil- 5 % (w/w) powder	5 % (w/w) suspension
1	4.31	4.40	4.26	4.16	5.26	4.76
2	4.32	4.39	4.61	4.24	5.07	4.83
3	3.95	4.00	3.82	4.11	5.16	5.05
Mean	4.23	4.30	4.07	4.18	5.18	4.91

Log initial viable count per 0.05 gm soil = 8.44

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.10	0.05	
Additive	5	3.14	0.628	18.5 xxx
Error	8	0.27	0.034	
Total	17	3.51		

APPENDIX TABLE XVIII

continued

Duncan's Multiple Range Test

Montmorillonite Concentration	140	1400	0	70	5% suspension	5% powder
Mean log viable count per 0.05gm. soil	4.07	4.18	4.23	4.30	4.91	5.18

APPENDIX TABLE XIX

Effect of various concentrations of montmorillonite added as a powder and as a suspension, on the survival of desiccated R.leguminosarum TA101 in soil.

	Log viable count per 0.05 gm soil						
		Montmorillonite concentration in -suspension (ugm/ml) -				%montmorillonite - powder in soil -	
Replication	control	17.5	175	1750	17500	5	10	20
1	4.46	4.46	4.67	3.72	3.25	5.28	5.86	6.27
2	4.09	4.33	4.79	3.86	3.36	5.27	6.08	5.82
3	4.33	3.87	4.70	2.96	3.50	5.31	5.77	5.70
Mean	4.33	4.28	4.73	3.65	3.38	5.29	5.94	6.00

Log initial viable count per 0.05 gm soil = 7.24

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.24	0.12	2.2 NS
Additive	7	20.59	2.94	54.4 xxx
Error	14	0.76	0.054	
Total	23	21.59		

Duncan's Multiple Range Test

Montmorillonite Concentration	**17500**	**1750**	**17.5**	**0**	**175**	**5%**	**10%**	**20%**
Mean log viable count per 0.05gm soil	3.38	3.65	4.28	4.33	4.73	5.29	5.94	6.00

APPENDIX TABLE XX

Effect of various additives on the survival of desiccated R.leguminosarum TA101 in soil.

Additive	Log viable count per 0.05 gm soil Replication Number 1	2	Mean
Control	4.86	4.64	4.77
PEG 400	1.0[x]	1.0[x]	1.00[x]
" 1500	3.04	2.20	2.83
" 6000	5.72	5.69	5.71
Glucose	6.38	6.43	6.41
Sucrose	6.54	6.61	6.61
Maltose	6.41	6.66	6.56
Glycerol	4.73	4.27	4.56
Montmorillionite (wet)	5.03	5.11	5.07
" (dried)	6.29	5.84	6.14
PVP (5%)	5.60	5.94	5.81
" (10%)	4.81	4.80	4.81
Inositol	4.45	4.90	4.73
Ammonium acetate 1%	4.86	4.90	4.88
" 5%	4.84	5.18	5.06
" 10%	4.33	5.06	4.85
Hydrophobic aerosil	4.99	4.74	4.89
Hydrophilic aerosil (wet)	2.08	2.77	2.56
Hydrophilic aerosil (dried)	3.40	3.14	3.28

x No recovery when diluted 1 in 10.

Log initial viable count per 0.05 gm soil = 7.92

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.007	0.007	
Additive	18	78.773	4.376	56.175 xxx
Error	18	1.402	0.0779	
Total	37	80.182		

Duncan's Multiple Range Test

Additive	Mean log viable count per 0.05 gm soil
PEG 400	1.00
Hydrophilic aerosil (wet)	2.56
PEG 1500	2.83
Hydrophilic aerosil (dried)	3.28
Glycerol	4.56
Inositol	4.73
Ammonium acetate 5%	4.88
Control	4.77
PVP (10%)	4.81
Hydrophobic aerosil	4.89
Ammonium acetate 1%	5.06
" " 10%	4.85
Montmorillonite (wet)	5.07
PEG 6000	5.71
PVP 5%	5.81
Montmorillonite (dried)	6.14
Glucose	6.41
Maltose	6.56
Sucrose	6.61

APPENDIX TABLE XX1

Effect of various additives on the survival of desiccated *Rhizobium* sp. SU343 in soil.

Additive	Log viable count per 0.05 gm soil		
	Replication Number		
	1	2	Mean
Control	3. 87	3. 98	3.97
Hydrophobic aerosil	3. 82	3. 32	3.75
Hydrophilic aerosil (wet)	1. 00[x]	1. 00[x]	1.00[x]
Hydrophilic aerosil (dried)	1. 08	1. 08	1.10
Glycerol	2. 53	2. 01	2.36
Montmorillonite (wet)	2. 79	2. 88	2.91
Montmorillonite (dried)	5. 13	4. 96	5.06
PEG 400	1. 00[x]	1. 00[x]	1.00[x]
" 1500	2. 07	2. 05	2.07
" 6000	4. 8	5. 23	5.10
Sucrose	6. 67	6. 82	6.75
Glucose	7. 08	6. 91	7.61
Maltose	6. 71	6. 73	6.72
Ammonium acetate 1%	4. 76	5. 20	5.06
" 5%	4. 58	4. 37	4.54
Inositol	5. 62	5. 40	5.61
PVP (10%)	4. 52	4. 49	4.60

x See footnote Appendix Table XX

Log initial viable count per 0.05 gm soil = 8.22

APPENDIX TABLE XX1

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.014	0.014	
Treatment	16	129.336	8.084	247.97 xxx
Error	17	0.498	0.0293	
Total	33	129.848		

Duncan's Multiple Range Test

Additive	Mean log viable count per 0.05 gm soil
Hydrophilic aerosil (dried)	1.10
PEG 1500	2.07
Glycerol	2.36
Montmorillonite (wet)	2.91
Hydrophobic aerosil	3.75
Control	3.97
Ammonium acetate 5%	4.54
PVP 10%	4.60
Ammonium acetate 1%	5.06
Montmorillonite (dried)	5.06
PEG 6000	5.10
Inositol	5.61
Sucrose	6.75
Maltose	6.72
Glucose	7.61

APPENDIX TABLE XX11

Effect of various additives on the survival of desiccated R.japonicum QA372 in soil.

Additive	Log viable count per 0.05 gm soil		
	Replication Number		
	1	2	Mean
Control	6. 43	6. 30	6.37
Montmorillonite (dried)	6. 10	6. 06	6.09
Montmorillonite (wet)	5. 80	5. 90	5.85
PEG 400	4. 00	4. 46	4.30
" 1500	6. 05	5. 87	5.97
" 6000	5. 91	6. 63	6.42
Glycerol	5. 41	5. 37	5.40
PVP (5%)	6. 76	6. 92	6.85
Maltose	7. 05	7. 05	7.05
Sucrose	7. 09	7. 16	7.12
Glucose	7. 09	7. 09	7.09
Hydrophilic aerosil (wet)	4. 10	4. 12	4.11
Hydrophilic aerosil (dried)	5. 14	4. 77	5.00
Hydrophobic aerosil	6. 48	6. 48	6.48
Ammonium acetate (5%)	5. 78	5. 72	5.75
Ammonium acetate (2.5%)	5. 91	5. 85	5.88

Log initial viable count per 0.05 gm soil = 7.97

APPENDIX TABLE XX11

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.014	0.014	
Additive	15	25.909	1.727	55.18xxx
Error	15	0.470	0.0313	
Total	31	26.393		

Duncan's Multiple Range Test

Additive	Mean log viable count per 0.05 gm soil
Hydrophilic aerosil (wet)	4.11
PEG 400	4.30
Hydrophilic aerosil (dried)	5.00
Glycerol	5.40
Ammonium acetate 5%	5.75
Montmorillonite (wet)	5.85
Ammonium acetate 2.5%	5.88
PEG 1500	5.97
Montmorillonite (dried)	6.09
PEG 6000	6.42
Control	6.37
Hydrophobic aerosil	6.48
PVP 5%	6.85
Maltose	7.05
Glucose	7.09
Sucrose	7.12

APPENDIX TABLE XX111

Effect of various additives on the survival of desiccated R.lupini UT12 in soil.

Additive	Log viable count per 0.05 gm soil. Replication Number 1	2	Mean
Control	5. 07	5. 31	5.23
Ammonium acetate	$<10^2$	$<10^2$	$<10^2$
Montmorillonite (dried)	4.84	4. 32	4.72
Montmorillonite (wet)	3.82	3. 32	3.67
Hydrophobic aerosil (dry)	4.09	3. 99	4.06
Hydrophilic aerosil (wet)	4.39	4. 18	4.32
Hydrophobic aerosil (dry)	5.53	5. 03	5.39
Hydrophobic aerosil (wet)	4.72	5. 02	4.92
PEG 400	$<10^3$	$<10^3$	$<10^3$
" 1500	$<10^3$	$<10^3$	$<10^3$
" 6000	$<10^3$	$<10^3$	$<10^3$
PVP 3%	5.92	6.03	6.00
" 10%	6.05	6.42	6.28
Glycerol	$<10^3$	$<10^3$	$<10^3$
Sucrose	6.62	6.82	6.73
Maltose	6.75	6.83	6.80
Glucose	6.70	6.89	6.82

Log initial viable count per 0.05 gm soil = 7.97

APPENDIX TABLE XXIII

continued

The Duncan's multiple range test was done only on those results used in the above analysis of variance.

Additive	Mean log viable count per 0.05 gm soil
Montmorillonite (wet)	3.67
Hydrophilic aerosil (dried)	4.06
Hydrophilic aerosil (wet)	4.32
Montmorillonite (dried)	4.72
Hydrophobic aerosil (wet)	4.92
Control	5.23
Hydrophobic aerosil (dried)	5.39
PVP 3%	6.00
PVP 10%	6.28
Sucrose	6.73
Maltose	6.80
Glucose	6.82

The treatment for which counts were not obtained (i.e. ammonium acetate; PEG 400, 1500, 6000; glycerol) were not included in the analysis of variance.

