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State of bacterial DNA after uptake into the roots of germinating barley seeds

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STATE OF BACTERIAL DNA AFTER UPTAKE INTO THE ROOTS OF GERMINATING BARLEY SEEDS

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I. INTRODUCTION

Asexual genetic transformation may be defined as genetic modification of an organism brought about by penetration of that organism by purified DNA from a genetically different organism. Asexual genetic transformation (hereafter to be called genetic transformation or simply transformation) was first discovered in 1928 by Frederick Griffith. He observed that when heat-killed Diplococcus pneumoniae (pneumococcus) cells of a virulent strain were mixed with live cells of an avirulent strain and injected into mice that some of the avirulent cells became virulent. It was not until 1944 that O. T. Avery, C. M. MacLeod, and M. McCarthy identified the transforming agent as DNA. They transformed nonencapsulated (rough) pneumococcus cells into encapsulated (smooth) cells by incubating cell free extracts from an encapsulated strain of pneumococcus with cells of a non-encapsulated pneumococcus strain. They culminated their work by isolating the transforming agent and identifying it as pure DNA.

Since the initial work of Griffith (1928), Avery, et al. (1944), the ability to be genetically transformed by purified DNA has been found in many organisms. The main bacterial genera which have strains capable of being genetically transformed by purified DNA are Pneumococcus, Streptococcus, Haemophilus, Neisseria, Bacillus, and Rhizobium (Spizizen, Reilly, and Evans, 1966). To a much lesser extent successful mammalian transformations have also been performed. Szybalska and Szybalski (1962) transformed cells from a mutant human cell line deficient in inosinate

pyrophosphorylase to inosinate pyrophosphorylase-producing cells by treatment with DNA extracted from wild type cells of the same line. L. M. Kraus (1961) incubated "sickle" erythrocytes with DNA isolated from the bone marrow of a man homozygous for hemoglobin A. After several days the erythrocytes began producing hemoglobin A.

Transformation of intact plants has not been very successful except for the work of Dieter Hess who has demonstrated transformation of flower color (Hess, 1969) and leaf shape (Hess, 1970) with Petunia hybrida. He demonstrated transformation of flower color by treating seedlings of a white flowering recessive mutant of Petunia hybrida with DNA from the leaves of a red flowering pure line of Petunia hybrida. The result upon flowering was that 27% of the seedlings produced red flowers. Selfing of the supposedly transformed petunias produced an F_1 which was also 100% red flowering. Selfing of F_1 petunias produced an F_2 which was also 100% red flowering. Vegetative reproduction of the F_1 also produced petunias which were 100% red flowering. Transformation of leaf shape was demonstrated by treatment of seedlings of the 34d10 mutant of Petunia hybrida (round leaves) with DNA from the cyanidin type Petunia hybrida (oblong leaves). The result after leaf production was plants all with round leaves. However, selfing of one of the treated plants produced a 3 to 1 segregation ratio of plants producing round leaves to plants producing oblong leaves. This is the expected result for the transplantation of a recessive trait. Hence, Hess has shown that transformation can occur in plants upon treatment with DNA from a mutant of the same species.

Of increasing interest are attempts to induce plant seedlings to take up bacterial DNA and incorporate that DNA into its own genome. The reason for wanting plants to incorporate bacterial DNA into their own genome might not be obvious at first. One potential that has been considered from the beginning in this laboratory, is the fact that some bacteria possess the ability to fix atmospheric nitrogen. Hence, the possibility of permanently giving a plant the capacity to fix its own nitrogen by way of a genetic transformation with bacterial DNA would be very important agriculturally, and therefore, very worthy of thorough investigation. The fact that nitrogen fixation genes can be transferred from one organism to another organism has been demonstrated by the genetic transfer of nitrogen fixation capability from Klebsiella pneumoniae to Escherichia coli by conjugation (Dixon and Postgate, 1972). It has also been shown that plant seedlings can take up foreign DNA. This was demonstrated in that a thiamine requiring mutant of Arabidopsis thaliana (mustard family) was given the ability to grow without thiamine by prior treatment with calf thymus DNA. The F_1 generation was able to grow without thiamine or DNA (Ledoux, Huart, and Jacobs, 1971).

The most active as well as the most successful research in plant uptake of bacterial DNA has been performed at Mol, Belgium, by L. Ledoux and his co-workers. They have shown that germinating barley seeds can take up Micrococcus lysodeikticus DNA through a laterally sectioned endosperm and translocate it to various parts of the plant, particularly the roots. This bacterial DNA (bouyant density, d = 1.731) can become integrated with the barley DNA (d = 1.702) in such a manner that the integrated combination can be replicated (Ledoux and Huart, 1968 and 1969). In their experiments, day-old germinating barley seeds were grown on drops

of M. lysodeikticus DNA for 6 to 12 hours, then on water for 72 hours, and finally on tritiated-thymidine for 3 hours. The roots were harvested and the DNA isolated and placed in a CsCl solution for ultracentrifugation. After ultracentrifugation, the fractions were collected dropwise and measured for optical density at 260 nm and radioactivity. An intermediate density peak was found at d = 1.712. Most of the radioactivity was also centered at this peak, thus indicating that the intermediate density DNA was being replicated since the tritiated-thymidine was added in the last three hours of growth, thereby labelling only newly formed DNA. In order to show that the intermediate density DNA was a combination of the barley DNA and the M. lysodeikticus DNA, the molecules at d = 1.712 were dialyzed against NaCl, ultrasonicated, mixed with a CsCl solution, and then ultracentrifuged. Fractions were collected and analyzed for radioactivity. The result was two radioactivity peaks: one at d = 1.702 and the other at d = 1.731. These are the bouyant densities of the parent DNAs. Furthermore, it was shown that the intermediate density peak represented a tightly, if not covalently, bound union of the two parent DNAs. This was accomplished by denaturing the intermediate peak at d = 1.712 by treatment at 100°C for 10 minutes. Slow cooling, which allows reannealing of DNA strands, resulted in the return of the single peak at d = 1.712, showing that the bacterial and barley DNAs remained together during denaturation. Fast cooling, which causes permanent strand separation, resulted in a single density peak at d = 1.727, corresponding to the expected density increase of 15 mg/ml (Stroun, Anker, and Ledoux, 1967) in going from double-stranded DNA to single-stranded DNA if one assumes the two DNAs to be covalently linked end-to-end. Similar results have also been obtained for barley and

Bacillus subtilis DNA (Ledoux and Huart, 1969) and for Solanum lycopersicum (tomato) and Agrobacterium tumefaciens DNA (Stroun, et al., 1967). Therefore, it has been well established that bacterial DNA can be taken up by some plants and become associated with the host DNA.

