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Isolation, purification, scanning electron microscopy and bacterial DNA uptake of plant protoplasts

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ISOLATION, PURIFICATION, SCANNING ELECTRON MICROSCOPY AND BACTERIAL DNA UPTAKE OF PLANT PROTOPLASTS

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CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION TO GENETIC ENGINEERING

Definition

There has been a great deal of excitement generated in the past few years about the possibility of genetic engineering. Genetic engineering includes any of a number of different genetic manipulations which by-pass the sexual cycle and result in an organism having a new combination of inherited traits (Heyn et al., 1974). In classical genetics offspring are produced by mating male and female and selection of varieties is achieved by ingenious breeding schemes, which include inbreeding. The number of possible combinations of genetic traits that can be brought together in a single organism is very much limited by the mating incompatibility between species as only closely related species can be crossed. Since genetic engineering offers the hope of overcoming this incompatibility, it would allow the introduction of traits and possibly even the production of organisms not before possible.

Benefits and Dangers

There is already a voluminous literature concerning possible consequences of genetic engineering both from the standpoint of possible benefits and inherent dangers (see for example, Berg, 1974; Danielli, 1974; Davis, 1974; Russell, 1974; Widdus and Ault, 1974; Berg, 1976). Some of the possible benefits frequently discussed are

in the areas of: (1) medicine, (2) agriculture and (3) industry. The inherent dangers of research in these areas, when so little is known about the actual biochemical mechanisms involved, such as gene regulation and control, have also been pointed out. In fact, in July, 1974, a distinguished panel of scientists under the auspices of the National Academy of Sciences sought a temporary moratorium on specific genetic experiments potentially threatening to human health (Russell, 1974). A meeting of a National Institutes of Health (NIH) committee followed in December, 1975, from which a set of guidelines for such research was drafted and are currently those recommended by NIH (Singer, 1976).

There are a number of benefits anticipated from using genetic engineering in the area of medicine. One of the most widely discussed is in the area of genetic therapy (particularly in humans). Of the 1500 distinguishable human genetic diseases, nearly 100 have already been identified with a specific enzyme deficiency and correction of the deficiency with direct treatment with genetic material has been suggested (Friedmann and Roblin, 1972). Even those metabolic diseases known to be polygenic have been discussed relative to genetic therapy along with some of the ethical questions such work would generate (Davis, 1970). In addition to genetic therapy, other benefits to medicine might be the development of a means of using microorganisms to synthesize such substances as hormones, enzymes, antibiotics or vitamins. It is hoped that these substances could be produced rather rapidly and gene products could be produced at a high rate (Widdus and Ault, 1974).

Another area likely to benefit from genetic engineering is the agricultural industry. Some of the genetic alterations suggested as future possibilities are: (1) the incorporation of genes for nitrogen fixation or improved nutritional quality and (2) the transfer of genes to confer disease, pest or herbicide resistance to plants. It has even been suggested that organisms suitable for "farming the seas," i.e., capable of using nutrients from the seas, could be developed (Danielli, 1972). The possibility of adding nitrogen fixation capability to plants has been particularly implicated as a possible solution to meet the world's population demand for a larger food supply. Danielli (1974) has reported that the increased world population expected over the next 20 years could be fed using the new crops being developed only if adequate fertilizer is available. Since there is no inexpensive process known for fixing nitrogen industrially, Danielli suggests that biological processes could be used to make fixed nitrogen available. One way to do this would be to develop better strains of nitrogen fixing bacteria or algae. Another way would be to transfer the nitrogen fixation gene "set" to crop plants. Preliminary work toward this end has been initiated by Dixon and Postgate (1972) with the transfer of genes for nitrogen fixation from Klebsiella pneumoniae to Escherichia coli. Recently another step in this direction has been taken with the intrageneric transfer of nitrogen fixation genes between the root nodule bacterium Rhizobium trifolii and Klebsiella aerogenes (Dunican and Tierney, 1974). A number of laboratories are trying to incorporate the nitrogen fixing genes into plants (Hardy and Havelka, 1975; Shanmugam and Valentine, 1975) but so far without reported success.

Industry is another area likely to benefit from genetic engineering studies. By converting to more biologically based industries, presently existing industries could be improved and even new industries developed. Lower pollution, higher conversion efficiency and higher accuracy or specificity of product would also be expected. Sewage or other effluent degradation, antibiotic production and fermentations could all be improved in efficiency (Widdus and Ault, 1974). Also, completely new industries such as the manufacture of totally new antibiotics and sequence-determined polymers or the desalination of water by genetically engineered microorganisms might arise (Danielli, 1971).

That dangerous and undesirable consequences might result from research in genetic engineering has been pointed out by many workers (Davis, 1974; Russell, 1974; Widdus and Ault, 1974; Berg, 1976). Two types of experiments that have been eschewed are those that involve insertion into bacteria of (1) bacterial genes which confer either resistance to antibiotics or ability to form bacterial toxins and (2) the genes of viruses. The potential danger of such experiments is that the bacteria endowed with these genes might escape and infect the population, particularly since the standard bacterium used is E. coli, a common inhabitant of the human intestinal tract. For these reasons, NIH has established a set of guidelines, as mentioned above, although the only method of control is the denial of funds in potentially dangerous research areas.

GENETIC ENGINEERING IN PROKARYOTES

Genetic engineering in prokaryotes via gene transfer has been known to occur for some time. Three processes for the exchange of genetic material are known to exist: transformation by uptake of DNA, transduction by means of viral carriers and conjugation by cell-to-cell contact. These processes serve as models for genetic engineering in higher organisms, so a discussion of pertinent facts known for the bacterial prokaryotes has been included to point out some of the problems that might be anticipated with higher organisms.

Transformation

Transformation was first discovered by Avery et al. (1944) with D. pneumoniae. Several other bacterial species, e.g., B. subtilis (Spizizen, 1958) and H. influenzae (Alexander and Leidy, 1951) were subsequently shown to take up purified DNA (isolated from the same species) and to incorporate it into their genomes with subsequent expression. Only recently has transformation been confirmed for E. coli (Cosloy and Oishi, 1973) in spite of the fact that several hundred workers must have looked for it over the last 25 years (Heyn et al., 1974). The difficulty in finding transformation for E. coli points out that transformation is a rare event and will take place only under special conditions (Tomasz, 1971).

The fact that transformation occurs after a rather complex set of events is shown by the information known so far for D. pneumoniae and E. coli. D. pneumoniae must be in a "competent" state to permit the exogenous DNA to enter. The exact conditions necessary for this

competent state are not fully known, but it seems that several macromolecular factors are necessary. These include a cationic activator, a receptor for the activator, an antigenic determinant and a surface agglutinin (Tomasz, 1973). The secret of transformation for E. coli appears to be the necessity of a loss of a gene function of the recipient cell. This gene codes for an ATP-dependent nuclease which destroys externally added DNA. Secondary mutations in other genes allow recombination of the exogenous DNA to occur without restoring the ability to produce the ATP-dependent nuclease. The information known for D. pneumoniae and E. coli should warn us that similar competency and inhibitory genetic factors may exist in higher organisms.

A further requirement for bacterial transformation seems to be the necessity of a certain degree of homology between host and donor DNAs as shown by the fact that the number of successful transformations increases with taxonomic similarity (Jones and Sneath, 1970). Such homologous base sequences may be necessary in order for the two DNA molecules to pair up (synapse), a process known to precede the actual formation of cross-overs between DNAs (Heyn et al., 1974). The requirement for a rather homologous DNA may also be that the DNA may then avoid the restriction systems within a given species (Glover, 1973). Restriction enzymes cause the breakdown of exogenous DNAs added to even closely related strains (E. coli B versus E. coli K 12) (Heyn et al., 1974). An exogenous DNA escapes degradation when a unique base sequence similar to that of the host DNA is recognized by the modification methylase. This methylase catalyzes a very specific methylation of certain bases of the DNA. Such restriction-modification systems have not yet been identified in eukaryotes but may be found to exist.

Problems are likely to be encountered in transformation studies with eukaryotes since competency, inhibitory genetic factors and homologous DNA requirements have all been found for prokaroytes. In spite of this, genetic engineering research has proceeded with higher organisms by using processes like transformation (see modification by uptake).

Transduction

Transduction is the transfer of genetic material from one bacterium to another, mediated by a virus. Occasionally a replicating virus picks up a piece of host DNA which can then be transmitted to a second host and incorporated into its genome with subsequent expression. The requirements for successful transduction seem to be the attachment of the virus to certain receptor sites on the cell membrane (Adams, 1959; Fenner, 1968) and the presence of specific viral genes, such as the int (for integrate) gene of the bacteriophage lambda which promotes integration of the infecting genome into the host chromosome (Gottesman and Weisberg, 1971). Transduction is a widespread phenomenon in the bacterial world, but so far plant DNA has not been found to occur in virus particles. Most plant viruses are RNA viruses with only a few known DNA viruses so the common occurrence of transduction by DNA viruses in plants seems doubtful. Something similar to transduction may occur in mammalian DNA viruses since host cell DNA has been detected in polyoma virus particles (Heyn et al., 1974). With the present technology, however, it is unlikely that a transducing system could be made operational on a quantitative basis for eukaryotic cells since the eukaryotic DNA is much larger than bacterial DNA and the

chance of a single gene being incorporated is very small (Heyn et al., 1974). It still might be possible to transfer prokaryotic genes to eukaryotic systems using viruses and some work has been carried out on this (see modification by viral facilitated transfer).

Conjugation

Conjugation in bacteria is a process resembling the sexual process of higher organisms. Male cells carrying a small circular DNA, F factor, can transfer this DNA to female recipient cells through narrow bridges. The F factor can be incorporated into the recipient genome or exist autonomously. No F-like factors have been described for eukaroytes although as Brinton (1972) has pointed out, they might be difficult to detect since conjugation in eukaryotes is unknown. However, fusion of eukaryotic cells has been carried out for genetic engineering purposes (see modification by fusion facilitated transfer).