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.013	0.013	
Additive	10	23.68	2.368	41.47 xxx
Error	10	0.571	0.0571	
Total	21	24.264		

APPENDIX TABLE XXIV

Effect of various additives on the survival of desiccated *R.leguminosarum* TA101 in soil.

Replication Number	Log viable count per 0.05 gm soil. Control	PVP	Montmorillonite	Montmorillonite + PVP	T	Maltose
1	4.81	6.02	5.73	4.96	1.0[x]	6. 40
2	4.47	6.02	5.46	4.83	1.0[x]	6. 35
3	4.84	6.08	5.73	5.07	1.52	6. 35
Mean	4.75	6.04	5.67	4.99	1.09	6. 38

Log initial viable count per 0.05 gm soil = 7.69

Log initial viable count per 0.05 gm soil when mixed with tetrabutylammonium bromide (T) = 6.95

x see footnote, table XX.

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.178	0.089	5. 298 x
Additive	5	53.645	10.729	638. 63 xxx
Error	10	0.168	0.0168	
Total	17	53.991		

APPENDIX TABLE XXIV

continued

Duncan's Multiple Range Test

Additive	T	Control	Montmorillonite + PVP	Montmorillonite	PVP	Maltose
Means of log viable counts per 0.05 gm. soil	1.09	4.75	4.99	5.67	6.04	6.38

APPENDIX TABLE XXV

Effect of various additives on the survival of desiccated *Rhizobium* sp. SU343 in soil.

Replication Number	Log viable count per 0.05 gm soil					
	Control	PVP	Montmorillonite	Montmorillonite + PVP	T	Maltose
1	4.37	6.19	5.80	5. 56	1.88	7. 05
2	4.80	6.26	5.75	5. 72	1.63	7. 07
3	4.74	6.21	6.24	5. 42	1.08	7. 03
Mean	4.68	6.22	6.06	5. 60	1.65	7. 05

Log initial viable count per 0.05 gm soil = 8.18

Log initial viable count per 0.05 gm soil when mixed with tetrabutylammonium bromide = 6.60

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	2	0.022	0. 011	
Additive	5	56.714	11.343	184. 44 xxx
Error	10	0.615	0.0615	
Total	17	57.351		

APPENDIX TABLE XXV

continued

Duncan's Multiple Range Test

Additive	T	Control	Montmorillonite + PVP	Montmorillonite	PVP	Maltose
Mean of log viable count per 0.05 gm. soil.	1.65	4.68	5.60	6.06	6.22	7.05

APPENDIX TABLE XXVI

Effect of various additives on the survival of desiccated *R. japonicum* QA372 in soil sampled over a period of 120 hrs.

Log viable count per 0.05 gm. soil.

Time (hrs.)	Rep. No.	Control	Mont-morillonite	PVP + Mont-morillonite	T[x]	PVP	Maltose
24	1	6.72	6.51	6.48	3.47	7.14	7.28
	2	6.79	6.18	6.39	3.68	7.08	7.31
48	1	6.58	6.21	6.40	3.17	6.98	7.28
	2	6.47	6.20	6.45	3.17	6.93	7.27
72	1	6.48	5.69	6.00	3.23	6.61	7.25
	2	6.36	6.07	6.30	2.91	6.80	7.18
96	1	5.72	5.68	6.29	2.96	6.80	7.08
	2	5.98	5.71	6.43	2.96	6.49	7.08
120	1	5.76	5.59	5.63	2.76	6.09	7.10
	2	5.96	5.59	5.64	2.77	6.06	7.15

Log initial viable count per 0.05 gm. soil = 8.12

Log initial viable count per 0.05 gm. soil when mixed with T = 8.01. x T = tetrabutylammonium bromide.

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.0	0. 0	
Time	4	3.978	0.9945	71.55 xxx
Additive	5	104.239	20.848	1499.86 xxx
Time x Additive	20	1.132	0.0566	4.0719xxx
Error	29	0.403	0.0139	
Total	59	109.752		

APPENDIX TABLE XXVI

continued

Duncan's Multiple Range Test

Additive

Time (hrs)	T	Montmorillonite	Montmorillonite + PVP	Control	PVP	Maltose
24	3.59	6.38	6.43	6.86	7.11	7.30
48	3.17	6.22	6.43	6.53	6.95	7.28
72	3.11	5.93	6.19	6.43	6.72	7.22
96	2.91	5.70	6.37	5.87	6.68	7.07
120	2.77	5.49	5.64	5.87	6.07	7.12

APPENDIX TABLE XXVI

continued

Duncan's Multiple Range Test

Control

Time (hrs)	96	120	72	48	24
Means[x]	5.87	5.87	6.43	6.53	6.86

Montmorillonite

Time (hrs)	120	96	72	48	24
Means[x]	5.49	5.70	5.93	6.22	6.38

Montmorillonite + PVP

Time (hrs)	120	72	96	48	24
Means[x]	5.64	6.19	6.37	6.43	6.43

Tetrabutylammonium bromide

Time (hrs)	120	96	72	48	24
Means[x]	2.77	2.91	3.11	3.17	3.59

PVP

Time (hrs)	120	96	72	48	24
Means[x]	6.07	6.68	6.72	6.59	7.11

Maltose

Time (hrs)	96	120	72	48	24
Means[x]	7.07	7.12	7.22	7.28	7.30

x Means = log viable counts per 0.05 gm. soil.

APPENDIX TABLE XXVII

Effect on survival of desiccation of R.leguminosarum TA101 in atmospheres of air and nitrogen, both in unamended and montmorillonite-amended soil samples.

Log viable count per 0.05 gm. soil.

Replication Number	--Air Dried -- samples		-- Nitrogen Dried -- samples	
	No. Montmor-illonite	Plus Montmor-illonite	No. Montmor-illonite	Plus Montmor-illonite
	Desiccator A		Desiccator C	
1	4.30	4.95	5.36	5.94
	4.34	4.70	5.34	5.89
	4.32	4.70	5.36	5.93
	3.85	4.78	5.18	5.87
2	3.86	4.53	5.28	5.73
	3.94	4.38	5.08	5.89
	3.95	4.61	5.30	5.92
	3.89	4.64	5.28	5.72
	Desiccator B		Desiccator D	
1	5.30	5.69	5.26	5.98
	5.34	5.76	5.42	6.03
	5.42	5.68	5.54	5.91
	5.23	5.74	5.32	5.85
2	5.18	5.85	5.45	5.81
	5.30	5.88	5.46	5.94
	5.15	5.81	5.40	5.91
	5.18	5.85	5.40	5.88

Log initial viable count per 0.05 gm. soil = 8.48

APPENDIX TABLE XXV11

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Gas	1	7.243	7.243	1. 33
Desiccator x gas	2	10.905	5.453	
Amended soil x unamended soil	1	4.935	4.934	102.8 xxx
Amended soil x gas	1	0.001	0.001	NS
Desiccator x amended soil	2	0.027	0.014	NS
Replication within desiccators	8	0.384	0.048	
Samples within replications	16	0.463	0.028	
Samples within samples	32	0.003	0.000	
Total	63	23.96		

Error mean square for main effects was the desiccator x gas interaction.

Error mean square for the sub-plots was the replications within desiccators.

APPENDIX TABLE XXV111

Growth of R.trifolii TP48 in yeast mannitol brothof different water activities obtained with NaCl.

Time (Hrs.)	Average log absorbance readings of 2 replicate tubes at a water activity of:- 0.999	0.997	0.995
0	0.07	0.07	0.07
16	0.13	0.098	0.08
24	0.40	0.17	0.09
28	0.49	0.24	0.09
40	0.67	0.39	0.096
48	0.77	0.45	0.10
52	0.81	0.49	0.10
65	0.86	0.54	-
72	0.89	0.55	-
88	0.89	0.55	-
112	0.92	0.54	0.10
116	0.92	0.55	-
137	0.93	0.55	-
157	0.93	-	-

APPENDIX TABLE XXIX

Growth of R.trifolii SU297/31A and R.trifolii SU297/32B in yeast mannitol broth of different water activities obtained with NaCl.

Bacterium	Time (hrs)	Average log absorbance readings of 2 replicate tubes at a water activity of:- 0.999	0.997	0.995
R.trifolii SU297/32B	0	0.07	0.08	0.09
	16	0.07	0.08	0.09
	24	0.13	0.05	0.07
	28	0.30	0.05	0.07
	40	0.38	0.05	0.06
	48	0.42	0.05	0.07
	52	0.45	0.05	0.07
	65	0.53	-	-
	72	0.53	-	-
	88	0.53	-	-
	112	0.53	-	-
	116	0.54	-	-
R.trifolii SU297/31A	0	0.06	0.04	0.05
	16	0.09	0.04	0.04
	24	0.11	0.04	0.04
	28	0.20	0.03	0.03
	40	0.40	0.03	0.02
	48	0.50	0.03	0.03
	52	0.55	0.04	0.03
	65	0.62	0.04	0.03
	72	0.84	-	-
	88	0.87	-	-
	112	0.90	-	-
	116	0.90	-	-

APPENDIX TABLE XXX

Growth of *Rhizobium meliloti* SU45 in yeast mannitol broth of different water activities obtained with NaCl.