Even though the work of Ledoux and his associates has clearly demonstrated that plants can take up bacterial DNA and translocate the DNA to the various parts of the plant, the question as to the condition of the bacterial DNA after uptake into the plant still remains to be answered. That is, does the bacterial DNA still exist in an undegraded form after uptake or has it been degraded to nucleotides or short polynucleotides. This is a basic question to solve since all hopes for transferring specific bacterial characteristics, such as nitrogen fixation ability to plants by way of plant uptake of bacterial DNA would be in vain if the bacterial DNA is extensively degraded while in the plant.

Ledoux's own work (Ledoux, 1969) provides some evidence that the bacterial DNA is not wholly degraded in the plant since he was able to recover bacterial DNA from barley roots that was of the proper density for the bacterial DNA and since he was able to detect apparently integrated recombinants of the two DNAs. More indicative of the condition of the bacterial DNA in the plant, however, would be whether this bacterial DNA can retain its biological activity as measured by transforming ability after removal from the plant. Considerable work on this aspect has been performed in this laboratory by Christine Ence (1972), but with results that are difficult to interpret.

In order to better establish the condition of bacterial DNA in the roots of barley seedlings after uptake of the bacterial DNA through the embryo, the research presented in this thesis has been performed.

Since biological activity is such a strong indicator of the physical state of DNA, one of the main goals of this research has been to recover transforming ability from bacterial DNA after uptake by germinating barley seeds. A second goal has been to repeat the results of Ledoux in detecting the presence of a replicating DNA of intermediate density between barley DNA and M. lysodeikticus DNA after the uptake of the M. lysodeikticus DNA by day-old germinating barley seeds. This work was important in order to show that similar results could also be obtained in this laboratory. There have been no attempts made to actually transform plants by bacterial DNA.

II. MATERIALS AND METHODS

Materials

Strains of *Bacillus subtilis* were obtained from Dr. James L. Farmer, Brigham Young University. The strains were maintained on nutrient agar plates under refrigeration at 4°C. *Micrococcus lysodeikticus* DNA was purchased from Miles Laboratories, Inc. Lysozyme, ribonuclease A, 5-bromouracil, and 5-bromodeoxyuridine were obtained from the Sigma Chemical Co. Thymidine-(methyl-^{3}H), specific activity 18.5 C/mmol, was obtained from Amersham/Searle Corporation. Cesium chloride, optical grade, was purchased from Schwarz/Mann. The scintillation fluors, 2,5-diphenyloxazole (PPO) and 1,4-bis(2-(4-methyl-5-phenyloxazolyl) benzene (di-Me-POPOP) were obtained from Nuclear Chicago. Penassay broth was obtained from Difco Laboratories.

Isolation of DNA

Bacterial DNA was isolated, purified, and sterilized according to the method of Marmur (1961) using the lysozyme procedure but omitting the isopropanol precipitation. In many cases the DNA was only partly purified in order to reduce degradation of the DNA. Sterile technique was used throughout this research.

Harvested barley roots were frozen and ground to a fine powder in a mortar with dry ice and acid-washed silica sand. The DNA was then isolated according to the method of Bendich and Bolton (1967). The ribonuclease treatment was not followed due to the small amount of DNA (0.8-1.5 mg) obtained.

Preparation of labelled bacterial DNA

Because of the very close bouyant densities of barley DNA and B. subtilis DNA, the B. subtilis DNA was heavy-labelled by substitution of 5-bromouracil for thymine in order to separate the two DNAs on a cesium chloride density gradient. Five hundred milliliters of Penassay broth were innoculated with B. subtilis, strain 168, thymine-requiring, and grown overnight in a 37°C shaker bath. The cells were then centrifuged and resuspended in 500 ml of a growth solution of growth medium (Anagnostopoulos and Spizizen, 1961) plus 3 µg/ml of thymine. This was then added to 4.5 liters of the same growth solution. Air was bubbled through the solution and the bacteria allowed to grow at 37°C. During late log phase 5 liters of a solution of growth medium plus 40 µg/ml of 5-bromouracil, 40 µg/ml of 5-bromodeoxyuridine, and 1.5 mCi (0.0192 mg) of tritiated thymidine were added to the growing cells. The cells were allowed to continue growing for 14 to 20 hours, after which they were collected in a Sharples centrifuge and the DNA isolated as described above.

Uptake of bacterial DNA by germinating barley seeds

Hordeum vulgare, var. "Bonneville," seeds were soaked in water for 1 hour, husked, and sterilized with 3.0% NaOCl for 30 min. The seeds were rinsed several times with water, placed between wet paper towels, and allowed to germinate 12 to 24 hours. The seedlings were then cut transversely in half and the sectioned surfaces placed on 10 µl drops of bacterial DNA solution (100 to 1000 µg/ml) which were on glass slides. After 8 to 16 hours of growth on the bacterial DNA, a second aliquot of 10 to 30 µl of the same bacterial DNA was added to each seedling in order to prevent depolymerization of the first DNA aliquot by

the plant nucleases (Ledoux and Huart, 1965). After 24 hours of growth on the second aliquot of bacterial DNA, the germinating seeds were placed on wet paper towels and grown for an additional 48 to 120 hours. The roots were then harvested and frozen until time for DNA isolation. In the cases where the seeds were grown on M. lysodeikticus DNA, the seedlings were treated with tritiated thymidine (25 μl/seedling) for 3 hours at the end of the 48 hours growth period on water. All growth was conducted in a dark, humid, enclosed container.

The procedure for uptake of bacterial DNA by germinating barley seeds just described allows the bacterial DNA to be taken up through the embryo rather than through the roots. This was done in order to increase the possibility of having the bacterial DNA enter the root cells rather than having the DNA located in intercellular spaces as would more likely be the case if the DNA was simply absorbed through the roots.

Density gradient analysis of DNA

A sample of DNA solution was diluted to a final volume of 3.00 ml with dilute standard saline-citrate (0.015 M NaCl plus 0.015 M Na citrate) solution in a 5-ml polyallomer centrifuge tube and the solution centrifuged at 30,000 rpm at 19°C in a Beckman model L3-40 preparative centrifuge. The oil was removed from the top of the centrifuge tube by a pipette and rolled paper toweling. The tube was then punctured at the bottom with a 20-gauge needle and the drops allowed to fall by gravity flow. Three or four drop fractions were then collected by an ISCO model 327 fraction collector. Sterile screw-cap test tubes were used to collect the drops when the fractions were to be tested for transforming ability. The gradient was determined by measuring the refractive index of selected fractions with a Bausch and Lomb refractometer kept at

25.0 ± 0.1°C with a Lauda model TK-30 ultrakryomat. The densities of the selected fractions were calculated by using the equation,

$$d_{25°C} = 10.8601\eta_D - 13.4974$$

where η is the refractive index at 25°C (Ifft, Voet, and Vinograd, 1961).