GENETIC ENGINEERING IN EUKARYOTES

Genetic engineering in eukaryotes has been carried out using prokaryotes as models. For example, the prokaryotes have a number of characteristics that have made them useful in elucidating molecular mechanisms for genetic engineering studies. They have extended haploid phases and small nutrient reserves which permit the immediate phenotypic expression of genetic variation. Large, homogeneous populations with short generation times can be grown on defined media which make possible the application of selective schemes to an enormous number of genomes (Chaleff and Carlson, 1974). These organizational features are now becoming available to higher plants. Cells of many plant

species can be cultured under defined conditions (Street, 1973), techniques exist for obtaining haploid cell lines (Chase, 1969; Nitsch, 1972; Smith, 1974) and whole plants can be regenerated from cultured cells of a few species (Vasil and Vasil, 1972; Reinert, 1973) or even whole plants regenerated from cells devoid of cell walls (protoplasts) (Heyn et al., 1974). Since microbiological methods for manipulating higher plants are becoming available, it should be possible to dissect the functioning of these more complex forms. Such information should allow beneficial changes to be made in crop plants, an area of research widely recognized for its importance (Nickell and Torrey, 1969; Cocking, 1973; Melchers and Labib, 1974; Smith, 1974) and an area relevant to the other chapters in this dissertation. For these reasons, the remainder of this introduction will be confined to the genetic engineering of plants. There are a number of excellent review articles on this subject (Hess, 1972; Melchers and Labib, 1973; Chaleff and Carlson, 1974; Heyn et al., 1974; Holl et al., 1974; Johnson and Grierson, 1974; Merril and Stanbro, 1974; Widdus and Ault, 1974). Three of these reviews also cover the literature on genetic engineering in animals (Hess, 1972; Merril and Stanbro, 1974; Widdus and Ault, 1974), an area that will not be discussed further.

Genetic engineering studies with microorganisms have been successful for the reasons indicated including the fact that suitable genetic markers have been available. These markers are an essential feature for assessing the success of modification experiments. The most suitable markers in microbial studies have been mutants differing from the wild strains with respect to nutritional requirements, toxin

or analogue resistance, or isoenzyme variation (Holl et al., 1974). Then, too, mutant selection schemes have allowed large-scale isolation of known biochemical lesions. The lack of available mutants and mutant selection schemes has retarded plant genetic studies, so progress has lagged far behind that in prokaryotic systems. Recently, a number of higher plant auxotrophs have been reported and reviewed (Chaleff and Carlson, 1974; Rice and Carlson, 1975). Some of these have been found in haploid cultures, an advantage recognized in microbial systems, e.g., streptomycin resistant petunia (Binding, 1972) and tobacco cells (Maliga et al., 1973). Plant mutants found so far have generally been identified in cultured cells and, therefore, have been fraught with difficulties since proliferated cell cultures are often accompanied by chromosomal aberrations, changes in ploidy and loss of totipotency (Chaleff and Carlson, 1974). Some of the mutants have also been found to have complex patterns of inheritance rather than patterns recognized in classical genetics. As these problems are worked out and suitable auxotrophic mutants and mutant selection schemes become available, rapid progress in the genetic engineering of plants can be expected.

A number of methods seem to be feasible for eliciting genetic alterations in plants. These are related to the processes discussed above for the prokaryotes and have found application in plant genetic engineering studies. The possible methods can be classified as follows: modification by uptake, modification by viral facilitated transfer and modification by fusion facilitated transfer.

Modification by Uptake

Synthetic or isolated genes would seem to be the best working material for uptake modification experiments, but the methods for obtaining genes are still limited. Only two structural genes have been synthesized, i.e., the DNA sequences of yeast alanyl-tRNA (Khorana et al., 1972) and E. coli precursor tyrosyl-tRNA (Khorana et al., 1976). Such syntheses are possible only for the few genes for which the base sequences have been determined, i.e., tRNAs, rRNAs and RNA transcripts of DNA segments (usually viral) (Widdus and Ault, 1974). The isolation of genes is limited to bacterial genes and genes for which the transcription product can be obtained, i.e., tRNAs, rRNAs and a very few mRNAs. Bacterial genes are not isolated cleanly but always contain some episomal or viral DNA as discussed above (see transduction). Genes complementary to transcription products have been produced using hybridization techniques and reverse transcriptases. Hybridization of RNAs with denatured DNAs followed by digestion with one of the numerous single-strand-specific nucleases (Sadowski and Bakyta, 1972) has resulted in isolation of a few genes. The major problem in the use of hybridization procedures at the moment is that RNA-DNA hybrids for genes of low reiteration cannot be obtained in a way that excludes DNA duplex formation (Widdus and Ault, 1974). Reverse transcriptases have been used to produce DNA chains complementary to a few mRNA templates, e.g., vaccinia virus mRNA and various hemoglobin mRNAs (Widdus and Ault, 1974). There is hope for producing more genes in the future by the use of these techniques as well as by the use of restriction enzymes which cleave at specific base sequences. As more

restriction enzymes are discovered and their base cleavage sites identified, it should be possible to isolate more genes by using the correct combination of these (Marx, 1973).

Although techniques are being developed for the synthesis and isolation of structural genes, there is still little known about the eukaroytic regulatory genes which may be necessary for their control. The nature of these regulatory genes will have to be determined before genes can be isolated or synthesized with their transcriptional control regions intact.

Once genes have been synthesized or isolated, it would be helpful if they could be mass produced. Bacterial plasmids which can be combined with prokaryotic or eukaryotic genes have the potential for replication at a high rate in bacteria, and this has generated a great deal of excitement (Morrow et al., 1974; Marx, 1976). Some workers have suggested this as a way of mass producing the nitrogen fixation gene "set" for transfer to plant cells, a very desirable feat since it would decrease the need for fertilizer (Shanmugam and Valentine, 1975) (see Benefits and Dangers).

Since the supply of available genes is very limited, workers have resorted to uptake of naked DNA, viruses, organelles and micro-organisms for genetic engineering studies. The ultimate goal of any of these modification experiments is the stable phenotypic expression of the new genetic information in the recipient organism. The progress made so far in whole plants or seeds, cell cultures, pollen grains and protoplasts from higher plants or eukaryotic algal cells is described below.

Ledoux and his colleagues at Mol began an investigation of DNA uptake by plants as long ago as 1961 (Ledoux and Huart, 1961). Ledoux et al. have reported the successful uptake of bacterial DNA by germinating barley (Ledoux and Huart, 1969), tomato plants (Stroun et al., 1967) and seeds of Arabidopsis thaliana (Ledoux and Huart, 1971; Ledoux et al., 1974). The fate of the DNA was investigated by autoradiography and CsCl density gradient centrifugation. These studies suggested a covalent end-to-end linkage of the bacterial and plant DNAs. In their more recent work, Ledoux and his co-workers have reported the repair of thiamine, thiazole or pyrimidine mutants of A. thaliana by treatment with calf thymus, E. coli or B. subtilis DNA (Ledoux et al., 1974). The offspring of such repaired plants showed an unexpected absence of segregation.

In a series of modification (transformation) experiments in Petunia hybrida, Hess similarly reported unusual segregation data for his supposedly transformed progeny (Hess, 1972). Hess treated white-flowering anthocyanin mutant seedlings with wild type (red-flowering line) DNA and analyzed the progeny for changes in leaf shape and flower color. After analyzing his results, Hess concluded that transformation had resulted in changes in both characteristics and explained his segregation data in terms of an exosome model like that proposed by Fox and Yoon (1970). According to this model, the incorporated exogenous DNA is associated with the original gene locus, but is not integrated into the linear chromosome, so mutually exclusive transcription of either allele can occur.

Although the Ledoux and Hess groups have claimed DNA-mediated alterations in whole plants or seeds, others have tried and

have not been as successful. Hotta and Stern (1971) were unable to repeat Ledoux's results using barley or tomato unless tissue was dessicated or X-irradiated, conditions they termed "physiologically abnormal". Bendich and Filner (1971) found rapid breakdown of bacterial DNA after uptake by pea seedlings, presumably by endogenous nuclease activity, with no covalent attachment of the type found by Ledoux. More recently, Kleinhofs et al. (1975) have offered alternative explanations for Ledoux's covalent integrated form, i.e., contaminating bacterial DNA and the techniques of DNA preparation. The fact that DNA is claimed to be mutagenic, and any alternations must be shown to be related to the genetic content of the DNA and nothing else, makes the interpretation of data even more difficult (Heyn et al., 1974). Thus, it can be concluded that the fate of DNA and the nature of its possible expression have not been unequivocally determined in these systems.

There have been reports of DNA uptake by cell cultures but no reports for integration or expression of the exogenous DNA. After application of radiolabeled bacterial DNA to A. thaliana callus cells and a study of the reassociation properties of the isolated plant DNA, Lurquin and Hotta (1975) concluded that if any bacterial DNA sequences were integrated into the host genome, they must have constituted less than 2 to 4% of the absorbed DNA. Two laboratories have studied bacterial DNA incorporation by tobacco suspension cultures with much the same results, i.e., no evidence for an integrated form of the type found by Ledoux was obtained, and most of the DNA taken up was present in a predominantly depolymerized state (Bendich and Filner, 1971; Heyn and Schilperoort, 1973). One

worker (L. Owens, personal communication) has looked for expression of exogenous DNA (col E1 plasmid carrying the kanamycin acetylase gene) in soybean and tobacco suspension cultures without success. Experiments with eukaryotic algal (C. reinhardi) cells in culture have given results similar to those found for tobacco suspension cultures, i.e., no evidence for integration of donor DNA into host DNA was obtained, and the exogenous DNA was extensively degraded (Lurquin and Behki, 1975). The breakdown products were found to be subsequently reutilized for host DNA synthesis in the algal cells.

The uptake of exogenous bacterial DNA and presence of the DNA in a highly polymerized state have been demonstrated in pollen grains although no integrated form has been found (Hess et al., 1974a). Pollen grains after DNA uptake are promising for genetic engineering studies since they can be induced to develop into haploid plants (Nitsch, 1974), which might then show the desired modification, or they could be used as DNA-carriers in the realm of a normal fertilization (Hess et al., 1974b). So far there is only one report for a genetic modification involving pollen grains. Mutant barley pollen grains injected with wild type barley DNA at a milk maturity stage showed modification in the waxy character of grains after development (Turbin et al., 1974). In addition to the work on DNA uptake, some experiments have been carried out on the uptake of bacteriophage by pollen grains. The bacteriophage was taken up and could be reisolated with a small fraction (about 0.01%) of the bacteriophage showing biological activity (Hess et al., 1974b). Bacteriophage may be useful in genetic engineering studies as described below (see modification by viral facilitated transfer).

Protoplasts are devoid of cell walls and for this reason seem to have an advantage in DNA uptake studies. Indeed, there have been reports of DNA uptake by protoplasts, e.g., ammi protoplasts took up about 2% of the exogenously supplied bacterial DNA (Ohyama et al., 1972), while petunia protoplasts took up about 0.047% of the exogenously supplied petunia DNA (Hoffmann, 1973). In the case of petunia, 85% of the associated radioactivity after radiolabeled DNA uptake was found to be nuclear associated (Hoffmann, 1973). A lower value (25%) was obtained for nuclei isolated from tobacco protoplasts treated with tobacco DNA (Uchimiya and Murashige, 1975). The DNA after uptake was found to be largely degraded for both higher plant (Ohyama et al., 1972; Gleba et al., 1974) and eukaryotic algal protoplasts (Lurquin and Behki, 1975). No evidence for an integrated form has been reported. There are two reports for attempted modifications in plant protoplasts using externally added DNA, but both are reports of negative results: (1) Lysine and hypoxanthine requiring tobacco protoplast mutants treated with wild type DNA were not corrected for the mutant variation (Carlson, 1972). (2) Soybean protoplasts unable to grow on mannitol medium were not altered in this ability after treatment with Azotobacter vinelandii DNA which contains the necessary enzymes for mannitol utilization (Holl et al., 1974). The fact that there have not been any successful genetic modifications so far is probably not surprising since plant protoplasts are so much more complex than the prokaryotic models for which modifications have been successful. Nevertheless, appropriate remedies should be found as reasons for these failures are discovered.