Bacterium	Time (hrs)	Average log absorbance readings of two replicate tubes at a water activity of:-					
		0.999	0.997	0.995	0.990	0.980	0.960
	0	0.0096	0.0091	0.0078	0.0078	0.0078	0.0082
	3	0.0094	0.0088	0.0078	0.0078	0.0078	0.0085
	6	0.0106	0.0096	0.0088	0.0082	0.0078	0.0082
	9	0.0129	0.0121	0.0112	0.0099	0.0078	0.0078
R.meliloti SU45	10	0.0140	0.0130	0.0124	0.0106	0.0082	0.0078
	11	0.0147	0.0139	0.0132	0.0112	0.0085	0.0078
	12	0.0154	0.0145	0.0140	0.0117	0.0082	0.0078
	13	0.0164	0.0158	0.0154	0.0126	0.0082	0.0078
	14	0.0171	0.0165	0.0160	0.0133	0.0085	0.0078
	24	0.0195	0.0190	0.0190	0.0181	0.0117	0.0082

APPENDIX TABLE XXXI

Growth of *Rhizobium meliloti* SU47A in yeast mannitol broth of different water activities obtained with NaCl.

Bacterium	Time (hrs)	Average log absorbance readings of two replicate tubes at a water activity of:- 0.099	0.997	0.995	0.990	0.980	0.960
	0	0.0099	0.0096	0.0099	0.0096	0.0101	0.0103
	3	0.0096	0.0094	0.0099	0.0096	0.0106	0.0103
	6	0.0108	0.0108	0.0120	0.0103	0.0106	0.0099
	9	0.0134	0.0137	0.0137	0.0124	0.0110	0.0099
R.meliloti	10	0.0145	0.0149	0.0150	0.0130	0.0116	0.0099
	11	0.0152	0.0157	0.0160	0.0136	0.0115	0.0096
	12	0.0161	0.0165	0.0166	0.0148	0.0115	0.0096
	13	0.0170	0.0171	0.0172	0.0157	0.0115	0.0096
	14	0.0174	0.0176	0.0178	0.0162	0.0115	0.0096
	24	0.0196	0.0198	0.020	0.0189	0.0115	0.0096

APPENDIX TABLE XXX11

Growth of R.lupini UT2 in yeast mannitol broth at various water activities obtained with NaCl.

Time (hrs)	Average log absorbance readings of 2 replicate tubes at a water activity of:- 0.999	0.997	0.995
0	0.070	0.040	0.040
13	0.070	0.070	0.040
17	0.075	0.070	0.040
21	0.080	0.070	0.038
26	0.094	0.080	0.040
37	0.21	0.100	0.040
43	0.34	0.160	0.037
46	0.42	0.230	0.037
51	0.52	0.350	0.038
62	0.68	0.540	-
69	0.77	0.640	-
77	0.85	0.710	-
89	1.02	0.50	-
95	1.10	0.80	-
100	1.15	0.85	0.038
105	1.15	0.85	-
110	1.19	0.85	-

APPENDIX TABLE XXXIII

Growth of R.lupini UT12 in yeast mannitol broth adjusted to different water activities obtained with NaCl.

Time (hrs)	Average log absorbance readings of 2 replicate tubes at a water activity of:- 0.999	0.997	0.995	0.990
0	0.0173	0.013	0.015	0.018
12	0.0137	0.018	0.014	0.020
25	0.0230	0.018	0.015	0.019
36	0.062	0.051	0.026	0.023
41	0.071	0.056	0.027	0.020
64	0.097	0.074	0.040	0.027
94	0.101	0.093	0.052	0.031
110	0.101	0.098	0.059	0.034

APPENDIX TABLE XXXIV

Growth of Rhizobium lupini (UT50) in yeast mannitol broth at various water activities as obtained with NaCl.

Time (hrs)	Average log absorbance readings of 2 replicate tubes at a water activity of:- 0.999	0.997	0.995
0	0.12	0.09	0.09
13	0.17	0.09	0.08
17	0.26	0.15	0.12
21	0.29	0.26	0.15
26	0.39	0.36	0.21
37	0.55	0.55	0.30
43	0.64	0.64	0.41
46	0.68	0.67	0.49
51	0.72	0.71	0.57
62	0.81	0.79	0.61
69	0.86	0.85	0.64
77	0.89	0.89	0.70
89	0.90	0.90	0.70
95	0.90	0.90	0.70
100	0.90	0.90	0.70

APPENDIX TABLE XXXV

Growth of *R. japonicum* QA372 in yeast mannitol broth adjusted to different water activities by the use of NaCl.

Time (hrs)	Average log absorbance readings of 2 replicate tubes at a water activity of:-				
	0.999	0.997	0.995	0.990	0.980
0	0.0064	0.0070	0.0070	0.0070	0.0070
12	0.0073	0.0074	0.0074	0.0066	0.0070
17	0.0089	0.0088	0.0080	0.0074	0.0070
20	0.0096	0.0095	0.0085	0.0074	0.0068
25	0.0106	0.0107	0.0091	0.0081	0.0065
35.5	0.0132	0.0130	0.0101	0.0085	0.0071
41	0.0140	0.0134	0.0101	0.0081	0.0070
43.5	0.0147	0.0139	0.0105	0.0085	0.0073
59.5	0.0171	0.0160	0.0112	0.0088	0.0068
73.5	0.0187	0.0181	0.0117	0.0096	0.0070
79.5	0.0189	0.0185	0.0119	0.0094	0.0070
95	0.0191	0.0188	0.0119	0.0096	0.0070
104	0.0192	0.0191	0.0123	0.0096	0.0070
109	0.0194	0.0192	0.0121	0.0099	0.0071
119	0.0194	0.0192	0.0125	0.0099	0.0072
131	0.0197	0.0195	0.0127	0.0099	0.0068
143	0.0197	0.0195	0.0130	0.0099	0.0069

APPENDIX TABLE XXXVIa

Survival of R.japonicum QA372, air dried in soil and rehydrated in media adjusted to different water activities with different solutes.

Solute	Water activity	Log viable count per 0.05 gm soil Replication Numbers 1	2	Mean
Nil	1.00	7.32	6.85	7.15
NaCl	0.995	7.40	7.19	7.31
	0.940	6.74	6.65	6.70
	0.860	6.50	6.48	6.52
KCl	0.995	7.10	7.13	7.11
	0.940	6.69	6.50	6.63
	0.860	6.83	6.69	6.78
Glycerol	0.995	6.87	6.88	6.89
	0.940	7.21	7.12	7.16
	0.860	6.69	7.11	6.97

Log initial viable count per 0.05 gm soil = 8.30

APPENDIX TABLE XXXVIa

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.028	0.028	1.094 NS
Treatment	9	1.260	0.140	5.469 xx
Error	9	0.230	0.0256	
Total	19	1.518		

Duncan's Multiple Range Test

Solute	Water activity	Mean log viable counts per 0.05 gm soil
NaCl	0.860	6.52
KCl	0.940	6.63
NaCl	0.940	6.70
KCl	0.860	6.78
Glycerol	0.995	6.89
Glycerol	0.860	6.97
Water	1.00	7.15
KCl	0.995	7.11
Glycerol	0.940	7.16
NaCl	0.995	7.31

APPENDIX TABLE XXXVIb

Survival of *R. trifolii* SU297/32B air dried in soil and rehydrated in media adjusted to different water activities with different solutes.

Log viable count per 0.05 gm soil

Solute	Water activity	Replication Number 1	2	Mean
Nil	1.00	5.74	5.43	5.62
NaCl	0.995	5.65	5.66	5.65
	0.940	4.85	5.09	4.99
	0.860	4.90	4.82	4.86
KCl	0.995	5.36	5.39	5.38
	0.940	4.99	4.88	4.95
	0.860	4.72	4.45	4.65
Glycerol	0.995	5.45	5.32	5.40
	0.940	5.13	5.25	5.19
	0.860	5.21	5.34	5.28

Log initial viable count per 0.05 gm soil = 8.30

APPENDIX TABLE XXXVIb

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.007	0.007	
Treatment	9	2.079	0.231	14.81 xxx
Error	9	0.140	0.0156	
Total	19	2.226		

Duncan's Multiple Range Test

Solute	Water activity	Mean log viable count per 0.05 gm soil
KCl	0.860	4.65
NaCl	0.860	4.86
KCl	0.940	4.95
NaCl	0.940	4.99
Glycerol	0.940	5.19
Glycerol	0.860	5.28
KCl	0.995	5.38
Glycerol	0.995	5.40
Nil	1.000	5.62
NaCl	0.995	5.65

APPENDIX TABLE XXXV11

Survival of freeze dried R. trifolii SU297/31A rehydrated in media adjusted to different water activities with different solutes.

Log viable count per 0.05 gm soil

Solute	Water Activity	Replication Number 1	2	Mean
Nil	1.00	5.00	4.98	5.02
NaCl	0.995	5.43	5.40	5.42
	0.940	3.08	2.98	3.05
	0.860	1.00[x]	2.06	1.76
Sucrose	0.995	5.35	5.22	5.30
	0.940	4.79	4.68	4.76
	0.860	4.26	3.85	4.11
Glycerol	0.995	5.92	5.57	5.88
	0.940	5.40	5.39	5.40
	0.860	4.34	4.22	4.29

x See footnote table XX

Log initial viable count per 0.05 gm soil = 8.22

APPENDIX TABLE XXXV11

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.003	0.003	NS
Treatment	9	30.598	3.3998	42.5 xxx
Error	9	0.732	0.0813	
Total	19	31.333		

Duncan's Multiple Range Test

Solute	Water Activity	Mean log viable count per 0.05 gm soil
NaCl	0.860	1.76
NaCl	0.940	3.05
Sucrose	0.860	4.11
Glycerol	0.860	4.29
Sucrose	0.940	4.76
Nil	1.000	5.02
Sucrose	0.995	5.30
Glycerol	0.940	5.40
NaCl	0.995	5.42
Glycerol	0.995	5.40

APPENDIX TABLE XXXVIIIa

Survival of freeze dried R.trifolii SU297/31A rehydrated in media adjusted to different water activities with different solutes.