In the cases where ultrasonication was performed before density gradient centrifugation, the DNA was diluted to 3.00 ml by dilute standard saline-citrate solution and ultrasonicated for 2 min. at 100 watts with a Branson Sonifier model 1850.

Detection of DNA

Absorbance.--After the addition of 0.7 ml of water to each fraction from CsCl density gradient centrifugation, the optical density of each fraction was measured at 260 nm on a Beckman DB or a Varian Techtron model 635 spectrophotometer.

Radioactivity.--To each CsCl gradient fraction 0.2 ml of 0.06 M HCl was added. The fractions were then heated to 70°C for 20 min. to hydrolyze the DNA in order to prevent precipitation in the fluor system used. The radioactivity of each fraction was counted in a Packard Tri-Carb liquid scintillation spectrometer after addition of 10 ml of "Bray's solvent" (Bray, 1960) to each fraction.

Assay for determination of biological activity of bacterial DNA

The ability to transform the competent mutant, B. subtilis, strain 168, tryptophan-requiring, was used as the assay for determining the biological activity of the B. subtilis DNA used throughout this research. The transformation procedures of Bott and Wilson (1967) were followed except that Penassay broth was used as the growth medium for

overnight cultures and dilutions were made with sterile physiological saline solution (0.9% NaCl), pH 7.0. The deoxyribonuclease step was also left out since transforming ability was not increased by its use. To each DNA sample and each CsCl density gradient fraction to be tested for transforming ability, 0.4 to 0.9 ml of competent bacterial broth containing about 5 x 10^8 bacteria/ml was added. The tubes were then shaken at 380 strokes per minute in a reciprocal shaker bath at 37°C for 30 min. Dilutions were made and plated on agar plates of the minimal medium of Anagnostopoulos and Spizizen (1961) which contains all the amino acids except tryptophan. CsCl fractions of DNA isolated from barley roots were not diluted.

III. RESULTS

Characterization of labelled *B. subtilis* DNA

B. subtilis, strain 168, thymine-requiring, was heavy-labelled and tritium-labelled by growth in the presence of 5-bromouracil, 5-bromodeoxyuridine and tritiated thymidine for various time periods of 2, 4½, and 14 hours. The DNA from each run was isolated and analyzed on a CsCl density gradient. The results of the bouyant density distribution of each run are given in Table 1. The main peaks were found at d = 1.776, d = 1.742, d = 1.717 and d = 1.702. According to Szybalski, *et al.*, (1960), the peaks of d = 1.776 and d = 1.742 should correspond respectively to substitution of 5-bromouracil for thymine in both stands and in one strand of double-stranded *B. subtilis* DNA. The peak at d = 1.702 is the normal density peak of *B. subtilis* DNA when not grown in the presence of a density label. The peak at d = 1.717 is probably due to partial, single-stranded substitution since its density is intermediate between that of single-strand substituted DNA and non-substituted DNA. The percentages shown in Table 1 were determined by dividing the radioactivity of each peak by the total radioactivity of all the peaks. In order to allow sufficient 5-bromouracil substitution to produce a minimum amount of *B. subtilis* DNA molecules with a density less than d = 1.742, growth periods of 14 to 20 hours in the presence of heavy label were used in preparing the density labelled *B. subtilis* DNAs used in this research.

TABLE 1.--Density distribution of *B. subtilis* DNA after growth[a] in the presence of 5-bromouracil and 5-bromodeoxyuridine

Growth time (hours)	Percentage of DNA at given densities			
	1.702	1.717	1.742	1.776
0	100	0	0	0
2	3.3	76	21	0.3
4.5	0.7	5	48	47
14	0.2	1	35	64

[a]See Materials and Methods.

Fig. 1 shows a typical CsCl density gradient distribution analysis for the DNA isolated from a 14-hour growth of B. subtilis, thymine-requiring, in the presence of 5-bromouracil, 5-bromodeoxyuridine, and tritiated thymidine. The peak at d = 1.765 occurred frequently and probably represents a preferred partial labelling of both DNA strands since its density is intermediate between that of 5-bromouracil substitution in two strands and in one strand of double-stranded B. subtilis DNA. The peaks at d = 1.703 and d = 1.692 represent non-labelled DNA molecules. Both peaks were often observed for the DNA isolated from B. subtilis cells grown in the absence of any label. The fact that the various peaks are quite broad leads one to believe that the labelling procedure is not an "all or none" process, especially since it is requisite that some thymine be present in order for the cells to survive. One would therefore expect slightly varying degrees of labelling of each strand since the DNA replication processes would have a mixture of both 5-bromodeoxyuridine and thymidine available.

In order to determine which 5-bromouracil substituted DNAs possessed biological activity, the transforming ability of each fraction was also measured. The maximum transforming ability was found at the densities corresponding to optical density and radioactivity peaks, particularly at d = 1.775, d = 1.765, d = 1.740, d = 1.715, d = 1.703, and d = 1.691. These results are illustrated in Fig. 2.

Thus it can be seen that labelling of B. subtilis DNA with 5-bromodeoxyuridine produces DNA molecules of several different densities, depending upon the extent of labelling. Each of the presumed DNA density gradient peaks demonstrated transforming ability, hence showing that the gradient peaks actually did represent undegraded molecules of DNA.