Protoplasts are known to take up particles larger than DNA, so modification experiments have been also carried out using organelles, viruses and microorganisms as possible sources of genetic material. Uptake of chloroplasts (Carlson, 1973; Potrykus, 1973; Bonnett and Eriksson, 1974) and nuclei (Potrykus and Hoffmann, 1973) by higher plant protoplasts has been demonstrated. The possible functioning of the incorporated chloroplasts in albino tobacco protoplasts has been reported with the regeneration of a green plant (Carlson, 1973), but a definitive interpretation awaits experiments with defined, chloroplast encoded markers (Chaleff and Carlson, 1974). Viral uptake and replication have been demonstrated for protoplasts from a number of sources and for several RNA-viruses (see recent review, Takebe, 1975), but there is only one report for expression of viral DNA (from T_3) in host protoplasts (Carlson, 1973). After barley protoplasts were infected with the bacteriophage T_3, the synthesis of two phage-specific enzymes that were not normally present in the plant was observed. The stability of this expression in subsequent culturing was not reported. A number of microorganisms, i.e., bacterium Rhizobium (Davey and Cocking, 1972), blue-green algal cells and yeast cells or protoplasts (Davey and Cocking, 1975), have been taken up into plant protoplasts but so far without reported genetic modification.

Modification by uptake does seem to be a promising genetic engineering method. Results with whole plants or seeds, cell cultures, pollen grains and protoplasts have all been encouraging even though there have been few reports of actual genetic modifications. As

suitable genetic markers, selective systems allowing unmistakable recognition of biological expression and isolated or synthetic genes become available, the information gained so far on uptake will be indispensable and it is hoped that progress with eukaryotes will someday match that already achieved with prokaryotes.

Modification by Viral Facilitated Transfer

Plant modification has been attempted using bacteriophages carrying bacterial genes, a process modeled after transduction in prokaryotes. Such bacteriophages may protect the bacterial DNA from endogenous nuclease activity and facilitate its entry into plant cells (Chaleff and Carlson, 1974). In one set of experiments, tomato and A. thaliana haploid cell cultures, normally unable to grow on lactose or galactose as the sole carbon source, were innoculated with bacteriophage λ or ϕ80 carrying the lac or gal operons, respectively. The cell cultures were able to survive on lactose or galactose after the uptake of the bacteriophage (Doy et al., 1973). In addition, Doy et al. (1973) claim to have shown the synthesis of the β-galactosidase responsible for the survival using the antibody-heat protection assay for the enzyme. However, they were not able to obtain a positive precipitin reaction between the antibody and plant extracts, so others do not consider this modification to have been fully confirmed (Smith et al., 1975). In a recent report of similar experiments, Johnson et al. (1973) conferred on sycamore cells the ability to grow on lactose as the sole carbon source using bacteriophage $\lambda_{plac}5$. They also used an immunological test for the presence of the bacterial β-galactosidase but were unable to obtain

positive results. The cultures were maintained through several cell generations, so the transfer seems to be long-lived although it is not certain that the transfer was permanent and stable. These two sets of experiments suggest that transfer of genetic information from bacteria to higher plants via bacteriophages is at least a possibility, although confirmation of this awaits further investigation.

Modification by Fusion Facilitated Transfer

In vitro fusion of plant protoplasts with subsequent regeneration of hybrid plants has been suggested as a method for producing greater genetic diversity in plants (Nickell and Torrey, 1969; Chaleff and Carlson, 1974; Heyn *et al.*, 1974; Rice and Carlson, 1975; Smith, 1974). Both intra- and interspecific fusions have been carried out using several techniques: suction of the protoplasts against a grid in perfusion micropipettes (Schenk and Hildebrandt, 1971), exposure of protoplasts to high concentrations of $NaNO_3$ followed by pressure on a cover glass (Potrykus, 1971) or centrifugation (Power *et al.*, 1970) and incubation with concanavalin A (Withers, 1973) with antisera (Hartmann *et al.*, 1973) and with polyethylene glycol (Kao and Michayluk, 1974). Progress has been limited so far by the unavailability of genetic markers which would unambiguously establish the hybrid character of the fusion products and by the lack of selection procedures to recover the hydrids obtained (Melchers and Labib, 1974). Progress has also been limited by the fact that only a few plants can be regenerated from protoplasts, and none of the regenerated plants are from protoplasts from cultivars of major crops.

The feasibility of genetic complementation after protoplast fusion has been shown with intra- and interspecific fusions. In one set of experiments, maize leaf protoplasts carrying chloroplast mutations were fused with normal maize protoplasts. The fusion product that resulted developed normal chloroplasts showing that genetic complementation could occur at the cellular level, i.e., without nuclear fusion (Giles, 1973). In a second set of experiments, protoplasts from two chlorophyll deficient light sensitive varieties of tobacco were fused with the resultant double heterozygote hybrid genes complementing to give resistance to high light intensity (Melchers and Labib, 1974). This was a complementation of two recessive, non-allelic genes to "normal" phenotype. In a third set of experiments, protoplasts from two different species, Petunia hybrida and Parthenocissus tricuspidata, were fused. Callus obtained from the fusion product was shown to possess parthenocissus chromosomes only, yet exhibited isoperoxidases of both species. However, the petunia-specific isoperoxidases were progressively lost after a year in culture (Power et al., 1975). The fact that genetic information from two species was expressed in a fusion product is exciting, but the fact that the modification was not genetically stable shows the need for further experimentation.

There has been one report for the production of a mature interspecific hybrid plant by fusing leaf protoplasts (Carlson, 1973). The species used were Nicotiana glauca (2n = 24), Nicotiana langsdorfii (2n = 18) and the amphiploid (2n = 42) of the tumorous hybrid between these species. In the medium used, protoplasts of the two species failed to proliferate into calluses, while a small percentage of

amphiploid protoplasts were able to form calluses. Plants derived from the growing calluses were indistinguishable from the sexually produced hybrid. The recovery and analysis of the hybrid plant depended on characteristics known to be unique to the sexually formed hybrid. As stated above, more widely applicable selective procedures must be developed before the method of protoplast fusion can be generalized for modification experiments.

EMPHASIS OF DISSERTATION

We have been studying higher plant protoplasts in our laboratory for a number of different reasons: (1) protoplasts are relatively homogeneous systems, (2) some can be cultured with high efficiency under sterile conditions or even a few (carrot, tobacco and petunia) regenerated into entire plants (Heyn et al., 1974), (3) protoplasts may not provide a barrier to uptake or fusion since they are devoid of cell walls and (4) mutant protoplasts should manifest almost immediate expression of any added genetic information since they exist singly as do microorganisms. We have been studying protoplasts isolated from barley because of its similarity to important cereal food crops and from tobacco because of the known ability of its protoplasts to be regenerated into fertile plants (Shepard and Totten, 1975).

The isolation and purification methods for tobacco protoplasts are well known (Shepard and Totten, 1975), but difficulty has been experienced in isolating and purifying protoplasts from the cereals. Thus, we have studied the effect of a number of variables, e.g., temperature, humidity, illumination and plant maturity, in

order to optimize protoplast yields from growing barley plants. We have also investigated techniques for obtaining barley protoplasts free from unwanted cellular organelles and debris in good yields.

We have examined tobacco and barley protoplasts by light and scanning electron microscopy (SEM) to further define the system by determining protoplast surface features and relative intactness. There are no previous reports for SEM of higher plant protoplasts even though it is obvious that SEM has enormous potential for adding to knowledge of processes of current interest, e.g., (1) protoplast cell wall formation, subsequent division and successive stages in plant regeneration, (2) mechanism of macromolecule, virus, organelle or microorganism uptake and (3) mechanism of protoplast fusion.

We have undertaken a study of bacterial DNA uptake by barley and tobacco protoplasts because of its anticipated contribution to genetic engineering studies. We chose bacterial DNA for uptake studies rather than the more complex genetic containing materials such as, chromosomes, bacteriophages, organelles or microorganisms. Bacterial DNAs, with their more simple structures and biochemical compositions, can be readily purified and their physical and biological properties determined before and after uptake. We have carried out such analyses and determined the efficiency, location, integrity and other fundamental characteristics of DNA uptake. Such information will be indispensable as suitable genetic markers and selective systems allowing unmistakable recognition of biological expression become available.

These areas of interest, i.e., isolation and purification of barley protoplasts, SEM of barley protoplasts and uptake of

bacterial DNA by barley and tobacco protoplasts, form the basis for the investigations described in this dissertation. Each of these three areas comprises a chapter and is presented as submitted for publication in scientific journals.

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CHAPTER II

ISOLATION AND PURIFICATION OF BARLEY MESOPHYLL PROTOPLASTS

Summary

Protoplasts were isolated from barley leaves using cellulase and macerozyme. Yields were found to vary widely depending on growth, isolation and purification conditions. The effect of such conditions was studied in detail in an attempt to optimize protoplast yields. Techniques are described for reproducibly obtaining protoplasts in high yields (about 3×10^6 per gram of tissue). Washing procedures for efficiently removing macromolecules from protoplast suspension medium after various manipulation procedures, such as DNA uptake, are described.

Introduction

The development of techniques which permit the isolation of viable protoplasts from many plant species has progressed rapidly during recent years (Wakasa, 1973) (Cocking, 1972). Tobacco (Nicotiana tabacum) and petunia (Petunia hybrida) provide a rather ideal working material (Nagata and Takebe, 1970) (Ohyama and Nitsch, 1972) (Durand et al., 1973) (Binding, 1974), giving protoplasts which can be induced to regenerate fertile plants in rather high yields (Shepard and Totten, 1975). On the other hand, cereal plants have proved to be much more challenging (Wakasa, 1973) (Evans et al., 1972) (Schaskolskaya et al., 1973). Rye (Secale cereale) has been reported to give several fold higher protoplast yields than wheat (Triticum aestivum), barley (Hordeum vulgare), or oats (Avena sativa) (Evans et al., 1972). Although protoplasts can be isolated in moderately high yields from these sources, they would appear to be "vulnerable" and easily "bursted" (Wakasa, 1973). Of the cereals, only wheat, rye and oat protoplasts have been seen to undergo division (Evans et al., 1972) (Galston et al., 1975), but as yet, plants have not been regenerated. One of the general problems in protoplast isolation lies in the difficulty of obtaining reproducibly high yields. It is becoming evident that plant growth conditions are critically important if high yields are to be consistently obtained. Temperature, humidity, illumination, irrigation, plant maturity and application of fertilizers all appear to be important variables (Shepard and Totten, 1975) (Binding, 1974) (Schaskolskaya et al., 1973). The importance of this publication lies chiefly in the fact that we have carefully controlled

such variables in an attempt to define conditions for barley protoplast isolation and purification which reproducibly give high yields, efficient removal of unwanted cellular organelles or debris, and good recoveries.