Solute	Water activity	Log viable count per 0.05 gm soil Replication No. 1	2	Mean
Nil	1.00	5.26	5.44	5.36
NaCl	0.995	5.16	4.93	5.05
	0.940	3.85	4.18	4.04
	0.860	1.00[x]	1.00[x]	1.00[x]
Sucrose	0.995	5.23	4.85	5.04
	0.940	3.85	4.18	4.05
	0.860	2.53	2.04	2.31
Glycerol	0.995	5.39	5.42	5.43
	0.940	4.71	4.93	4.82
	0.860	3.49	3.69	3.63

x see footnote table XX

Log initial viable count per 0.05 gm soil = 8.20

APPENDIX TABLE XXXVIIIa

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.002	0.002	
Treatment	9	37.441	4.16	97.65 xxx
Error	9	0.383	0.0426	
Total	19	37.829		

Duncan's Multiple Range Test

Solute	Water Activity	Mean log viable count per 0.05 gm soil
NaCl	0.860	1.00
Sucrose	0.860	2.31
Glycerol	0.860	3.63
NaCl	0.940	4.04
Sucrose	0.940	4.05
Glycerol	0.940	4.82
Sucrose	0.995	5.04
NaCl	0.995	5.05
Nil	1.000	5.36
Glycerol	0.995	5.43

APPENDIX TABLE XXXVIIIb

Survival of freeze dried R.lupini UT12 rehydrated in media adjusted to different water activities with different solutes.

Solute	Water Activity	Log viable count per 0.05 gm soil: Replication No. 1	2	Mean
Nil	1.00	7.09	7.04	7.07
NaCl	0.995	7.05	7.12	7.09
	0.940	6.45	6.26	6.17
	0.860	4.61	5.40	5.17
Sucrose	0.995	7.03	6.82	6.94
	0.940	6.25	6.26	6.27
	0.860	5.50	5.70	5.62
Glycerol	0.995	6.96	6.97	6.97
	0.940	6.87	6.56	6.75
	0.860	6.28	6.38	6.33

Log initial viable count per 0.05 gm soil = 8.40

APPENDIX TABLE XXXVIIIb

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.009	0.009	
Treatment	9	8.421	0.9357	20.04xxx
Error	9	0.420	0.0467	
Total	19	8.850		

Duncan's Multiple Range Test

Solute	Water Activity	Mean log viable count per 0.05 gm soil
NaCl	0.860	5.17
Sucrose	0.860	5.62
Sucrose	0.940	6.27
Glycerol	0.860	6.33
NaCl	0.940	6.17
Glycerol	0.940	6.75
Sucrose	0.995	6.94
Glycerol	0.995	6.97
Nil	1.00	7.07
NaCl	0.995	7.09

APPENDIX TABLE XXXVIIIc

Survival of freeze dried Rhizobium sp. CB736 rehydrated in media adjusted to different water activities with different solutes.

		Log viable count per 0.05 gm soil.		
		Replication No.		
Solute	Water Activity	1	2	Mean
Nil	1.000	7.34	7.38	7.36
NaCl	0.995	7.50	7.49	7.49
	0.940	7.38	7.36	7.37
	0.860	5.25	5.35	5.30
Sucrose	0.995	7.48	7.50	7.49
	0.940	7.38	7.36	7.37
	0.860	7.16	7.31	7.24
Glycerol	0.995	7.49	7.44	7.47
	0.940	7.33	7.35	7.34
	0.860	7.11	7.14	7.13

Log initial viable count per 0.05 gm soil = 8.20

APPENDIX TABLE XXXVIIIc

continued

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.003	0.003	
Treatment	9	7.881	0.876	461.0xxx
Error	9	0.017	0.0019	
Total	19	7.901		

Duncan's Multiple Range Test

Solute	Water Activity	Mean log viable count per 0.05 gm soil
NaCl	0.860	5.30
Glycerol	0.860	7.13
Sucrose	0.860	7.24
Glycerol	0.940	7.34
Nil	1.00	7.36
Sucrose	0.940	7.37
NaCl	0.940	7.43
Glycerol	0.995	7.47
Sucrose	0.995	7.49
NaCl	0.995	7.50

APPENDIX TABLE XXXIX

Desiccation induced repairable damage of *E.coli* M13 dried on glass beads.

Log viable count per 0.05 gm glass beads.

Time after rehydration (hrs)	Control Replication No.			Lysozyme Replication No.		
	1	2	Mean	1	2	Mean
0	6.62	6.60	6.62	5.27	5.38	5.35
0.25	6.76	6.65	6.72	6.33	6.37	6.35
0.5	6.65	6.72	6.69	6.40	6.41	6.40
0.75	6.81	6.68	6.76	6.44	6.42	6.43
1.00	6.69	6.71	6.71	6.40	6.41	6.40
1.50	6.67	6.66	6.68	6.45	6.40	6.42
2.00	6.72	6.79	6.76	6.40	6.43	6.42

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.0000143	0.0000143	
Lysozyme	1	1.3817	1.3817	626.18 xxx
Time	6	1.2034	0.2006	90.89 xxx
Lysozyme x Time	6	0.8334	0.1389	62.95 xxx
Error	13	0.02869	0.00221	
Total	27	3.4472		

APPENDIX TABLE XXXIX

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	0	1.5	0.5	1.0	0.25	2.00	0.75
Mean x	6.62	6.68	6.69	6.71	6.72	6.76	6.76

2 Lysozyme treatment

Time after rehydration	0	0.25	0.5	2.0	0.75	1.0	1.50
Mean x	5.35	6.35	6.40	6.42	6.43	6.41	6.42

x Mean log viable count per 0.05 gm glass beads.

3

Time after rehydration (hrs)	Mean log viable counts per 0.05 gm glass beads for:- Control	Lysozyme
0	6.62	5.35
0.25	6.72	6.35
0.5	6.69	6.40
0.75	6.76	6.43
1.0	6.71	6.40
1.50	6.68	6.42
2.00	6.76	6.42

APPENDIX TABLE XL

Desiccation-induced repairable damage of *E.coli* M13 dried on glass beads.

Time after rehydration (hrs)	Log viable count per 0.05 gm glass beads								
	Control Replication No.			Lysozyme Replication No.			Deoxyribonuclease Replication No.		
	1	2	Mean	1	2	Mean	1	2	Mean
0	7.11	7.16	7.14	6.59	6.60	6.60	6.93	6.96	6.95
0.25	7.08	7.12	7.10	6.91	6.95	6.93	7.10	7.09	7.10
1.00	7.08	7.07	7.08	6.99	6.87	6.93	7.19	7.21	7.20
2.00	7.10	7.13	7.12	7.02	7.06	7.04	7.20	7.06	7.13

APPENDIX TABLE XL

continued

Analysis of variance of control versus lysozyme log data

Source of	D.F	Sum of Squares	Mean Square	F
Replication	1	0.00123	0.00123	0.68
Lysozyme	1	0.2025	0.2025	112.72xxx
Time	3	0.0819	0.0273	15.2 xxx
Lysozyme x Time	3	0.1132	0.0377	20.995 xxx
Error	7	0.0126	0.0018	
Total	15	0.4114		

Analysis of variance of control versus deoxyribonuclease log data above.

Source of Variation	D.F	Sum of Squares	Mean	F
Replication	1	0.0	0.0	0.0
Deoxyribonuc-lease	1	0.0009	0.0009	0.488
Time	3	0.0213	0.00711	3.857
DNA x Time	3	0.04985	0.01662	9.0167 x
Error	7	0.0129	0.00184	
Total	15	0.08498		

APPENDIX TABLE XL

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	1.0	0.25	2.0	0.0
Mean [x]	7.08	7.10	7.12	7.14

2 Lysozyme

Time after rehydration (hrs)	0	0.25	1.0	2.0
Mean [x]	6.63	6.93	6.93	7.04

3 Deoxyribonuclease

Time after rehydration (hrs)	0	0.25	2.0	1.0
Mean [x]	6.95	7.10	7.13	7.20

x Mean log viable count per 0.05 gm glass beads.

4

Time after rehydration (hrs.)	Mean log viable counts per 0.05 gm. glass beads for:- Deoxyribonuclease	Control	Lysozyme
0	6.95	7.14	6.63
0.25	7.10	7.10	6.93
1.0	7.20	7.08	6.93
2.0	7.13	7.12	7.04

APPENDIX TABLE XL1

Desiccation-induced repairable damage of *E.coli* M13 dried on glass beads.

Log viable counts per 0.05 gm glass beads

Time after rehydration (hrs)	Control Replication No.			Lysozyme Replication No.		
	1	2	Mean	1	2	Mean
0	5.34	5.32	5.33	4.29	4.32	4.31
0.25	5.45	5.44	5.44	5.16	5.21	5.19
0.5	5.46	5.44	5.45	5.37	5.28	5.33
0.75	5.49	5.43	5.46	5.30	5.32	5.31
1.0	5.41	5.42	5.42	5.35	5.33	5.34
1.50	5.47	5.42	5.45	5.35	5.29	5.32

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.0020	0.0020	2.39 NS
Lysozyme	1	0.5163	0.5163	598.83 xxx
Time	5	1.031	0.206	239.12 xxx
Lysozyme x Time	5	0.6613	0.1323	15.342xxx
Error	11	0.00948	0.00086	
Total	23	2.2199		

APPENDIX TABLE XLI

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	0	1.0	0.25	0.5	1.50	0.75
Means[x]	5.33	5.42	5.44	5.45	5.45	5.46

2 Lysozyme treatment

Time after rehydration (hrs)	0	0.25	0.75	1.50	0.5	1.00
Means[x]	4.31	5.19	5.31	5.32	5.33	5.34

x Mean log viable counts per 0.05 gm glass beads.