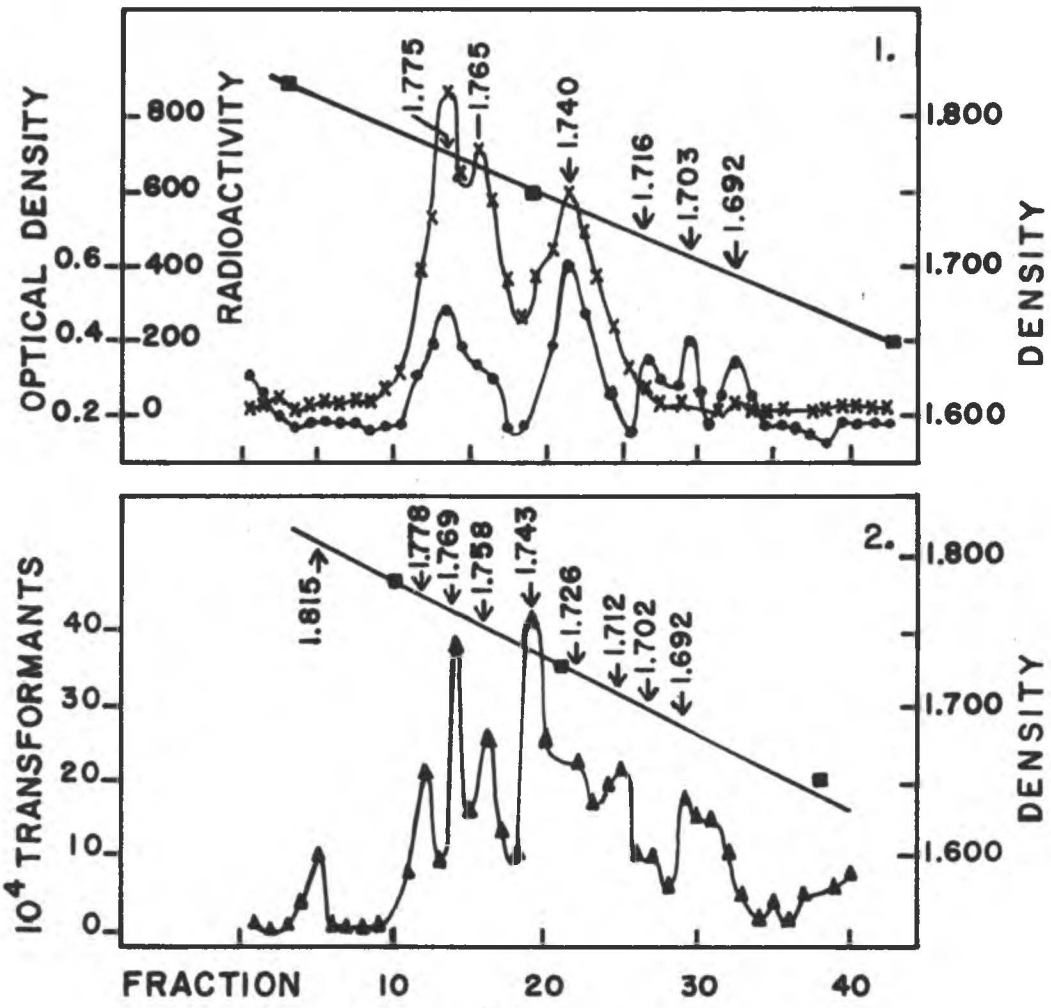

Fig. 1.--CsCl density gradient centrifugation of DNA isolated from B. subtilis cells grown in the presence of 5-bromodeoxyuridine, 5-bromouracil,and tritiated thymidine. (×) Radioactivity, counts per minute; (●) optical density, 260 nm; (■) bouyant density, grams per cubic centimeter.

Fig. 2.--CsCl density gradient centrifugation of DNA isolated from B. subtilis cells grown in the presence of 5-bromodeoxyuridine, 5-bromouracil,and tritiated thymidine. (▲) Transforming ability, transformants per 0.4 ml; (■) bouyant density, grams per cubic centimeter.

Uptake of *B. subtilis* DNA by germinating barley seeds

Uptake of radioactive, heavy-labelled *B. subtilis* DNA by germinating barley seeds was studied under a variety of conditions in order to determine the method that would allow uptake of the most DNA. The results are summarized in Tables 2 and 3. DNA_a was isolated from the roots of barley seedlings which were grown on heavy *B. subtilis* DNA in the usual manner as described in the Materials and Methods section. DNA_e was isolated from the roots of seedlings that were sectioned very close to the embryo rather than in the middle of the seed, everything else being the same. DNA_c was obtained from the roots of sectioned barley seedlings that were allowed to grow continually on drops of DNA rather than removing them from the DNA solution to wet paper towelling for the 72-hour growth period as was usually done. DNA_r was isolated from the roots of seedlings which were repeatedly placed on fresh drops of heavy-labelled *B. subtilis* DNA at 18½, 24, 25, and 37 hours after the beginning of germination. DNA_d was isolated from the roots of sectioned barley seedlings which were allowed to dry in the open atmosphere for 30 minutes before placing them on the initial drops of DNA and for 2 hours before adding the second aliquot of DNA (see Materials and Methods). Also, the seeds were sectioned 2 mm from the end opposite the embryo.

For each of the DNA preparations just described, Table 2 shows the percentage of the bacterial DNA available to the barley seeds that was recovered from the roots. The ratio of the amount of *B. subtilis* DNA recovered from the roots compared to the amount of *B. subtilis* DNA made available to the seeds for uptake was used to determine the percentage of bacterial DNA available for uptake that was recovered from the roots.

TABLE 2.--Percentage of available bacterial DNA found in the roots of barley seedlings after uptake of bacterial DNA by 50 germinating barley seeds.

Type of DNA	*B. subtilis* DNA available to 50 seeds (μg)	*B. subtilis* DNA recovered from the roots (μg)	Percentage of *B. subtilis* DNA found in the roots
DNA_a	1750	8.6	0.49
DNA_e	1750	8.9	0.51
DNA_c	1345	13.3	1.0
DNA_r	1120	13.9	1.2
DNA_d	840	19.5	2.3

TABLE 3.--Percentage bacterial DNA found in the DNA isolated from barley roots after uptake of bacterial DNA by 50 germinating barley seeds.

Type of DNA	Total DNA isolated from the roots (µg)	*B. subtilis* DNA isolated from the roots (µg)	Percentage bacterial DNA in the total root DNA
DNA_a	520	8.6	1.7
DNA_e	670	8.9	1.3
DNA_c	800	13.3	1.7
DNA_r	960	13.9	1.4
DNA_d	1500	19.5	1.3

Table 3 shows the percentage of the DNA isolated from the roots of 50 barley seedlings that was B. subtilis DNA. The ratio of the amount of B. subtilis DNA recovered from the roots compared to the total amount of DNA recovered from the roots was used to determine the percentage of DNA isolated from the roots that was B. subtilis DNA.

The amount of B. subtilis DNA recovered from the barley roots was determined on the basis of the known radioactivity per microgram of the B. subtillis DNA used for uptake. The radioactivity of the DNA isolated from the barley roots was assumed to be due to bacterial DNA only and not to any reincorporation of bacterial DNA degradation products into barley DNA. In order for this to be a valid assumption, it must be shown that the radioactivity occurs where there is bacterial DNA. As illustrated in Fig. 3, most of the radioactivity does occur at the characteristic density gradient peaks for the heavy-labelled B. subtilis DNA used in this research of d = 1.776, d = 1.766, d = 1.745, d = 1.722, and d = 1.710. About 10% of the radioactivity occurred at density positions characteristic for barley DNA (d = 1.702 and d = 1.692); however, B. subtilis DNA that has not been heavy-labelled also has similar density positions (see Fig. 1). Therefore, the assumption that the radioactivity of the DNA isolated from the roots was due only to bacterial DNA was probably valid.