Materials and Methods

Growth Conditions

Hordeum vulgare var. Bonneville was planted in high density, 2 to 3 cm deep, in vermiculite in 10 cm tapered plastic pots. They contained four 1 cm holes through which 10 cm long cotton wicks were pulled. Each pot was subsequently placed in a plastic reservoir jar and 600 ml of modified Hoagland's (Huffaker et al., 1966) nutrient (diluted 1:2) poured slowly over the vermiculite. Liquid levels in the reservoirs were maintained with distilled water. For optimum protoplast yields, plants were grown in darkness at 27°C for 5 to 7 days before placing them in the light (4000-10,000 lux) produced by a combination of fluorescent and tungsten lamps.

Protoplast Isolation

Leaves were excised just above the coleoptile, weighed, cut with a razor blade into approximately 1 mm^2 pieces (stripping of lower epidermis was not required) and placed in the protoplast release solution: 4% cellulase R-10 and 1% macerozyme R-10 (Yakult Biochemicals); 0.01 M potassium citrate, pH 5.6; and sucrose, mannitol or glucose, 0.3-0.65 M. Leaf pieces, 0.75 g/6.25 ml enzyme solution, were incubated at room temperature for 1-6 hr in 50 ml erlenmeyer

flasks. At the end of the incubation period the protoplast slurry was squeezed through four layers of cheesecloth and counted in a hemacytometer.

Although during the course of most of this work barley protoplasts free from microorganisms were difficult to obtain, treatment with 0.53% NaOCl (10% Clorox) for 20 min and then 70% ethanol for 3 min with sterile distilled water rinses after each was very effective in controlling contamination problems.

Protoplast Purification

Protoplasts were washed in modified Murashige and Skoog's nutrient (Nagata and Takebe, 1971), containing either mannitol or sucrose, by discontinuous gradient centrifugation as follows: (a) sedimentation (600 x g, 5 min) through a fresh solution of mannitol (0.45 M) onto a 0.5 M sucrose "pad" or (b) floatation through a fresh solution of sucrose (0.45 M) overlayed with 0.45 M mannitol. In both cases protoplasts were found at the mannitol-sucrose interface. Swinging bucket and fixed angle centrifugation were employed but the former proved to give the best results.

As a measure of protoplast purity, ^{125}I-DNA (*Micrococcus luteus* or *Bacillus subtilis*) was prepared by the method of Commerford (1971) and added at various stages of the purification procedure.

Results and Discussion

Growth Conditions

Whereas the sequential method of macerozyme followed by cellulase treatment (Nagata and Takebe, 1971) did not give release of protoplasts from barley leaves, their use in concert (Power and

Cocking, 1970) provided a method for variable release depending on growth and isolation conditions (Evans et al., 1972) (Otsuki and Takebe, 1969) (Wakasa, 1973). Under conditions of low continuous illumination (2000 lux) and high humidity (80-90%) Schaskolskaya et al. (1973) obtained good protoplast yields in young barley plants, but the percentage of "morphologically intact" protoplasts varied significantly over the 12 day test period. Only 7 to 8 day old plants could be used in experiments. Our results confirm these findings, i.e., protoplast yields may vary considerably during plant maturation. Wakasa (1973) found that protoplasts isolated from young seedlings were "vulnerable" while those isolated from plants over 30 days were "intact." During initial protoplast isolation experiments little attention was paid to growth conditions, and yields were very unpredictable ranging from 0 - 6 x 10^6 protoplasts per gram of tissue. At that time good yields had been obtained from greening dark grown plants. These were tested further to determine growth conditions which reproducibly favored high yields.

As seen in Fig. 1, 6 day dark grown plants rapidly became resistant to protoplast release upon continuous illumination (10,000 lux), maximum yields (4 x 10^6 protoplasts/g) being obtained in less than one days growth in the light (between 6-18 hr). Changing to a diurnal 12 hr light-dark cycle increased dramatically the period over which good yields could be obtained (2-5 days). A further decrease in day length (8 hr light, 16 hr dark) and light intensity (6000 lux) extended the period over which high yields could be obtained to at least 4 weeks, Fig. 2. The data in Fig. 2 were taken from experiments carried out over more than a year under rather similar conditions and

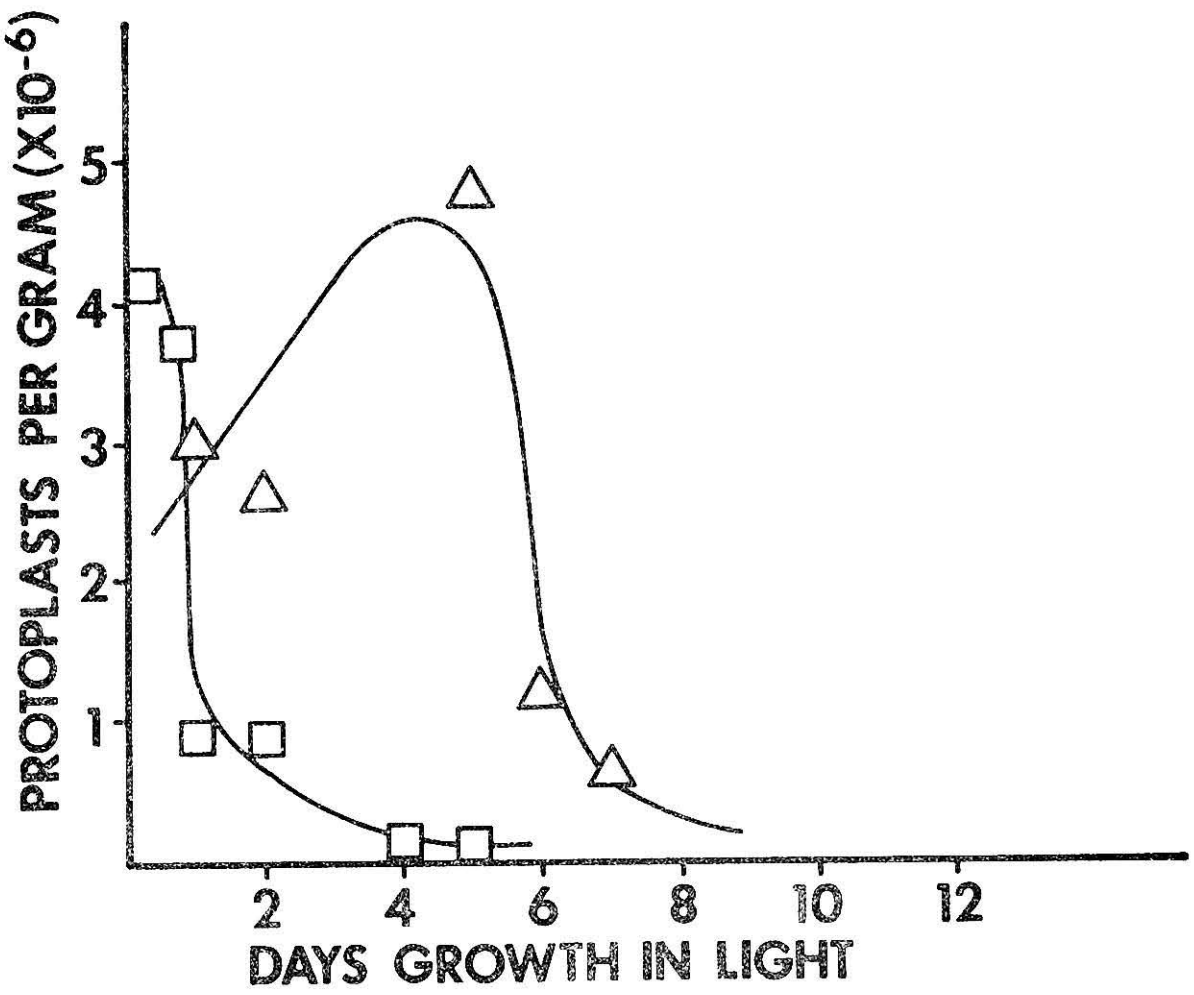

Fig. 1.--Effect of illumination conditions on barley protoplast yield. Six day old etiolated plants were exposed to light (10,000 lux), continuously (□), 12 hr light-dark cycles (△) for various times and protoplasts isolated by floatation in sucrose as described in the Methods.