3

Time after rehydration (hrs)	Mean log viable counts per 0.05 gm. glass beads for:-	
	Control	Lysozyme
0	5.33	4.31
0.25	5.44	5.19
0.5	5.45	5.33
0.75	5.46	5.31
1.00	5.42	5.34
1.50	5.45	5.32

APPENDIX TABLE XLII

Effect of low temperature on repair of desiccation-induced damage of *E.coli* M13 dried on glass beads.

Log viable counts per 0.05 gm. glass beads.

Time after rehydration (hrs)	Control Replication No. 1	2	Means	Lysozyme Replication No. 1	2	Means	Actinomycin D Replication No. 1	2	Means
0	4.89	4.84	4.87	3.20	3.13	3.17	4.07	4.16	4.12
0.25	4.79	4.88	4.84	3.63	3.51	3.57	4.45	4.40	4.43
0.5	4.91	4.88	4.90	4.11	3.83	3.97	4.66	4.50	4.58
0.75	4.80	4.87	4.84	4.20	4.05	4.13	4.67	4.66	4.67
1.0	4.88	4.90	4.89	4.34	4.28	4.31	4.69	4.67	4.68
1.5	4.91	4.87	4.89	4.60	4.60	4.60	4.75	4.76	4.76
2.0	4.87	4.84	4.86	4.90	4.85	4.88	4.88	4.87	4.88

APPENDIX TABLE XLII

continued

Analysis of variance of control versus lysozyme treatments.

Source of Variation	D.F	Sum of Squares	Mean Square	F
Rep.	1	0.0175	0.0175	4.1139 NS
Lysozyme	1	4.2432	4.2432	997.50 xxx
Time	6	2.1131	0.3522	82.793 xxx
Lysozyme x Time	6	2.0276	0.3379	79.413 xxx
Error	13	0.0553	0.0043	
Total	27	8.4568		

Analysis of variance of control versus actinomycin D treatments.

Source of Variation	D.F	Sum of Squares	Mean Square	F
Rep.	1	0.00051	0.00051	0.242 NS
Actinomycin D	1	0.5544	0.5544	261.27 xxx
Time	6	0.3909	0.0652	30.71 xxx
Actinomycin x Time	6	0.3670	0.06117	28.83 xxx
Error	13	0.0276	0.00212	
Total	27	1.3405		

APPENDIX TABLE XLII

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	0.25	0.75	2.0	0.0	1.0	1.5	0.5
Mean[x]	4.84	4.84	4.86	4.87	4.89	4.89	4.90

2 Lysozyme treatment

Time after rehydration (hrs)	0.	0.25	0.5	0.75	1.0	1.5	2.0
Mean[x]	3.17	3.57	3.97	4.13	4.31	4.60	4.88

3 Actinomycin D treatment

Time after rehydration (hrs)	0	0.25	0.5	0.75	1.0	1.5	2.0
Mean[x]	4.12	4.43	4.58	4.67	4.68	4.76	4.88

x Mean log viable counts per 0.05gm glass beads.

4

Time after rehydration (hours)	Mean log viable counts per 0.05 gm glass beads for:- Lysozyme	Control	Actinomycin D
0	3.17	4.87	4.12
0.25	3.57	4.84	4.43
0.5	3.97	4.90	4.58
0.75	4.13	4.84	4.67
1.0	4.31	4.89	4.68
1.5	4.60	4.89	4.76
2.0	4.88	4.86	4.88

APPENDIX TABLE XLIII

Repair of desiccation-induced damage of *E.coli* M13 dried on glass beads and rehydrated in yeast mannitol broth.

	Log viable count per 0.05 gm. glass beads.					
Time after rehydration (hrs)	Control Replication No.			Lysozyme Replication No.		
	1	2	Means	1	2	Means
0	5.54	5.58	5.56	4.74	4.78	4.76
0.25	5.70	5.56	5.63	5.25	5.13	5.19
0.5	5.65	5.66	5.66	5.27	5.19	5.23
0.75	5.50	5.58	5.54	5.39	5.19	5.29
1.0	5.68	5.77	5.73	5.48	5.49	5.49
2.0	5.71	5.70	5.71	5.51	5.71	5.61

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Rep.	1	0.000267	0.000267	NS
Lysozyme	1	0.8438	0.8438	134.6 xxx
Time	5	0.6162	0.1232	19.67 xxx
Lysozyme x Time	5	0.2996	0.0599	9.56 xxx
Error	11	0.0689	0.00627	
Total	23	1.8287		

APPENDIX TABLE XLIII

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	0.75	0	0.25	0.5	2.0	1.0
Mean[x]	5.54	5.56	5.63	5.66	5.71	5.73

2 Lysozyme treatment

Time after rehydration (hrs)	0	0.25	0.5	0.75	1.0	2.0
Mean[x]	4.76	5.19	5.23	5.29	5.49	5.61

x Mean log viable count per 0.05 gm glass beads.

3

Time after rehydration (hrs.)	Mean log viable counts per 0.05 gm glass beads for:- Control	Lysozyme
0	5.56	4.76
0.25	5.63	5.19
0.5	5.66	5.23
0.75	5.54	5.29
1.0	5.73	5.49
2.0	5.71	5.61

APPENDIX TABLE XLIV

Repair of desiccation-induced damage of *E.coli* M13 dried on glass beads and rehydrated in yeast mannitol broth.

	Log viable count per 0.05 gm. glass beads					
Time after rehydration (hrs)	Control Replication No. 1	2	Means	Lysozyme Replication No. 1	2	Means
0.0	5.14	4.99	5.07	4.49	4.43	4.46
0.25	5.11	5.04	5.08	4.98	4.88	4.93
0.5	5.25	4.95	5.10	5.03	4.92	4.98
0.75	5.34	5.13	5.24	5.24	4.99	5.12
1.0	5.38	5.15	5.27	5.38	5.06	5.22

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Rep.	1	0.162	0.162	36.0 xxx
Lysozyme	1	0.216	0.216	48.07 xxx
Time	4	0.550	0.138	30.57 xxx
Lysozyme x Time	4	0.203	0.0507	11.266 xxx
Error	9	0.041	0.0045	
Total	19	1.172		

APPENDIX TABLE XLIV

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	0	0.25	0.5	0.75	1.0
Mean[x]	5.07	5.08	5.10	5.24	5.27

2 Lysozyme Treatment

Time after rehydration (hrs)	0	0.25	0.5	0.75	1.0
Mean[x]	4.46	4.93	4.98	5.12	5.22

x Mean log viable count per 0.05 gm glass beads.

3

Time after rehydration (hrs)	Mean log viable count per 0.05 gm glass beads for:-	
	Control	Lysozyme
0	5.07	4.46
0.25	5.08	4.93
0.5	5.10	4.98
0.75	5.24	5.12
1.0	5.27	5.22

APPENDIX TABLE XLV

Desiccation-induced repairable damage of R.leguminosarum TA101 dried on glass beads.

	Log viable count per 0.05 gm glass beads					
Time after rehydration (hrs)	Control Replication No.			Lysozyme Replication No.		
	1	2	Means	1	2	Means
0	3.07	3.21	3.16	2.99	2.97	2.99
0.5	3.00	2.91	2.98	2.68	2.75	2.74
1.0	2.93	2.86	2.95	2.85	2.89	2.88
2.0	3.02	2.96	3.00	2.94	3.01	2.99
19.0	3.16	2.98	3.10	3.05	2.93	3.03

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.0024	0.0024	
Lysozyme	1	0.0541	0.0541	10.82 xx
Time	4	0.1481	0.0370	7.41 xx
Lysozyme x Time	4	0.0363	0.00908	1.82
Error	9	0.0450	0.0050	
Total	19	0.2859		

APPENDIX TABLE XLV

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	1.0	0.5	2.0	19.0	0
Mean[x]	2.95	2.98	3.00	3.10	3.16

2 Lysozyme treatment

Time after rehydration (hrs)	0.5	1.0	0	2.0	19.0
Mean[x]	2.74	2.88	2.99	2.99	3.03

x Mean log viable count per 0.05 gm glass beads.

3 Time after rehydration (hrs)	Mean log viable counts per 0.05 gm glass beads for:- Control	Lysozyme
0	3.16	2.99
0.5	2.98	2.74
1.0	2.95	2.88
2.0	3.00	2.99
19.0	3.10	3.03

APPENDIX TABLE XLVI

Desiccation-induced repairable damage of R.leguminosarum TA101 dried on glass beads.

Log viable count per 0.05 gm glass beads.

Time after rehydration (hrs)	Control Replication Number 1	2	Mean	Lysozyme Replication Number 1	2	Mean
0	3.63	3.68	3.66	3.38	3.42	3.41
0.5	3.67	3.70	3.69	3.50	3.56	3.54
1.0	3.71	3.73	3.72	3.59	3.67	3.64
1.5	3.56	3.73	3.66	3.48	3.56	3.53
2.0	3.59	3.61	3.61	3.59	3.61	3.61
4.0	3.67	3.71	3.70	3.54	3.64	3.59
20.0	3.56	3.59	3.57	3.58	3.55	3.57

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.018	0.018	16.2 xxx
Lysozyme	1	0.0772	0.0772	69.45 xxx
Time	6	0.0549	0.00916	8.25 xxx
Lysozyme x Time	6	0.0457	0.00762	6.85 xxx
Error	13	0.0145	0.00111	
Total	27	0.2103		

APPENDIX TABLE XLVI

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	20.0	2.0	1.5	0	0.5	4.0	1.0
Mean[x]	3.57	3.61	3.66	3.66	3.69	3.70	3.72

2 Lysozyme treatment

Time after rehydration (hrs)	0	1.5	0.5	20.0	4.0	2.0	1.0
Mean[x]	3.41	3.53	3.54	3.57	3.59	3.61	3.64

x Mean log viable count per 0.05 gm glass beads

3

Time after rehydration (hrs)	Mean log viable count per 0.05gm glass beads for:- Control	Lysozyme
0	3.66	3.41
0.5	3.69	3.54
1.0	3.72	3.64
1.5	3.66	3.53
2.0	3.61	3.61
4.0	3.70	3.59
20.0	3.57	3.57

APPENDIX TABLE XLVII

Desiccation-induced repairable damage of *R.leguminosarum* TA101 dried on glass beads and rehydrated in Hambleton's (1971) medium.