It can be seen from Table 3 that the percentage of the total DNA isolated from the roots that is B. subtilis DNA is relatively constant. Table 2 shows that the more bacterial DNA that was available to the seeds, the lower was the recovery of bacterial DNA from the roots. Comparison of Table 3 to Table 2 shows that although the percentage of bacterial DNA found in the DNA isolated from the roots is relatively

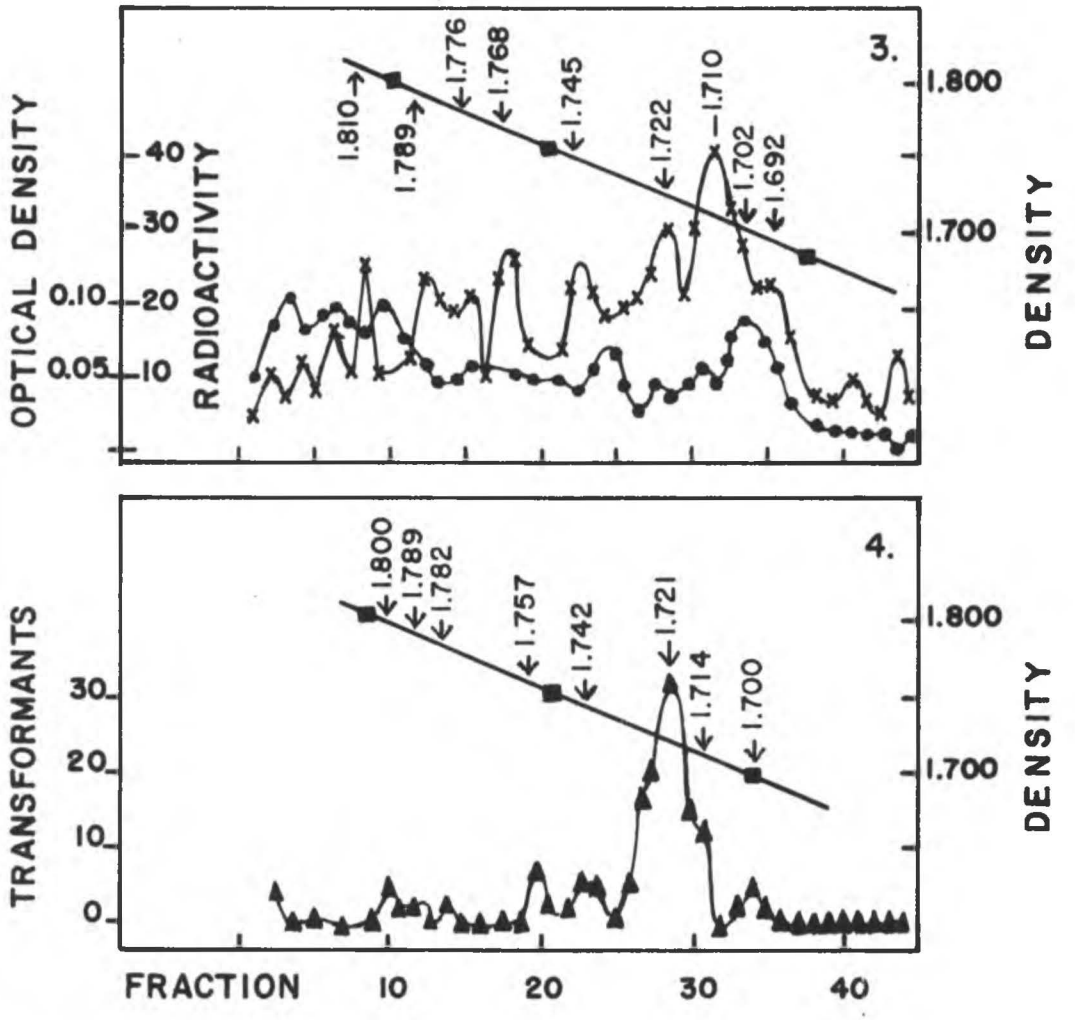

Fig. 3.--CsCl density gradient centrifugation of DNA isolated from the roots of 19-hour germinated barley seeds grown for 10 hours on tritiated, 5-bromouracil-substituted B. subtilis DNA, 24 hours on a second aliquot of the same B. subtilis DNA, and 72 hours on water. (✖) Radioactivity, counts per minute; (●) optical density, 260 nm; (■) bouyant density, grams per cubic centimeter.

Fig. 4.--CsCl density gradient centrifugation of DNA isolated from the roots of 19-hour germinated barley seeds grown for 10 hours on tritiated, 5-bromouracil-substituted B. subtilis DNA, 24 hours on a second aliquot of the same B. subtilis DNA, and 72 hours on water. (▲) Transforming ability, transformants per ml; (■) bouyant density, grams per cubic centimeter.

constant, the amount of bacterial DNA made available to the seeds for uptake varied considerably.

Analysis of barley root DNA after uptake of *B. subtilis* DNA

The CsCl density gradient profile of barley DNA after uptake of radiolabelled, heavy-labelled *B. subtilis* DNA is illustrated in Fig. 3. As expected, the radioactivity peaks of d = 1.775, d = 1.765, and d = 1.740, which are the main peaks of heavy-labelled *B. subtilis* DNA (see Fig. 1), were also present after uptake into the barley roots. Two new peaks at d = 1.722 and d = 1.710 also occurred. Since they are radioactive, they could represent integrated forms of barley DNA and *B. subtilis* DNA. ["Integration is a term used by Ledoux (Ledoux and Huart, 1968) to describe general binding of a foreign DNA molecule to an endogenous DNA molecule. It does not connote a specific type of bonding such as covalent bonding.] The fact that optical density peaks do not occur at the radioactivity peaks is due to the very small amount (1.3%) of radioactive bacterial DNA in the roots. The highest radioactivity peak in Fig. 1 has a radioactivity of 41 cpm. From the known radioactivity of the DNA used for uptake (17,500 cpm per optical density unit), this represents 0.0023 optical density units, which would be too low for detection.

The density gradient analysis profile shown in Fig. 3 was rather typical, especially with respect to the positions of the peaks. The profile illustrated in Fig. 3 was presented in this thesis because it represents the DNA preparation that gave the most successful transformation results (Fig.4).

Transforming ability of bacterial DNA after uptake into barley roots

The presence of barley DNA inhibits the transforming ability of B. subtilis DNA especially for the ratio of barley DNA to bacterial DNA found in the roots after DNA uptake (Ledoux and Huart, 1972). Therefore, in order to measure the transforming ability of the bacterial DNA found in the roots, the bacterial DNA had to be separated from the barley DNA. The method of CsCl density gradient ultracentrifugation was chosen as the means to bring about this separation. For this purpose the bacterial DNA needed to be heavy-labelled since the bouyant densities of barley DNA and B. subtilis DNA are nearly the same and hence would not be able to be separated on the basis of bouyant density.