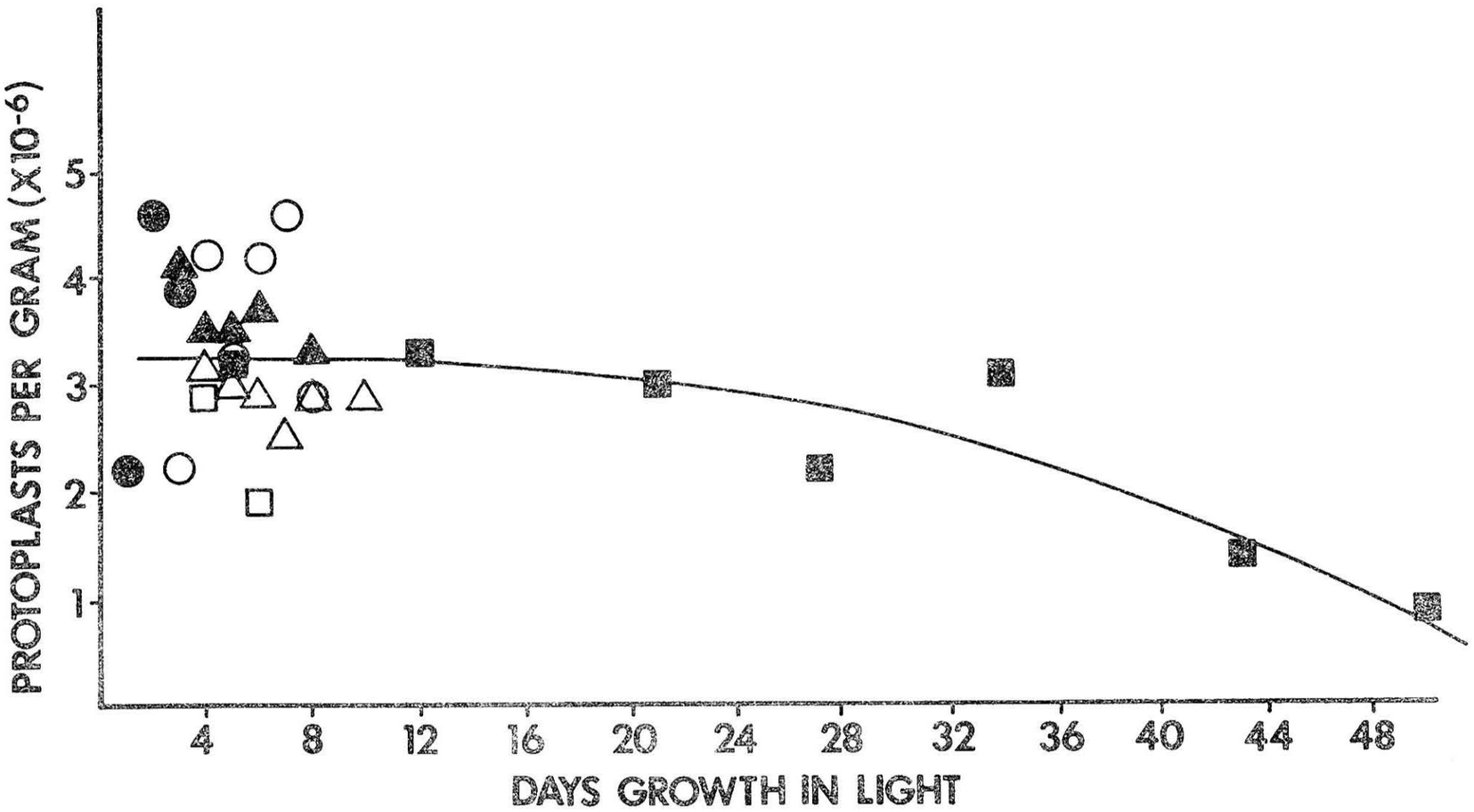

Fig. 2.--Effect of illumination conditions on barley protoplast yield. Six day old etiolated barley plants were exposed to light (6,000 lux, 8 hr light-16 hr dark) for periods up to 50 days, and protoplasts isolated as in Fig. 1. Like symbols represent data taken from a single experiment.

demonstrate that yields varying by a factor of about 2 can be expected, and that a gradual decrease in protoplast release (about 4-fold) was obtained between the 25 and 50 days of illumination. However, even after nearly two months, yields were far superior to those previously reported (Evans et al., 1972) (Wakasa, 1973). A major advantage of these conditions is that plants can be grown in the laboratory (27°C under the lights) with no precautions taken relative to room temperature or humidity, except as provided by the building central air conditioning system.

Recently it was reported that high humidity favored good protoplast yields and viability in tobacco (Shepard and Totten, 1975). Barley plants grown under such a regime were also an excellent source of tissue for protoplast isolation, using sucrose or mannitol as osmoticum, Fig. 3. However, the period during which good yields could be expected was limited to a few days, average yields decreasing from about 4 to 2 x 10^6 protoplasts/g between 6 and 12 days.

Isolation Conditions

The similarity of protoplast yields, when either sucrose or mannitol was used as osmoticum, was evident from the experiment just mentioned and many other experiments, Fig. 3, 4, and Table 1. This similarity was most obvious in kinetic experiments involving protoplast release as a function of time, Fig. 4. This similarity of sucrose and mannitol was in contrast to the work of Schaskolskaya et al. (1973) who found sucrose did not provide good yields. Only about half as many protoplasts were obtained when glucose was used as the osmoticum. Barley protoplast instability in the crude macerozyme and cellulase enzyme system was evident in sucrose after about 4 hours.

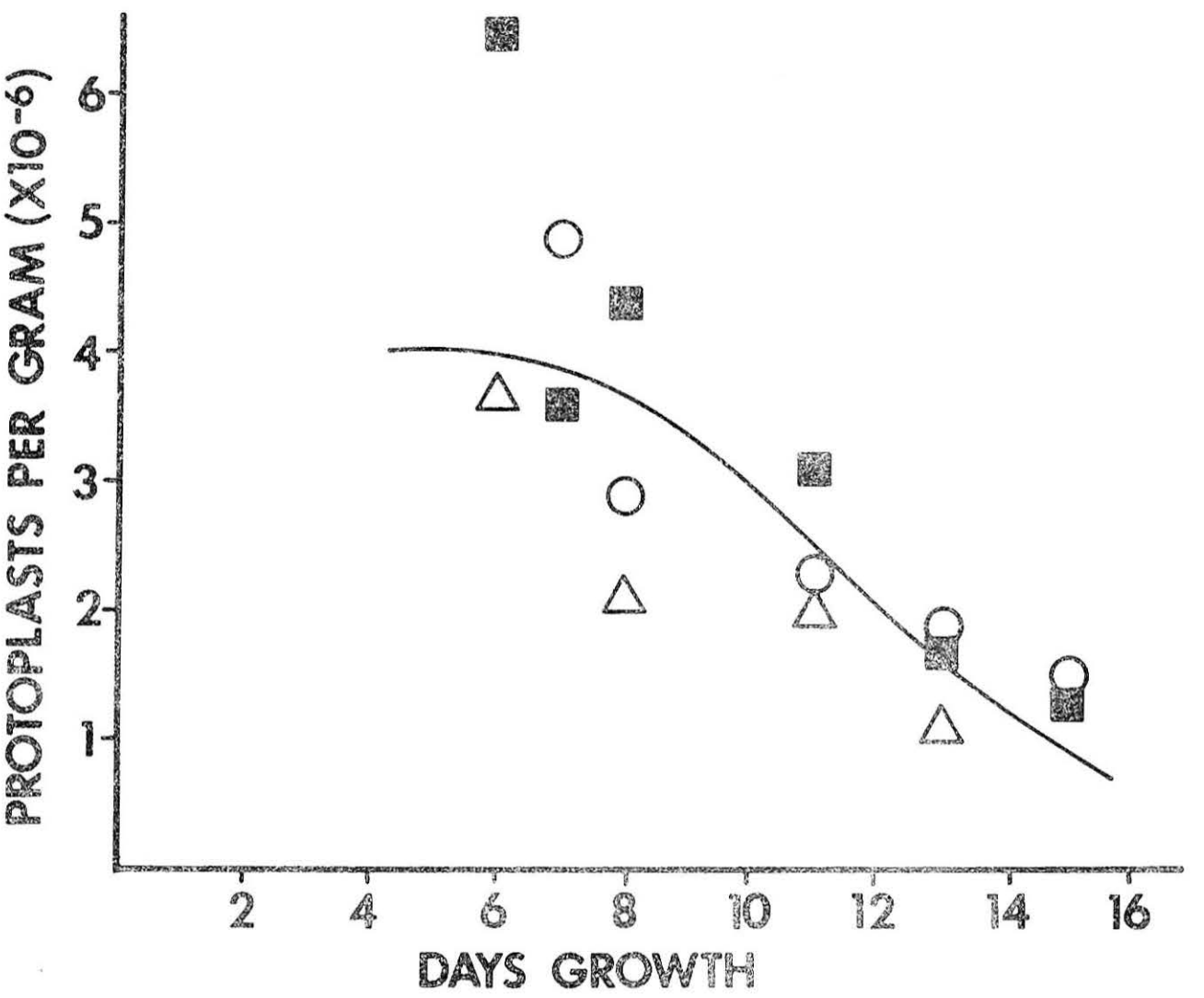

Fig. 3.--Effect of high humidity during illumination on barley protoplast yields when isolated under various osmotic conditions. Plants were germinated and grown in the light (10,000 lux, 16 hr light-8 hr dark). Isolation procedures were otherwise the same as Fig. 2. (○) = 0.2 M sucrose, (△) = 0.3 M sucrose, (■) = 0.5 M mannitol.

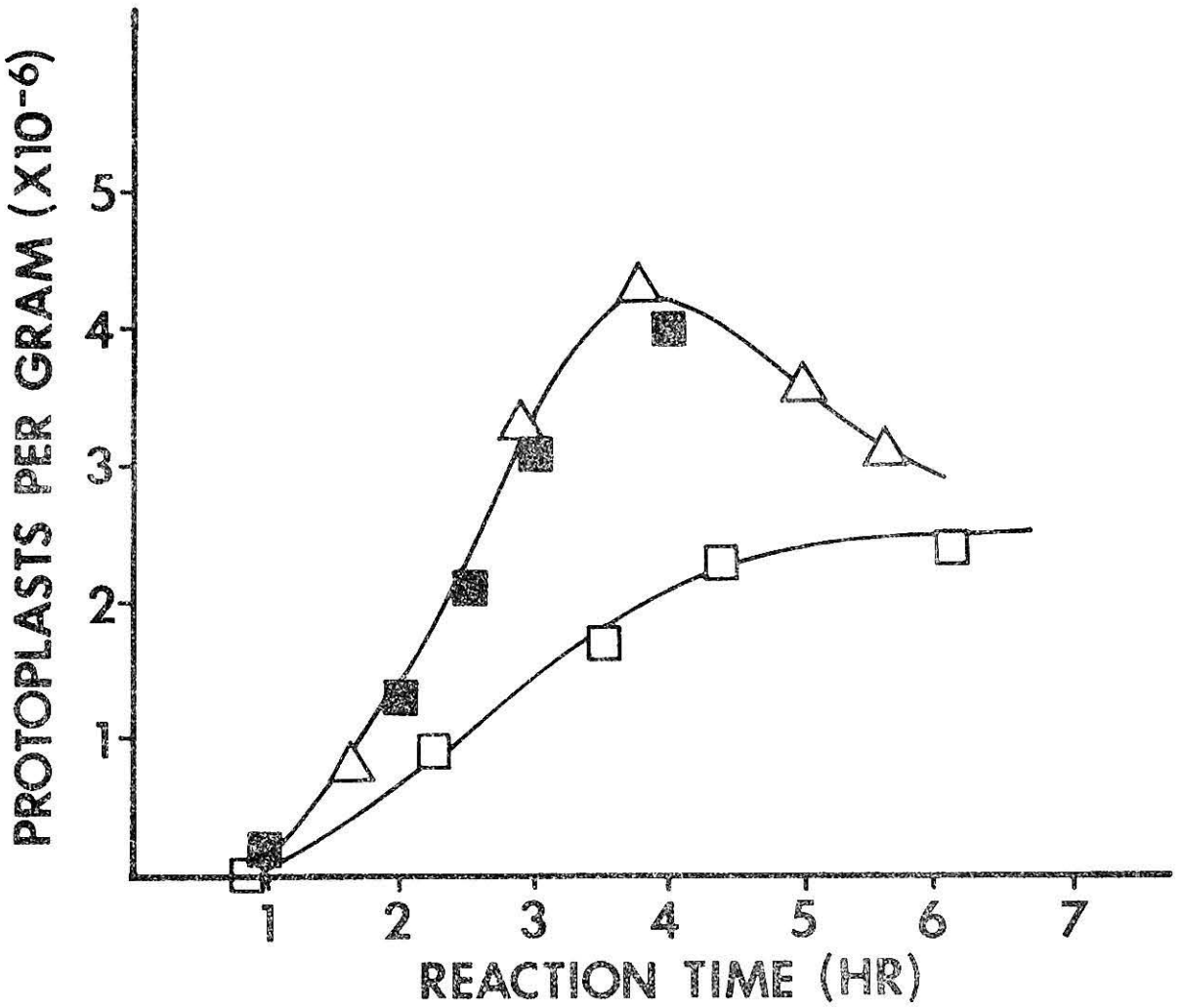

Fig. 4.--Kinetics of protoplast release using various osmotica, procedures otherwise as described in Fig. 2. (△) = 0.3 M sucrose, (■) = 0.5 M mannitol, (□) = 0.3 M glucose.