Log viable count per 0.05 gm. glass beads.

Time after rehydration (hrs)	Control Replication No. 1	2	Mean	Lysozyme Replication No. 1	2	Mean
0	3.30	3.90	3.69	3.18	3.60	3.44
0.5	3.51	3.90	3.75	3.52	3.88	3.74
1.0	3.56	3.94	3.79	3.56	3.98	3.82
1.5	3.65	3.97	3.84	3.45	3.95	3.77
2.0	3.62	4.04	3.88	3.61	4.02	3.86

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F
Replication	1	0.89	0.0804	291. 6 xxx
Lysozyme	1	0.0245	0.0205	6. 707 x
Time	4	0.2537	0.0634	20. 77 xxx
Lysozyme x Time	4	0.0364	0.0091	2.978 NS
Error	9	0.0275	0.00305	
Total	19	1.228		

APPENDIX TABLE XLVII

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	0	0.5	1.0	1.5	2.0
Means[x]	3.69	3.75	3.79	3.84	3.88

2 Lysozyme treatment

Time after rehydration (hrs)	0	0.5	1.5	1.0	2.0
Means[x]	3.44	3.74	3.77	3.82	3.86

x Mean log viable count per 0.05 gm glass beads.

3

Time after rehydration (hrs)	Mean log viable count per 0.05 gm glass beads for:- Control	Lysozyme
0	3.69	3.44
0.5	3.75	3.74
1.0	3.79	3.82
1.5	3.84	3.77
2.0	3.88	3.86

APPENDIX TABLE XLVIII

Desiccation-induced repairable damage of R.meliloti CC131 dried on glass beads.

Log viable count per 0.05 gm glass beads.

Time after rehydration (hrs)	Control Replication No. 1	2	Mean	Lysozyme Replication No. 1	2	Mean
0	4.76	4.71	4.74	4.35	4.50	4.43
0.5	4.83	4.87	4.85	4.53	4.64	4.60
1.0	4.84	4.77	4.81	4.64	4.67	4.66
1.5	4.75	4.71	4.73	4.65	4.66	4.66
2.0	4.77	4.74	4.76	4.53	4.70	4.63
4.0	4.70	4.70	4.70	4.75	4.60	4.68
19.0	4.70	4.57	4.64	4.70	4.58	4.65

Analysis of variance (based on log data)

Source of Variation	D.F	Sum of Squares	Mean Square	F	
Replication	1	0.00023	0.000229	0.0459	NS
Lysozyme	1	0.1317	0.1317	26.46	xxx
Time	6	0.0633	0.01055	2.12	NS
Lysozyme x Time	6	0.0830	0.01384	2.78	NS
Error	13	0.0647	0.004975		
Total	27	0.3429			

APPENDIX TABLE XLVIII

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	19.0	4.0	1.5	0	2.0	1.0	0.5
Means[x]	4.64	4.70	4.73	4.74	4.76	4.81	4.85

2 Lysozyme treatment

Time after rehydration (hrs)	0	0.5	2.0	19.0	1.0	1.5	4.0
Means[x]	4.41	4.60	4.62	4.65	4.66	4.66	4.68

x Mean log viable count per 0.05 gm glass beads.

3

Time after rehydration (hrs)	Mean log viable count per 0.05gm glass beads for:- Control	Lysozyme
0	4.74	4.41
0.5	4.85	4.60
1.0	4.81	4.66
1.5	4.73	4.66
2.0	4.75	4.63
4.0	4.70	4.68
19.0	4.64	4.65

APPENDIX TABLE XLIX

Desiccation-induced repairable damage of R. japonicum QA372 dried on glass beads.

Log viable count per 0.05 gm glass beads.

Time after rehydration (hrs)	Control Replication No. 1	2	Mean	Lysozyme Replication No. 1	2	Mean
0	5.74	5.73	5.74	5.67	5.59	5.64
0.25	5.78	5.76	5.77	5.78	5.77	5.78
0.5	5.81	5.75	5.78	5.77	5.70	5.74
1.0	5.86	5.86	5.86	5.83	5.85	5.84
2.0	5.78	5.74	5.76	5.80	5.79	5.80
5.0	5.84	5.79	5.82	5.81	5.76	5.79
7.0	5.87	5.81	5.84	5.84	5.82	5.83
20.0	5.74	5.75	5.75	5.67	5.72	5.70

Analysis of variance (based on log data)

Source of Variation	D.F.	Sum of Squares	Mean Square	F
Replication	1	0.005	0.0050	7.813 x
Lysozyme	1	0.0061	0.00605	9.453 xx
Time	7	0.08699	0.01243	19.417 xx
Lysozyme x Time	7	0.0122	0.001736	2.712 NS
Error	15	0.0096	0.00064	
Total	31	0.11979		

APPENDIX TABLE XLIX

continued

Duncan's Multiple Range Test

1 Control

Time after rehydration (hrs)	0	20	2.0	0.25	0.5	5.0	7.0	1.0
Means[x]	5.74	5.75	5.76	5.77	5.78	5.82	5.84	5.86

2 Lysozyme treatment

Time after rehydration (hrs)	0	20.0	0.5	0.25	5.0	2.0	7.0	1.0
Means[x]	5.64	5.70	5.74	5.78	5.79	5.80	5.83	5.84

x Mean log viable count per 0.05 gm glass beads.

3

Time after rehydration (hrs)	Mean log viable count per 0.05 gm. glass beads for:- Control	Lysozyme
0	5.74	5.64
0.25	5.77	5.78
0.5	5.78	5.74
1.0	5.86	5.84
2.0	5.76	5.80
5.0	5.82	5.79
7.0	5.84	5.83
20.0	5.76	5.70

APPENDIX TABLE L

Adsorption - Desorption Isotherm for serum albumin.

Jacket temperature = 40°C

Temperature °C	Relative Humidity (%)	Weight of water per 100 gm albumin	
		Sorption	Desorption
3	10	3.5	4.36
13	20	5.82	6.60
19	30	7.27	-
24	40	-	10.63
25	43.3	9.29	-
27.5	50	10.29	12.20
31.25	60	12.50	13.76
33.5	70	14.48	15.66
35	75	17.90	17.90

Comparison of the mean of the experimental data for both sorption and desorption isotherms with that of Bull (1944).

Relative Humidity %	Mean experimental weight (gm of water per 100 gm of albumin)	Values from Bull (1944) (gm of water per 100 gm of albumin)
10	3.93	3.90
20	6.21	6.00
50	11.25	11.25
60	13.13	13.08
70	15.07	14.90
75	17.90	18.80

APPENDIX TABLE LI

Adsorption of water vapour by Ca^{2+}-montmorillonite.

Jacket temperature = 40°C.

Temperature °C	Relative Humidity (%)	Grams water sorbed per 100 gm. Ca^{2+}-montmorillonite
0	8.28	4.49
8	14.53	5.76
12	19.00	7.22
16	24.63	8.49
20	31.68	9.85
24	40.44	11.32
28	51.23	12.78
32	64.45	14.73
34	72.11	17.62
36	80.54	21.85
38	89.82	25.00

Dry weight of sample was 0.1025 gm.

APPENDIX TABLE LII

Sorption of water vapour by R.trifolii SU297/32B (jacket temperature = 40°C).

Temperature °C	Relative Humidity (%)	Grams water sorbed per 100 gm. bacteria
3	10.0	2.85
8	14.53	2.85
12	19.00	3.81
16	24.6	4.23
20	31.68	5.50
22	35.83	6.24
24	40.44	7.08
26	45.44	7.93
28	51.23	8.46
30	57.51	9.83
32	64.45	10.47
34	72.11	14.48
36	80.54	17.13
38	89.82	21.25
40	100.0	34.14

Dry weight of cells was 0.1509 gm.

APPENDIX TABLE LIII

Sorption of water vapour by *R. japonicum* QA372

Jacket temperature = 40°C.

Temperature °C	Relative Humidity (%)	Grams of water per 100 grams of bacteria	
		Sorption	Desorption
0	8.28	0.3195	-
4	11.02	0.532	-
8	14.53	0.745	-
12	19.00	1.07	-
16	24.63	1.49	-
20	31.68	2.02	3.30
24	40.44	2.66	-
28	51.23	3.94	-
32	64.45	5.62	6.81
34	72.11	7.67	9.16
36	80.54	13.52	-
38	89.82	26.20	-

Dry weight cells was 0.0935 gms.

APPENDIX TABLE LIV

Sorption of water vapour by *R.trifolii* SU297/32B

Jacket temperature = 40°C

Temperature °C	Relative Humidity (%)	Grams water sorbed per 100 gms.bacteria
7	13.75	3.87
10	16.64	4.15
12	19.00	4.42
14	21.66	4.79
16	24.63	5.25
18	27.96	5.62
20	31.68	6.18
22	35.83	6.73
24	40.44	7.37
26	45.55	8.39
28	51.23	9.31
30	57.51	10.23
32	64.45	11.06
34	72.11	14.34
36	80.54	17.70
38	89.82	24.39

Dry weight of cells was 0.1085 gm.