Fig. 4 shows the results of an attempt to detect the transforming ability of the B. subtilis DNA recovered from the barley DNA_e preparation after ultracentrifugation on a CsCl density gradient. The peaks of transforming ability correspond very closely to the radioactivity peaks in Fig. 3 and with the peaks of transforming ability of heavy-labelled B. subtilis DNA in Fig. 2. DNA_e was also used for the results in Fig. 3. The peak of maximum transforming ability at d = 1.722 corresponds to the second highest radioactivity peak, and the shoulder at d = 1.714 in Fig. 4 corresponds to the maximum radioactivity peak. The high density peaks in Fig. 4 are also found in Fig. 3. Selected colonies from the transformants of the gradient fractions in Fig. 4 were examined under a microscope after Gram staining and found to appear like B. subtilis. Aliquots of the broth in which the competent mutant bacteria were growing and the barley DNA_e were plated onto agar plates in order to detect any contamination. There was none.

On the basis of the transforming ability of the B. subtilis DNA used for uptake (300 transformants/microgram of DNA per milliliter of competent bacterial broth) the amount of B. subtilis DNA in the DNA isolated from the roots (2.0 μg), and the degree by which CsCl ultracentrifugation lowers transforming ability (35%, as determined with heavy-labelled B. subtilis DNA), it was determined that about 400 transformants would have been expected for the results shown in Fig. 4; however, there were only 162 transformants, about 40% of the calculated amount. Thus, translocation through the endosperm to the roots and recovery of the DNA lowered the transforming ability of the B. subtilis DNA by 60%.

Analysis of barley root DNA
after uptake of M. lysodeikticus DNA

Because of the numerous bouyant density peaks that arise in the ultracentrifugation of heavy-labelled B. subtilis DNA, it was not possible to positively determine the presence of any intermediate density peaks that might be the result of association of the bacterial DNA with the barley DNA. In order to determine in this laboratory whether germinating barley seeds can take up bacterial DNA in such a way as to produce an integrated DNA combination with a bouyant density intermediate between barley DNA and the bacterial DNA, one-day germinating, sectioned barley seeds were grown on M. lysodeikticus DNA, which has a considerably higher density (d = 1.731) than barley DNA (d = 1.702). Fig. 5 shows the results of CsCl density gradient analysis of DNA from the roots of barley seedlings grown on M. lysodeikticus DNA. The largest peak represents barley DNA. The shoulder peak at d = 1.715 probably represents the desired peak of intermediate density. Ledoux and Huart (1969) report the density of their intermediate density peak to be d = 1.712 ± 0.002. The small peak at d = 1.731 represents M. lysodeikticus DNA.

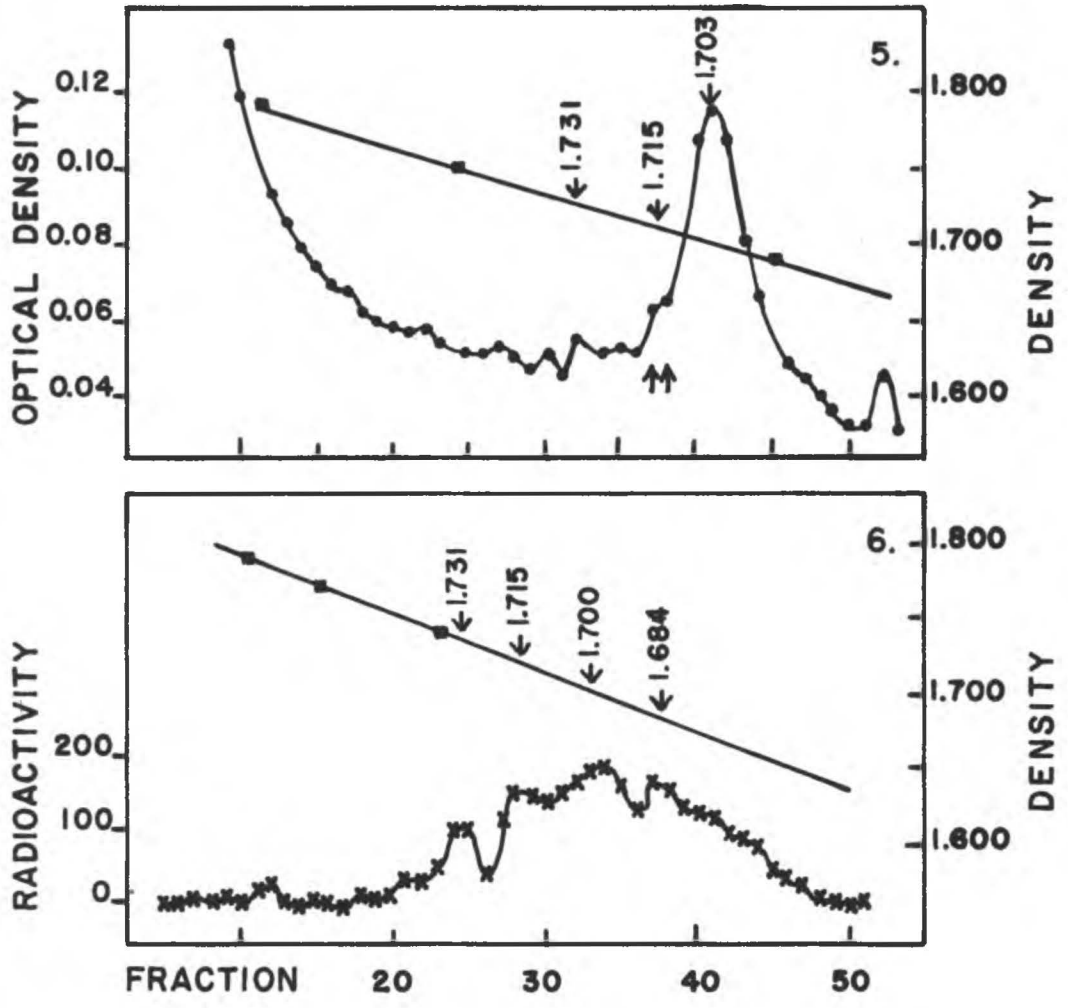

Fig. 5.--CsCl density gradient centrifugation of DNA isolated from the roots of 1-day germinated barley seeds grown for 11 hours on M. lysodeikticus DNA, 84 hours on water, and 3 hours on tritiated thymidine. (•) Optical density, 260 nm; (■) bouyant density, grams per cubic centimeter.

Fig. 6.--CsCl density gradient centrifugation of fractions 36 and 37 in Fig. 5 (see arrows) after sonication of the fractions. (✖) Radioactivity, counts per minute; (■) bouyant density, grams per cubic centimeter.

Note the small size of this peak. Ledoux and Huart (1969) also report that the majority of the M. lysodeikticus DNA taken up by barley seedlings in their research was found under the peak of intermediate density.