TABLE 1.--Effect of Osmolarity and Various Osmotica on Protoplast Release

Osmoticum		Yield Protoplasts/gram x 10^{-6}(a,b)
Mannitol	0.30 M	2.5
	0.45 M	3.5
	0.50 M	3.3
	0.70 M	1.2
Sucrose	0.30 M	3.5
	0.45 M	3.5
	0.55 M	3.2
	0.65 M	.2
Sorbitol	0.50 M	3.5
	0.60 M	2.1
Glucose	0.30 M	1.9
Lactose	0.45 M	.4

[a]4 hr incubations.

[b]Barley plants germinated 5 3/4 days in the dark and then grown in the light with 8 hr of light per day.

Similar instability has been observed in mannitol. Further purification of cellulase using the method of Schenk and Hildebrandt (1969) did not increase yield or stability. Although protoplast yields were very similar using either sucrose or mannitol as osmoticum, protoplasts prepared in sucrose were considerably more polar with respect to chloroplast distribution than their mannitol counterparts.

The effect of osmotic strength on the kinetics of protoplast release is shown in Fig. 5. Good yields were not obtained from barley unless osmotic conditions were correctly chosen. Increasing concentrations of 0.45, 0.55 and 0.65 M sucrose gave progressively lower yields over the 4 to 6 hr reaction period. The enormous sensitivity of yields on osmolarity, i.e., nearly a 20-fold decrease between 0.34 and 0.32 M observed by Schaskolskaya et al. (1973), was not consistent with our results although 0.65 M gave significantly lower yields than 0.55 M sucrose. The higher osmolarity was very destructive to protoplasts, as determined by the number of fragments in the protoplast solution. Growth conditions have been reported to be an important variable in determining osmotic conditions for optimum protoplast release (Shepard and Totten, 1975). This could explain why Schaskolskaya et al. (1973) results are at variance with those mentioned above. They obtained no intact protoplasts in 0.45 M mannitol. A comparison of mannitol, sucrose, sorbitol, glucose and lactose at various osmolarities, during a 4 hr treatment with macerozyme and cellulase is shown in Table 1. These results also showed the similarity of mannitol and sucrose relative to protoplast release, as

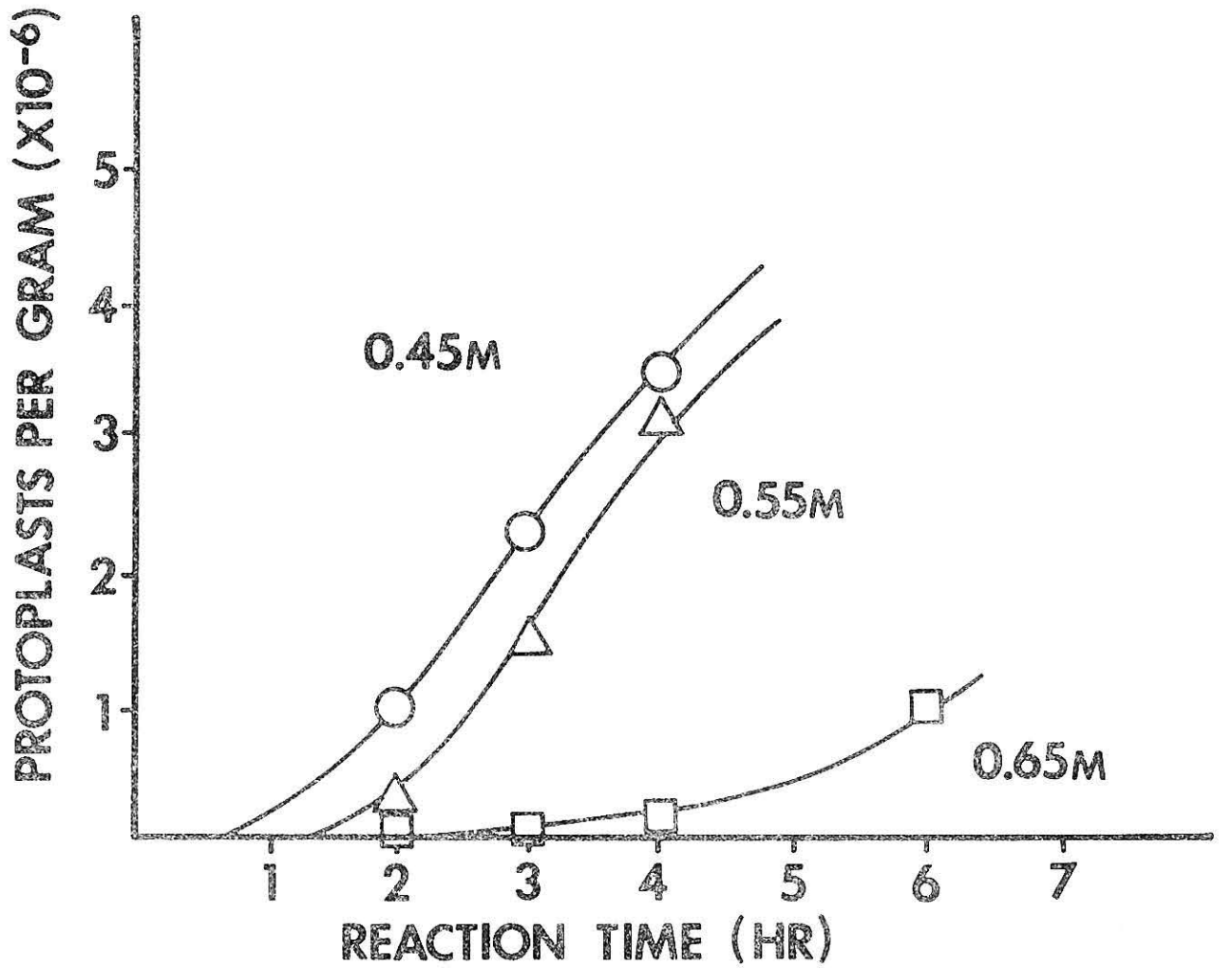

Fig. 5.--Kinetics of protoplast release under various osmotic conditions. Same illumination conditions as in Fig. 2. (○) = 0.45 M sucrose, (△) = 0.55 M sucrose, (□) = 0.65 M sucrose.

mentioned. Sorbitol also gave satisfactory yields while glucose and lactose have repeatedly given poor protoplast release. The reason for these differences is not obvious.

An effect of solution depth during protoplast release has been observed, shallow solutions favoring higher yields (Table 2), with plants grown under a number of different conditions. No exceptions were found to this general rule. Containers were chosen which provided sufficient area so as to give solution depths of 4-6 mm, e.g., 0.75 g tissue per 6.25 ml protoplast release enzymes in a 50 ml erlenmeyer flask.

Various concentrations of macerozyme and cellulase were found to make a difference in protoplast release. Although more rapid release was obtained at higher concentrations, more protoplast damage was also obtained giving lower overall yields. This may have been due to contaminants in the partially purified enzymes which destabilized the plasma membrane (Cocking, 1972). The concentrations of cellulase and macerozyme which proved to be suitable for barley were similar to those used with many other plants: 4% cellulase and 1% macerozyme. Cellulase Onozuka SS and crude macerozyme gave good yields with tobacco and safflower, but partially purified enzymes (R-10) were required for protoplast release in barley.

When increasing amounts of leaf tissue were added to a constant amount of enzyme solution, larger protoplast quantities were obtained, Fig. 6. Yield per gram of tissue was highest between 0.2 and 0.6 gram/reaction (3 and 10% (w/v)) depending on reaction time. If amount of tissue is not a consideration, 30% (w/v) solutions work well giving about twice as many protoplasts as obtained with an 8% (w/v) solution.

TABLE 2.--Effect of Standard Protoplast Release Solution Depth on Protoplast Yield

Reaction Depth (mm)	Yield Protoplasts/ g x 10^{-6}	Plant Growth Conditions Days Dark Grown	Days Light	Light Intensity (Lux)
6	2.2	7	3	4000
18	1.8	7	3	4000
6	3.1	6	4	4000
18	1.6	6	4	4000
6	2.6	5 3/4	6	6000
10	0.91	5 3/4	7	6000
6	2.9	5 3/4	6	6000
10	0.99	5 3/4	9	6000
6	2.5	5 3/4	7	6000
10	0.83	5 3/4	7	6000