APPENDIX TABLE LV

Sorption of water vapour by R.leguminosarum TA101

Jacket temperature = 40°C

Temperature °C	Relative Humidity (%)	Grams water sorbed per 100 grams bacteria
0	8.275	2.44
4	11.019	3.30
8	14.53	3.79
12	19.00	4.03
16	24.63	4.88
20	31.68	5.98
24	40.44	7.20
28	51.23	9.04
32	64.45	11.72
34	72.11	16.03
36	80.54	19.36
38	89.82	26.74

Dry weight of cells = 0.0819 gm.

APPENDIX TABLE LVI

Sorption of water vapour by R.lupini UT2

Jacket temperature = 40°C

Temperature °C	Relative Humidity (%)	Grams water sorbed per 100 gms. bacteria
0	8.28	0.131
4	11.019	-
8	14.53	0.393
12	19.00	0.655
16	24.63	0.917
20	31.68	1.70
24	40.44	2.49
28	51.23	3.41
32	64.45	4.66
34	72.11	6.68
36	80.54	9.24
38	89.82	24.64

Dry weight of cells was 0.0763 gm.

APPENDIX TABLE LVII

Sorption of water vapour by R.lupini UT12

Jacket temperature = 40°C

Temperature °C	Relative Humidity (%)	Grams water sorbed per 100 gms bacteria
0	8.275	0.983
4	11.019	1.405
8	14.53	1.550
12	19.00	1.830
16	24.63	2.39
20	31.68	2.81
24	40.44	3.65
28	51.23	4.80
32	64.45	5.76
34	72.11	8.01
36	80.54	11.61
38	89.82	24.68

Dry weight was 0.0712 gm.

APPENDIX TABLE LVIII

Sorption of water vapour by R.lupini UT12

Jacket temperature = 40°C

Temperature °C	Relative Humidity (%)	Grams water sorbed per 100 gms. bacteria
6.0	12.67	1.03
8.5	15.0	1.14
12.0	19.0	1.37
14.0	21.66	1.49
16.0	24.63	1.71
18.0	27.96	1.94
20.0	31.68	2.06
22.0	35.83	2.40
24.0	40.44	2.97
26.0	45.55	3.43
28.0	51.23	3.54
30.0	57.51	4.60
32.0	64.45	5.72
34.0	72.11	7.72
36.0	80.54	10.55
38.0	89.82	18.17

APPENDIX TABLE LIX

Adsorption of water vapour by R.leguminosarum TA101 that had been disrupted with Triton x-100 and either dialysed or not dialysed.

Jacket temperature = 40°C.

Temperature °C	Relative Humidity (%)	Grams water sorbed/100 gms. bacteria.	
		Disrupted and Not Dialysed	Disrupted and Dialysed
0	0.0828	-	1.33
8	0.1453	2.48	2.66
12	0.1900	2.92	-
16	0.2463	3.54	4.55
20	0.3168	4.52	5.65
24	0.4044	5.76	7.43
28	0.5123	7.66	10.20
32	0.6445	9.92	12.86
34	0.7211	12.14	24.06

Dry weight undialysed cells = 0.1129 gms.

" " dialysed cells = 0.0902 gms.

APPENDIX TABLE LX

Water adsorption isotherm of R.lupini UT12 that had been disrupted with Triton X100 and a portion of the suspension dialysed, the rest was not dialysed.

Jacket temperature = 40°C.

Temperature °C	Relative Humidity (%)	Grams water adsorbed/100 gms. bacteria	
		Not dialysed	dialysed
0	8.28	0.094	0.63
8	14.53	0.421	1.27
16	24.63	1.17	2.32
24	40.44	-	3.80
30	57.51	3.78	5.39
34	72.11	5.99	7.81

Initial dry weight of undialysed bacterial mass = 0.1068 gm.

Initial dry weight of dialysed bacterial mass = 0.0947 gm.

APPENDIX TABLE LXI

Application of B.E.T. equation to water sorption data for *R.trifolii* SU297/32B

Relative vapour pressure P/P_o	$(1-P/P_o)$	W (gm)	$W(1-P/P_o)$	$\frac{P/P_o}{W(1-P/P_o)}$
0.1375	0.8625	3.87	3.3379	0.0412
0.1664	0.8336	3.15	3.4594	0.0481
0.19	0.8100	4.42	3.5802	0.0531
0.2166	0.7834	4.79	3.7525	0.0578
0.2463	0.7537	5.25	3.9570	0.0623
0.2796	0.7204	5.62	4.0487	0.0691
0.3168	0.6832	6.18	4.2222	0.0751
0.3583	0.6417	6.73	4.3187	0.0830
0.4044	0.5956	7.37	4.3896	0.0922
0.4555	0.5445	8.39	4.5684	0.0997
0.5123	0.4877	9.31	4.5405	0.1129
0.5751	0.4249	10.23	4.3468	0.1323
0.6445	0.3555	11.06	3.9319	0.1640
0.7211	0.2789	14.34	3.9995	0.1803
0.8054	0.1946	17.70	3.4445	0.2339
0.8982	0.1018	24.39	2.4829	0.3618

W = weight of water adsorbed at vapour pressure, P, (see Appendix Table LIV)

P_o = the saturation vapour pressure.

APPENDIX TABLE LXII

Application of B.E.T. equation to water sorption data for R.trifolii SU297/32B

Relative vapour pressure P/P_o	$(1-P/P_o)$	(W_{gm})	$W(1-P/P_o)$	$\frac{P/P_o}{W(1-P/P_o)}$
0.1000	0.9000	2.85	2.565	0.039
0.1453	0.8547	2.85	2.436	0.0596
0.1900	0.8100	3.81	3.086	0.0616
0.2463	0.7540	4.23	3.189	0.0771
0.3168	0.6832	5.50	3.758	0.0843
0.3583	0.6417	6.24	4.004	0.0895
0.4044	0.5956	7.08	4.217	0.0959
0.4544	0.5456	7.93	4.327	0.1050
0.5123	0.4877	8.46	4.1259	0.1242
0.5751	0.4249	9.83	4.1768	0.1377
0.6445	0.3555	10.47	3.7221	0.1732
0.7211	0.2789	14.48	4.039	0.1785
0.8054	0.1946	17.13	3.3335	0.2416
0.8982	0.1018	21.23	2.1633	0.4152

W = weight of water adsorbed at vapour pressure, p, (see Appendix Table LII)

P_o = saturation vapour pressure.

APPENDIX TABLE LXIII

Application of B.E.T. equation to water sorption data for *R. leguminosarum* TA101.

Relative vapour pressure P/P_o	$(1-P/P_o)$	W (gms)	$W(1-P/P_o)$	$\frac{P/P_o}{W(1-P/P_o)}$
0.0828	0.9172	2.44	2.2380	0.0370
0.1102	0.8898	3.30	2.9363	0.0375
0.1453	0.8547	3.79	3.239	0.0449
0.1900	0.8100	4.03	3.264	0.0582
0.2463	0.7537	4.88	3.6781	0.0670
0.3168	0.6832	5.98	4.0855	0.0775
0.4044	0.5956	7.20	4.2883	0.0943
0.5123	0.4877	9.04	4.4088	0.1162
0.6445	0.3555	11.72	4.1665	0.1547
0.7211	0.2789	16.03	4.4708	0.1613
0.8054	0.1946	19.36	3.7675	0.2138
0.8982	0.1018	26.74	2.7221	0.3300

W=weight of water adsorbed at vapour pressure, P, (see Appendix Table LV)

P_o = the saturation vapour pressure.

APPENDIX TABLE LXIV

Application of B.E.T. equation to water sorption data for R. japonicum QA372.

Relative vapour pressure P/P_o	$(1-P/P_o)$	W (gms)	$W(1-P/P_o)$	$\frac{P/P_o}{W(1-P/P_o)}$
0.0828	0.9172	0.3195	0.2931	0.2825
0.1102	0.8898	0.532	0.4734	0.2328
0.1453	0.8547	0.745	0.6368	0.2282
0.1900	0.8100	1.07	0.8667	0.2192
0.2463	0.7537	1.49	1.1230	0.2193
0.3168	0.6832	2.02	1.3801	0.2296
0.4044	0.5956	2.66	1.5843	0.2553
0.5123	0.4877	3.94	1.9215	0.2666
0.6445	0.3555	5.62	1.9979	0.3226
0.7211	0.2789	7.67	2.1392	0.3371
0.8054	0.1946	13.52	2.6310	0.3061
0.8982	0.1018	26.20	2.6672	0.3368

W = weight of water adsorbed at vapour pressure, P, (see Appendix Table LIII)

P_o = the saturation vapour pressure.

APPENDIX TABLE LXV

Application of B.E.T. equation to water sorption data for *R.lupini* UT12

Relative vapour pressure P/P_o	$(1-P/P_o)$	W (gm)	$W(1-P/P_o)$	$\frac{P/P_o}{W(1-P/P_o)}$
0.1267	0.8733	1.03	0.8995	0.1409
0.1500	0.8500	1.14	0.9690	0.1548
0.1900	0.8100	1.37	1.1097	0.1712
0.2166	0.7834	1.49	1.1673	0.1856
0.2463	0.7537	1.71	1.2889	0.1911
0.2796	0.7204	1.94	1.3976	0.2001
0.3168	0.6832	2.06	1.4074	0.2251
0.3583	0.6417	2.40	1.5401	0.2327
0.4044	0.5956	2.97	1.7690	0.2286
0.4555	0.5445	3.43	1.8677	0.2439
0.5123	0.4877	3.54	1.7265	0.2968
0.5751	0.4249	4.60	1.9546	0.2943
0.6445	0.3555	5.72	2.0335	0.3170
0.7211	0.2789	7.72	2.1531	0.3350
0.8054	0.1946	10.55	2.0531	0.3923
0.8982	0.1018	18.17	1.8497	0.4856

W= weight of water adsorbed at vapour pressure, P, (see Appendix Table LVIII)

Po = the saturation vapour pressure.