In order to determine whether or not the peak at d = 1.715 truly represents a combination of barley DNA and M. lysodeikticus DNA, fractions 36 and 37 in Fig. 5, encompassing the density range of d = 1.711 to d = 1.716, were sonicated and ultracentrifuged in a CsCl density gradient in order to see if the intermediate density DNA molecules could be broken up into the suspected parent DNA densities of d = 1.702 and d = 1.731. Fig. 6 shows the results of this sonication and ultracentrifugation. It can be seen that the parent DNA densities do appear after sonication. The density peak which reoccurred at d = 1.715 probably represents intermediate density DNA molecules which were not broken up into the parent DNA densities. The density peak at d = 1.684 has often occurred as a barley DNA peak.

IV. DISCUSSION

Uptake of *B. subtilis* DNA by germinating barley seeds

Tables 2 and 3 show two interesting facts: (1), that variations in the method of inducing germinating barley seeds to take up bacterial DNA has little effect on the amount of bacterial DNA taken up, and (2), that the percentage of bacterial DNA found in the total root DNA is independent of the amount of bacterial DNA made available for uptake. In other words, there appears to be a saturation point for uptake of bacterial DNA into barley roots.

The seedlings from which DNA_e was isolated were sectioned as near to the embryo as possible without cutting the embryo. DNA_d was obtained from seedlings that were sectioned about two millimeters from the end opposite the embryo. In both cases, however, the percentage of bacterial DNA found in the DNA from the roots was the same (1.3%). Hence, the position of cutting the seedlings did not have much to do with DNA transport to the roots. Also, repeated additions of bacterial DNA or continual growth of the sectioned seedlings on bacterial DNA did not significantly alter the percentage of bacterial DNA found in the roots (1.4% for the repeated additions, 1.7% for the continual growth).

As noted from comparison of the far-right hand columns of Tables 2 and 3, there was considerable variation in the percentage of available bacterial DNA taken up by the barley seedlings, whereas the percentage DNA from the roots that was bacterial DNA remained relatively constant. This was especially demonstrated in the cases of barley DNA_e and barley

DNA_d. In both cases the percentage of DNA from the roots that was bacterial DNA was the same (1.3%); however, the roots from which DNA_d was isolated took up four times the percentage of available bacterial DNA (2.3%) as did the roots from which DNA_e was prepared (0.51%).

Hence, there must be some type of saturation point for bacterial DNA uptake into barley roots. This saturation point has been confirmed by Ledoux and Huart (1965) who report that the maximum amount of M. lysodeikticus DNA they could isolate from the roots of sectioned barley seedlings grown on M. lysodeikticus DNA was 1 to 2 % of the total root DNA.

Evidence that bacterial DNA is not extensively degraded upon uptake into barley roots

The results obtained in this research have established at least four indications that bacterial DNA is not extensively degraded after uptake by germinating barley seeds and translocation through the roots. Three of the observations are physical while the fourth, and most important one, is biological. These four observations will now be discussed.

Density gradient distribution before uptake and after uptake.--Figs. 1 and 2 show the results of CsCl density gradient distribution of B. subtilis DNA labelled with 5-bromodeoxyuridine. As previously mentioned, the array of peaks is due to different degrees of heavy labelling of the B. subtilis DNA strands. They were quite reproducible from one DNA preparation to another. Figs. 3 and 4 show the results of density gradient distribution after uptake of the heavy-labelled B. subtilis DNA into the barley roots. All of the optical density and radioactivity density gradient peaks that occurred before uptake also appeared after uptake. Most of the peaks of transforming ability before uptake were also present after uptake. The coincidence of the positions of the B.

subtilis DNA density peaks before uptake and after uptake by barley seeds shows that the *B. subtilis* DNA was not degraded by host nucleases to a significant extent since otherwise the bacterial DNA would have been broken down to nucleotides and short polynucleotides and barley DNA would have been the only DNA present on the density gradient.

Radioactivity in the bacterial DNA density positions.--A further line of evidence indicating that the bacterial DNA for the most part was not degraded to an appreciable extent in the barley roots is the fact that after isolation of DNA from the roots and separation of the various DNAs on a CsCl density gradient at least 78% of the radioactivity was found in the expected density gradient positions for the various heavy-labelled *B. subtilis* DNA molecules. If it is assumed that the optical density peaks at d = 1.702 and d = 1.692 in Fig. 3 represent only barley DNA (which is not completely true since non-heavy, radiolabelled *B. subtilis* DNA would also be present at these densities), then the maximum percentage of the radioactivity found in the barley DNA density gradient positions would be 22% of the total DNA radioactivity. In addition, nearly 100% of the radioactivity of the DNA aliquot placed on the CsCl gradient was found in the density peaks, thus eliminating the presence of radioactive nucleotides and short polynucleotides. Hence, the majority of the *B. subtilis* DNA was not degraded and the degradation products not reutilized in synthesis of plant DNA since most of the DNA radioactivity was found to be due to *B. subtilis* DNA.

Hotta and Stern (1971) performed experiments very similar to those presented here except that the bacterial DNA was taken from *M. lysodeikticus*. Their results indicated that the radiolabelled bacterial DNA was broken down in the plant and the degradation products reutilized

in synthesis of barley DNA such that most of the radioactivity was found in the barley DNA density position. This is in direct conflict with the results just reported and those to be presented immediately as well as those of Ledoux and Huart (1969) who found most of the radioactivity located at a density position intermediate between M. lysodeikticus DNA and barley DNA. Just why this discrepancy in location of radioactivity exists is confusing since the same organisms were used by both groups of investigators. The only reported difference between the procedures used was that Ledoux and Huart (1969) isolated the total DNA from the barley roots whereas Hotta and Stern (1971) isolated only the DNA present in the root nuclei. Whether this variation in DNA isolation could be the cause of the discrepancy in their results, it is not known. However, the results presented in this thesis (discussed below) agree with those of Ledoux and Huart (1969).

Formation and replication of an intermediate DNA.--A third observation showing that the bacterial DNA is not degraded in the barley roots is the occurrence of a density gradient peak intermediate between M. lysodeikticus DNA and barley DNA that apparently undergoes replication. These experiments were an attempt to repeat the work of Ledoux and Huart (1969) who convincingly claim that the density gradient peak intermediate between M. lysodeikticus DNA and barley DNA represents a dovalently linked, end-to-end recombination of the two DNAs. Fig. 5 shows the barley DNA peak (d = 1.703), the M. lysodeikticus DNA peak (d = 1.731), and the intermediate DNA peak (d = 1.715). As also reported by Ledoux and Huart (1969), the majority of the M. lysodeikticus DNA was found under the intermediate density peak, thus showing that the bacterial DNA in some way formed a relatively strong association with the bacterial DNA rather

than being degraded. As evidence that the peak of intermediate density actually does represent an association of barley DNA and bacterial DNA, the fractions containing the supposed intermediate density DNA peak (shown in Fig. 5 by the arrows) were sonicated and put on a CsCl density gradient. The expected parent densities of d = 1.731 and d = 1.700 appeared along with some remaining, undissociated intermediate density DNA. Therefore, the DNA found under the intermediate density peak did consist of a combination of M. lysodeikticus DNA and barley DNA.