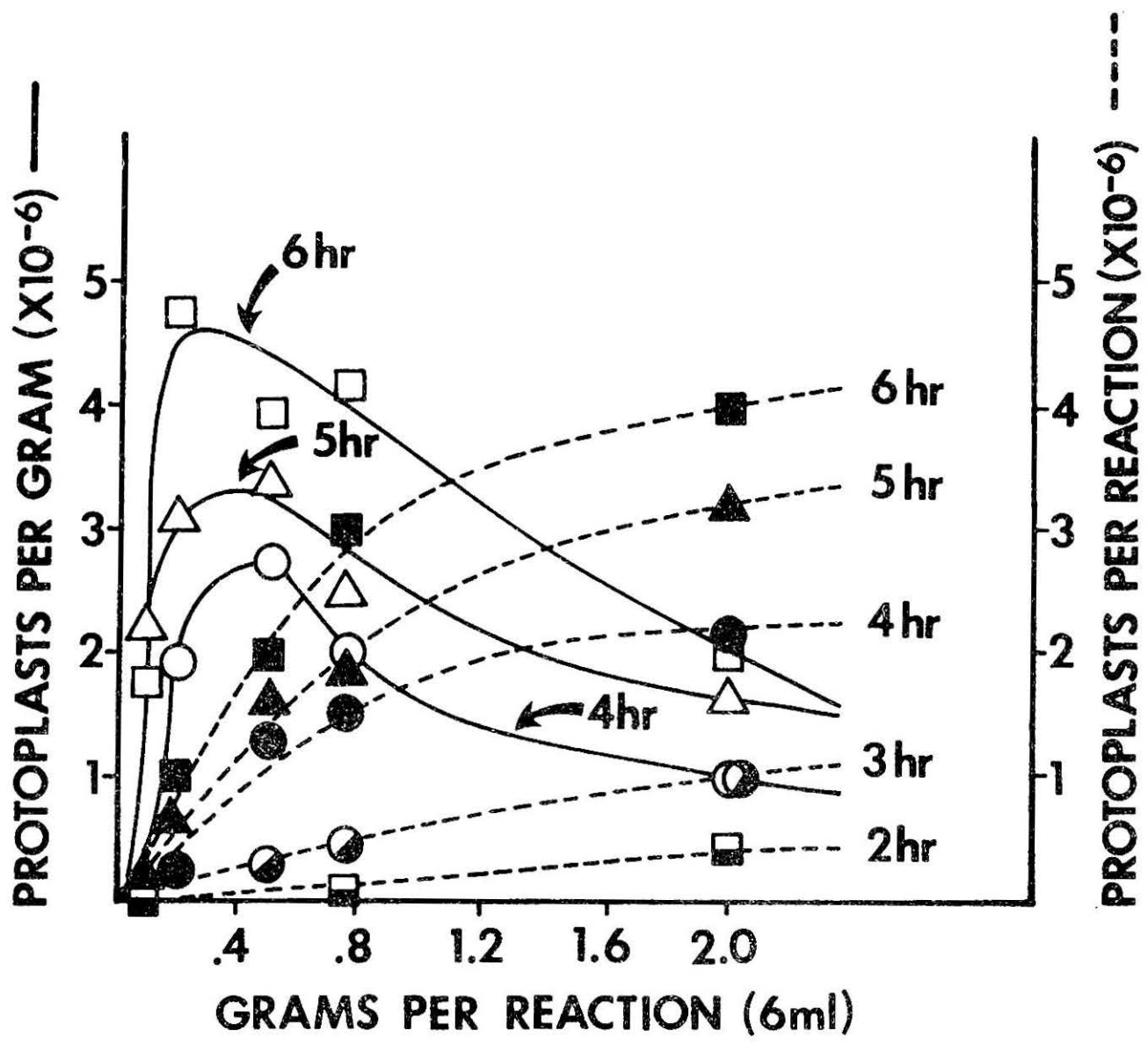

Fig. 6.--Effect of increasing concentrations of leaf tissue on protoplast yields. Solid line represents protoplasts per gram and dashed line total protoplasts per reaction. Reaction volumes were constant (6 ml) and reaction times were as shown.

It is obvious that the techniques which have been worked out for barley vary somewhat from day to day and from one pot of plants to another and are very dependent of growth conditions. For this reason, data which was to be compared directly was obtained during a single experiment with protoplasts isolated from leaves harvested at the same time, from the same pot of plants. The barley protoplast isolation techniques also worked well with safflower (Carthamus tinctorius), but not well with corn (Zea maize) and poorly with tomato (Lycopersicon esculentum) grown under various conditions, Fig. 7.

A technique which in some cases has proved to be a valuable substitute for slicing leaves, prior to treatment with protoplast releasing enzymes, involves brushing lower leaf surfaces with carborundum (240 mesh). This is particularly useful when large quantities of protoplasts are required, and has been successfully used with cow pea (Beier and Bruening, 1975), and in our laboratory with tobacco, barley and safflower, providing essentially complete protoplast release upon subsequent treatment with macerozyme and cellulase. However, brushing with carborundum does not provide protoplast release from tomato leaves with these enzymes. Raising the temperature during enzyme treatment from 21 to 30° decreases the time required for protoplast release by nearly two-fold with no apparent increase in damage.

Protoplast Purification

As important as yield, in the considerations above, was protoplast recovery after various manipulation procedures. Barley protoplasts were not very stable if left in the enzyme solution for more than a few hours, Fig. 4. Furthermore these enzyme solutions are

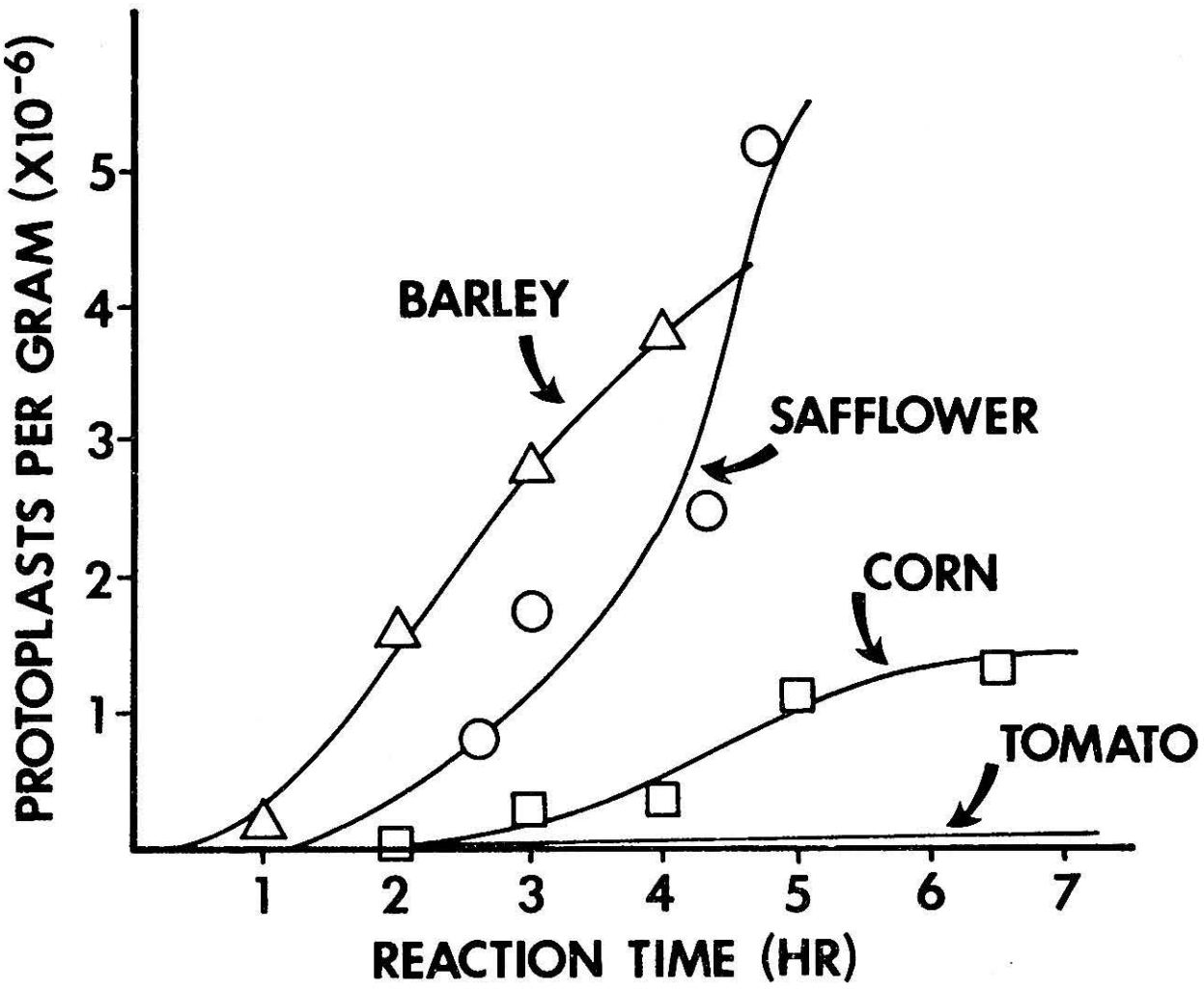

Fig. 7.--Kinetics of protoplast release from various plants using conditions as described in Fig. 2.

contaminated with nucleases, as also observed by Cocking et al. (1969), which create an even greater problem in DNA uptake experiments. We have shown, for example, that a 600-fold dilution of the protoplast release enzyme solution decreased the transforming ability of Bacillus subtilis DNA 30% after 2 hr. Conventional washing techniques for removing such contaminants involving dilution followed by recovery by sedimentation or floatation, repeated several times, have not proven to be satisfactory because of significant losses of barley protoplasts during repeated steps of the procedure. Efficiency of washing was greatly improved by application of one or more of the following simple discontinuous gradient centrifugation techniques: (a) Underlaying the protoplast suspension with a sucrose pad prior to sedimentation in mannitol prevents formation of a compact protoplast pellet. Intact barley protoplast recoveries from such compact pellets are very poor. Debris and nuclei sediment through such sucrose pads, while protoplasts are found in a narrow zone at the interface. (b) Overlaying suspensions of barley protoplasts with mannitol during floatation in sucrose increases recoveries. Although this step is not required with tobacco and safflower protoplasts, it greatly improves recovery of barley protoplasts during isolation by floatation. The reason for this phenomenon is not immediately obvious, but probably involves destabilization of barley protoplast membranes by the air-water interface during centrifugation. If protoplasts are allowed to float slowly overnight in the cold (4°C), the mannitol overlayer is not necessary. (c) Rather than diluting protoplasts during washing procedures, a fresh sucrose solution of slightly lower density (0.45 M) layered over the protoplasts suspension (0.5 M sucrose) followed by a

mannitol (0.45 M) overlayer, greatly increases washing efficiency. Protoplasts are floated up through the fresh uncontaminated solution during centrifugation. The amber color of the macerozyme and cellulase remains in the 0.5 M sucrose. Approximately equal volumes of protoplasts and fresh overlayered sucrose provide a 10-100 fold decrease in ^{125}I-DNA added to the protoplasts just prior to centrifugation, depending on the volume in which the protoplasts are recovered, as opposed to a 2-10 fold decrease which is obtained upon simple solution and floatation. Increasing the volume of the overlayered sucrose wash solution relative to that of the protoplast suspension greatly improves this purification. For example, a ratio of 5:1 gives approximately a 1000-fold decrease in ^{125}I-DNA remaining with the protoplasts.