APPENDIX TABLE LXVI

Application of B.E.T. equation to water sorption data for R.lupini UT12

Relative vapour pressure P/P_o	$(1-P/P_o)$	W (gm)	$W(1-P/P_o)$	$\frac{P/P_o}{W(1-P/P_o)}$
0.0828	0.9172	0.983	0.9016	0.0918
0.1102	0.8898	1.405	1.2502	0.0882
0.1453	0.8547	1.550	1.3248	0.1097
0.1900	0.8100	1.830	1.4823	0.1282
0.2463	0.7537	2.390	1.8013	0.1367
0.3168	0.6832	2.810	1.9198	0.1650
0.4044	0.5956	3.650	2.1739	0.1860
0.5123	0.4877	4.800	2.3410	0.2188
0.6445	0.3555	5.760	2.0477	0.3147
0.7211	0.2789	8.010	2.2340	0.3228
0.8054	0.1946	11.610	2.2590	0.3565
0.8982	0.1018	24.680	2.5123	0.3575

W = weight of water adsorbed at vapour pressure, P, (see Appendix Table LVII)

P_o = the saturation vapour pressure.

APPENDIX TABLE LXVII

Application of B.E.T. equation to water sorption data for *R. lupini* UT2

Relative vapour pressure P/P_o	$(1-P/P_o)$	W (gm)	$W(1-P/P_o)$	$\frac{P/P_o}{W(1-P/P_o)}$
0.0828	0.9172	0.131	0.1202	0.6889
0.1102	0.8898	-	-	-
0.1453	0.8547	0.393	0.3359	0.4326
0.1900	0.8100	0.655	0.5306	0.3581
0.2463	0.7537	0.917	0.6911	0.3564
0.3168	0.6832	1.70	1.1614	0.2728
0.4044	0.5956	2.49	1.483	0.2727
0.5123	0.4877	3.41	1.663	0.3081
0.6445	0.3555	4.66	1.6566	0.3890
0.7211	0.2789	6.68	1.8631	0.3870
0.8054	0.1946	9.24	1.798	0.4479
0.8982	0.1018	24.64	2.5084	0.3581

W = weight of water adsorbed at vapour pressure, p, (see Appendix Table, LVI)

P_o = the saturation vapour pressure.

APPENDIX TABLE LXVIII

Application of B.E.T. equation to water adsorption data of Ca^{2+}-montmorillonite.

Relative vapour pressure P/Po	(1-P/Po)	W (gm)	W(1-P/Po)	$\frac{P/Po}{W(1-P/Po)}$
0.0820	0.9172	4.49	4.027	0.0201
0.1453	0.8547	5.76	4.923	0.0295
0.1900	0.8100	7.22	5.848	0.0325
0.2463	0.7537	8.49	6.40	0.0385
0.3168	0.6832	9.85	6.73	0.0471
0.4044	0.5956	11.32	6.74	0.0600
0.5123	0.4877	12.78	6.23	0.0822
0.6445	0.3555	14.73	5.24	0.1230
0.7211	0.2789	17.62	4.91	0.1429
0.8054	0.1946	21.85	4.25	0.1895
0.8982	0.1018	25.00	2.55	0.3522

W = weight of water adsorbed at vapour pressure, P,
(see Appendix Table LI)

Po = the saturation vapour pressure.

APPENDIX TABLE LXIX

Values of $W/_{p/p_0}$ for various species of Rhizobium used for the graphical integration of Bangham's free energy equation.

Values of $W/_{p/p_0}$ used for the graphical integration of the Bangham free energy equation.

Vapour pressure P/Po	R. lupini UT12	R. trifolii SU297/32B	R. trifolii SU297/32B
0.100	-	-	28.5
0.1267	8.13	-	-
0.1375	-	28.15	-
0.1453	-	-	19.62
0.150	7.60	-	-
0.1664	-	24.94	-
0.1900	7.21	23.26	20.05
0.2166	6.88	22.12	-
0.2463	6.94	21.32	17.20
0.2796	6.94	20.10	-
0.3168	6.50	19.51	17.36
0.3583	6.70	18.78	17.42
0.4044	7.34	18.23	17.51
0.4555	7.53	18.42	17.45
0.5123	6.91	18.17	16.51

APPENDIX TABLE LXIX

continued

Vapour Pressure P/P_0	R.lupini UT12	R.trifolii SU297/32B	R.trifolii SU297/32B
0.5751	8.00	17.79	17.09
0.6445	8.88	17.16	16.25
0.7211	10.71	19.89	20.08
0.8054	13.10	21.98	21.27
0.8982	20.23	27.15	23.66

W=weight of water adsorbed at vapour pressure, P.

P_0 =saturation vapour pressure.

Values of W for R.lupini UT12 are in Appendix Table LVIII.

Values of W for R.trifolii SU297/32B are in Appendix Table LIV.

Values of W for R.trifolii SU297/32B are in Appendix Table LII.

APPENDIX TABLE LXX

Values of $W/P/p_o$ for various species of *Rhizobium* and calcium - montmorillonite used for the graphical integration of Bangham's free energy equation.

Values of $w/_{P/Po}$ used for the graphical integration of the Bangham free energy equation.

Vapour pressure P/P_o	Calcium-Montmorillonite	*R.lupini* UT2	*R.lupini* UT12	*R.japonicum* QA372	*R.leguminosarum* TA101
0.0828	53.63	1.58	11.87	3.86	29.47
0.1102	-	-	12.75	4.83	29.95
0.1453	39.90	2.71	10.67	5.13	26.08
0.1902	37.25	3.45	9.63	5.63	21.21
0.2463	34.47	3.72	9.71	6.05	19.81
0.3168	31.09	5.37	8.87	6.38	18.88
0.4044	27.99	6.16	9.03	6.58	17.80
0.5123	24.95	6.66	9.37	7.69	17.65
0.6445	22.86	7.23	8.94	8.72	18.19
0.7211	24.44	9.26	11.11	10.64	22.23
0.8054	27.13	11.47	14.42	16.79	24.04
0.8982	27.84	27.43	27.48	29.17	29.77

W = weight of water adsorbed at vapour pressure, P.

Po= saturation vapour pressure

Values of W for montmorillonite are in Appendix Table LI.
" " " " *R.lupini* UT2 " " " " LVI.
" " " " *R.lupini* UT12 " " " " LVII.
R.japonicum QA372" " " " LIII.
" " " " *R.leguminosarum* TA101 " " LV

APPENDIX TABLE LXXI

Evaluation of Bangham's free energy equation at various P/Po values to determine the surface energy changes ($A \triangle F$) per gm. associated with adsorption of water by rhizobia and Ca^{2+}-montmorillonite.

Relative humidity (%)	Surface energy changes ($A \triangle F$), cal./gm.							
	R. trifolii	SU297/31A	R. leguminosarum TA101	R. lupini UT12		R. lupini UT2	R. japonicum QA372	calcium-montmorillonite
10	25.9	37.3	38.0	17.3	10.4	3.5	3.5	72.5
20	211.7	179.2	205.5	82.9	55.3	13.8	34.5	362.6
30	347.0	292.8	333.2	141.6	93.2	34.5	67.3	576.7
40	471.3	408.5	452.4	195.1	134.7	65.6	103.6	773.5
50	578.7	519.7	571.5	250.3	179.6	103.6	145.0	954.1
60	725.1	628.5	683.7	305.6	224.5	143.3	193.4	1110.1
70	819.4	725.1	808.0	362.6	279.7	186.5	246.9	1270.7
80	954.8	871.9	958.2	442.0	352.2	249.7	329.8	1426.1
90	1120.5	1022.1	1141.2	581.8	490.3	376.4	483.4	1622.9

APPENDIX TABLE LXXII

Evaluation of Bangham's free energy equation at various P/Po values in order to determine the free energy changes per m^2 associated with adsorption of water to rhizobia and Ca^{2+}-montmorillonite.

Relative Humidity (%)	Free energy changes (ΔF), cal/m^2							
	R.trifolii SU297/31A	R.leguminosarum TA101	R.lupini UT12		R.lupini UT2	R.japonicum QA372	Ca^{2+}-montmorillonite
10	15.46	22.55	23.70	18.16	12.75	4.98	3.51	29.86
20	126.37	108.35	128.17	87.18	68.01	19.93	35.08	149.28
30	207.18	177.63	207.83	148.93	114.77	49.81	68.41	237.43
40	281.39	246.97	282.19	205.23	165.79	94.64	105.24	318.47
50	345.51	314.19	356.50	263.35	221.05	149.44	147.34	392.83
60	432.91	379.96	426.51	321.47	276.31	206.72	196.45	457.09
70	489.19	438.41	504.06	381.41	344.32	268.99	250.83	523.20
80	570.00	527.14	597.76	464.95	433.59	360.14	335.02	587.18
90	668.95	617.97	711.93	612.07	603.63	542.96	491.13	668.22

APPENDIX TABLE LXXIII

Application of the Bradley theory to the isotherm data for fast - and slow - growing rhizobia.

Vapour Pressure p/p_0	log log p_0/p + 2	Mean weight of water adsorbed gms/100 gms bacteria.	
		Fast-growing rhizobia	Slow-growing rhizobia
0.0828	2.034	2.44	0.48
0.1102	1.981	3.30	0.97
0.1453	1.923	3.32	1.02
0.1900	1.858	4.09	1.23
0.2463	1.784	4.79	1.45
0.3168	1.698	5.89	2.15
0.4044	1.595	7.22	2.94
0.5123	1.463	8.94	3.92
0.6445	1.281	11.08	5.44
0.7211	1.153	14.95	7.52
0.8054	0.989	18.06	11.23
0.8982	0.667	24.12	23.42