Evidence that the intermediate density DNA undergoes replication is shown by the fact that the intermediate density DNA is radioactive. Tritiated thymidine was added to the barley seedlings only three hours before harvesting the roots; hence, only DNA that was being replicated in those three hours would be radioactive.

Recovery of transforming ability of bacterial DNA after uptake into barley roots.--A very important evidence that the bacterial DNA present in the barley is in a non-degraded condition relative to its state before uptake is the fact that after separation from the barley DNA on a CsCl density gradient, the density peaks characteristic of the heavy-labelled B. subtilis DNA were able to transform a competent, trytophan-requiring mutant of B. subtilis. This is illustrated in Fig. 3. It is known that in order for competent B. subtilis mutants to be transformed, the transforming DNA must be double-stranded and must have a minimum molecular weight of ten million (Hayes, 1970). It has also been determined that the mean molecular weight of B. subtilis DNA changes from about one billion before isolation to about twenty-five million after removal of protein and RNA (Hayes, 1970). Hency, in order for the B. subtilis DNA molecules taken up by the barley seedlings to have

transforming ability, they must have retained their double-stranded structure as well as a molecular weight of at least ten million. The fact that 40% of the expected transforming ability was recovered (see Results) indicates that at least 40% of the B. subtilis DNA molecules were not substantially degraded.

Although the results of Fig. 4 give strongly supporting evidence that bacterial DNA can retain its biological activity after uptake into barley roots, it must be pointed out that transforming ability of heavy-labelled B. subtilis DNA after uptake by germinating barley seeds and separation on a CsCl density gradient was observed in only two out of twelve attempts. The results of Ledoux and Huart (1972) in attempting to detect transforming ability of B. subtilis DNA after recovery from barley roots (discussed below) show, however, that the attempts reported in this thesis to recover transforming ability from the B. subtilis DNA after uptake into barley roots were quite successful. The fact that transforming ability of the B. subtilis DNA after seedling uptake was not observed as frequently as hoped for probably indicates that there was some breakdown of the bacterial DNA in the barley roots or at least in the DNA isolation procedure. This is supported by the observation that only 40% of the expected transforming ability was obtained when transformation did occur. However, the extent of breakdown could be relatively minor since transforming ability was detectable 16% of the time and since degradation of the bacterial DNA to any size less than one-half of its size before uptake could put the molecular weight of the bacterial DNA too low to have transforming ability (less than the minimum molecular weight of ten million required for transforming ability). Such a degree of degradation would be quite easy to envision since the

process of DNA isolation from B. subtilis and subsequent purification causes each DNA molecule to be broken down into about 40 fragments (Hayes, 1970).

A further reason why recovery of transforming activity from B. subtilis DNA after uptake into barley roots might be difficult to observe is mentioned by Ledoux and Huart (1972), who were able to recover only 0.3% of the expected transforming ability of B. subtilis DNA after uptake into barley roots. He states that the loss of transforming ability of B. subtilis DNA is not due so much to absorption into barley tissues as it is due to the procedures for isolating the DNA from the barley root homogenate. He arrived at this conclusion by adding some radioactive B. subtilis DNA to barley root homogenate, isolating the DNA from the mixture, separating the DNAs on a CsCl density gradient, and testing for transforming ability. Only one percent of the expected transforming ability was recovered while eighty-five percent of the radioactivity was recovered. Hence, nearly all of the transforming ability was lost even though most of the bacterial DNA was recovered, thus showing that the DNA isolation procedures destroyed most of the transforming ability of the bacterial DNA. These results have also been confirmed in this laboratory by Bronwyn Hughes (1973).

V. CONCLUSIONS

The main conclusions that can be drawn from this research are that sectioned, germinating barley seeds translocate only a saturating amount of bacterial DNA to the roots and that this bacterial DNA remains principally undegraded over a several-day period while in the seedlings. This final conclusion was supported by four main lines of evidence: (1) the *B. subtilis* DNA still retained its transforming ability under certain experimental conditions after uptake into and isolation from the roots of barley seedlings grown on *B. subtilis* DNA labelled with 5-bromodeoxyuridine; (2) the 5-bromouracil-substituted *B. subtilis* DNA taken up by the barley seedlings had the same density gradient distribution before uptake as after uptake; (3) most of the radioactivity recovered from the barley roots grown on radioactive, heavy-labelled *B. subtilis* DNA was found at the heavy-labelled *B. subtilis* DNA positions upon CsCl density gradient analysis; (4) barley seedlings that had taken up *M. lysodeikticus* DNA produced a new DNA form that underwent replication and which had a bouyant density intermediate between the bouyant densities of barley DNA and *M. lysodeikticus* DNA.

The goal of this research has been to determine whether bacterial DNA can exist in the roots of barley seedlings after uptake through the embryo without being substantially degraded. As mentioned in the introduction to this thesis, the most sought after evidence to show that the bacterial DNA was not degraded while in the seedlings was recovery of the transforming ability of the *B. subtilis* DNA used for uptake. It is felt

by the author that the attempts to recover the transforming activity of the *B. subtilis* DNA as well as the attempts to provide physical proof of the condition of the bacterial DNA have been successful enough to provide respectable evidence for the undegraded state of bacterial DNA present in the roots of barley seedlings that had taken up bacterial DNA through the embryo.

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STATE OF BACTERIAL DNA AFTER UPTAKE INTO THE ROOTS OF GERMINATING BARLEY SEEDS

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ABSTRACT

Germinating barley seeds have been shown to take up Bacillus subtilis DNA labelled with 5-bromouracil and tritiated thymidine and retain the DNA in the root system without substantially degrading it. The percentage of bacterial DNA recovered from the total root DNA was independent of the amount of bacterial DNA available for uptake. Four observations indicated the stability of bacterial DNA after uptake into barley roots: (1) the bacterial DNA retained its transforming ability after recovery from the roots; (2) the labelled B. subtilis DNA showed similar CsCl density gradient patterns before uptake and after uptake; (3) most of the radioactivity of the total root DNA was found at the labelled B. subtilis DNA density positions upon CsCl gradient analysis; (4) barley seedlings that had taken up Micrococcus lysodeikticus DNA produced a new DNA form that underwent replication and which had a bouyant density intermediate between the bouyant densities of barley DNA and M. lysodeikticus DNA (a verification of the results of Ledoux and Huart).