The following example serves as an illustration of the improved washing or purification obtained by discontinuous gradient centrifugation. Barley protoplasts were mixed with 3.9×10^4 cpm of ^{125}I-DNA, total volume of 5 ml in 0.5 M sucrose. A discontinuous gradient was formed by overlayering these protoplasts with 6 ml of 0.45 M sucrose and then 1.0 ml of 0.45 M mannitol. After centrifugation (see Methods) protoplasts were found at the mannitol-sucrose interface. Radioactivity of fractions (0.4 to 2.0 ml) was determined as shown in Fig. 8 (A). Protoplasts were found between 9.1 and 11.6 ml. Only negligible radioactivity (34 cpm) was found in the mannitol layer above the protoplasts (11.6 to 12 ml). Since 3.9×10^4 cpm (5 ml) was added to the gradient and 2.2×10^3 cpm (2.5 ml) were still contaminating the protoplasts after centrifugation, an overall purification of almost 20-fold was obtained. By mixing the original 6 ml with 5 ml of fresh sucrose and recovering protoplasts in the same volume (2.5 ml)

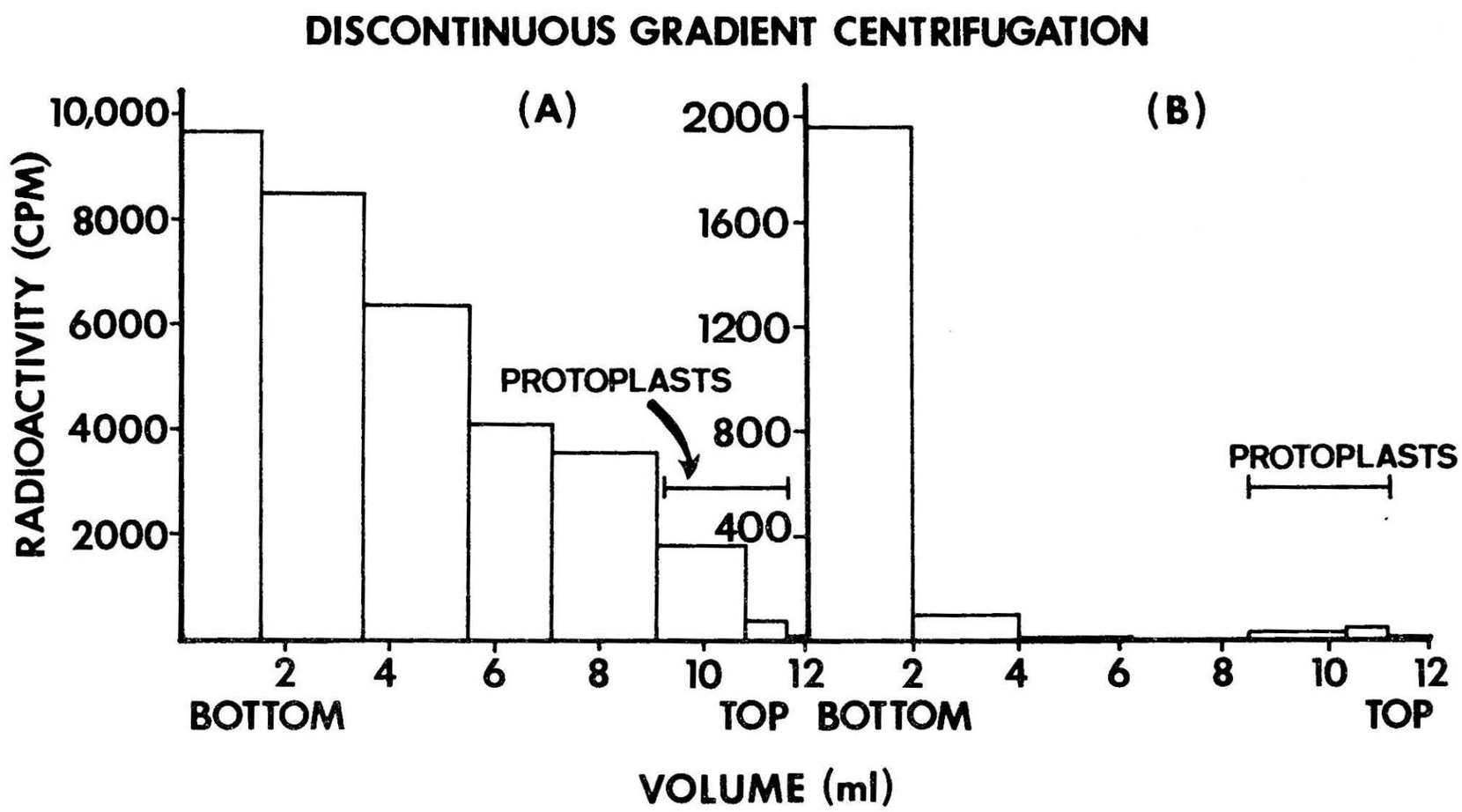

Fig. 8.--Distribution of ^{125}I-DNA after discontinuous gradient centrifugation of protoplasts mixed with ^{125}I-DNA. (A) 6 ml protoplasts (0.5 M sucrose) floated up through 5 ml 0.45 M sucrose. (B) 2 ml protoplasts (0.5 M sucrose) floated up through 9 ml of 0.45 M sucrose. See Methods.

only about a 4-fold purification could be obtained (3.9×10^4 cpm divided by 3.9×10^4 cpm per 11 ml x 2.5 ml = 4.4-fold). Thus, dilution would have to be repeated a second time in order to be comparable with the discontinuous gradient technique ($4.4^2 = 19.4$). In this example is was not possible to determine if radioactivity was associated with protoplasts because of high background resulting from ^{125}I-DNA carried along with the protoplasts.

However, decreasing the protoplast volume relative to the sucrose overlayer greatly increased the washing efficiency. This was accomplished by first sedimenting the protoplasts out of the radioactive medium followed by discontinuous density gradient centrifugation. In this case 3.9×10^4 cpm (^{125}I-DNA) were mixed with protoplasts in 0.45 M mannitol. This was layered over 0.5 ml of 0.53 M sucrose followed by centrifugation, Fig. 9 (A) (B). The protoplasts (2 ml) were transferred to a second centrifuge tube, overlayered with 0.45 M sucrose, and then 0.45 M mannitol, Fig. 9 (C), and centrifuged as before. The distribution of counts is shown in Fig. 8 (B). The last centrifugation step resulted in a 1000-fold purification. Coupled with a nearly 20-fold decrease in counts during the first centrifugation, an overall washing efficiency of about 20,000-fold was obtained. In this case removal of ^{125}I-DNA was sufficient to clearly visualize radioactivity associated with protoplasts, since background was negligible. Protoplast recovery was about 60%.

Protoplast Stability

The increased washing efficiency characteristic of the discontinuous gradient centrifugation system, described above, decreased the number of steps required to remove contaminants from

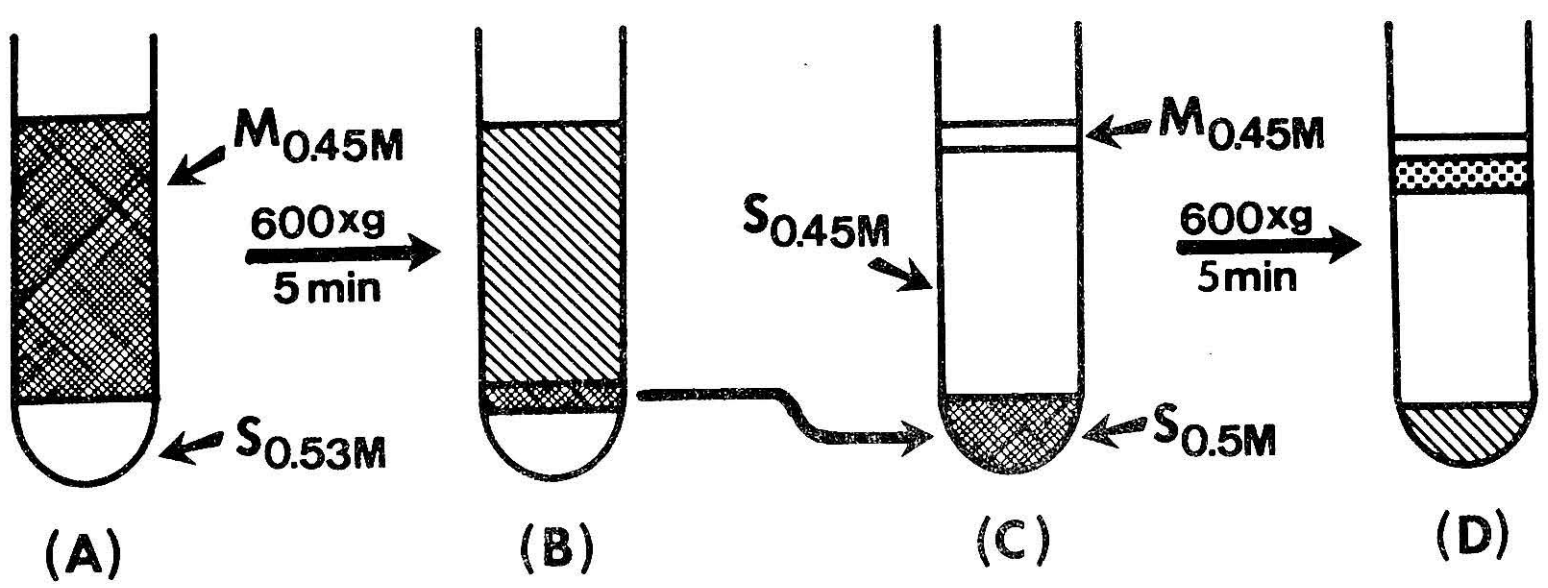

Fig. 9.--Schematic drawing illustrating techniques used in discontinuous gradient centrifugation. S = sucrose, M = mannitol. (A) → (B) Sedimentation of protoplasts in 0.45 M mannitol, ^{125}I-DNA solution. (B) → (C) collection of protoplasts from sucrose-mannitol interface and transfer to discontinuous sucrose gradient. (C) → (D) Floatation of protoplasts through sucrose (0.45 M).

fragile barley protoplasts. This greatly facilitated higher yields. Losses which were experienced during these and other related procedures were due primarily to: changes in the centrifugation properties of protoplasts, protoplasts sticking to the walls of glass centrifuge tubes and protoplast fragmentation. These are probably related events.

Age and growth conditions are probably important variables in determining protoplast stability during various manipulation procedures. Under conditions of low light intensity (4000 lux), recovery of protoplasts from greening barley leaves, after two centrifugation steps, increased nearly two-fold between 1 and 3 days in the light in one experiment and between 3 to 6 days in the light in another, Fig. 10. Increasing the light intensity (6000 lux) in a third experiment gave much smaller increases between 4 and 8 days. From these results it would appear that protoplasts isolated from very young tissue are inherently more unstable, in greening barley leaves, and that this instability is more rapidly overcome at higher light intensities. This is consistent with the data of Wakasa (1973) as mentioned above, and may also explain the results of Schaskolskaya et al. (1973) who obtained poor recoveries and extreme osmotic sensitivity of protoplasts isolated from very young plants grown under low light (2000 lux). After about one week in the light (8 hr light-16 hr dark, 6000 lux), there appears to be no reproducible trend in stability. Recoveries average about 60%, ranging between 45 and 90% from day to day and from one pot of plants to another.

Acknowledgments

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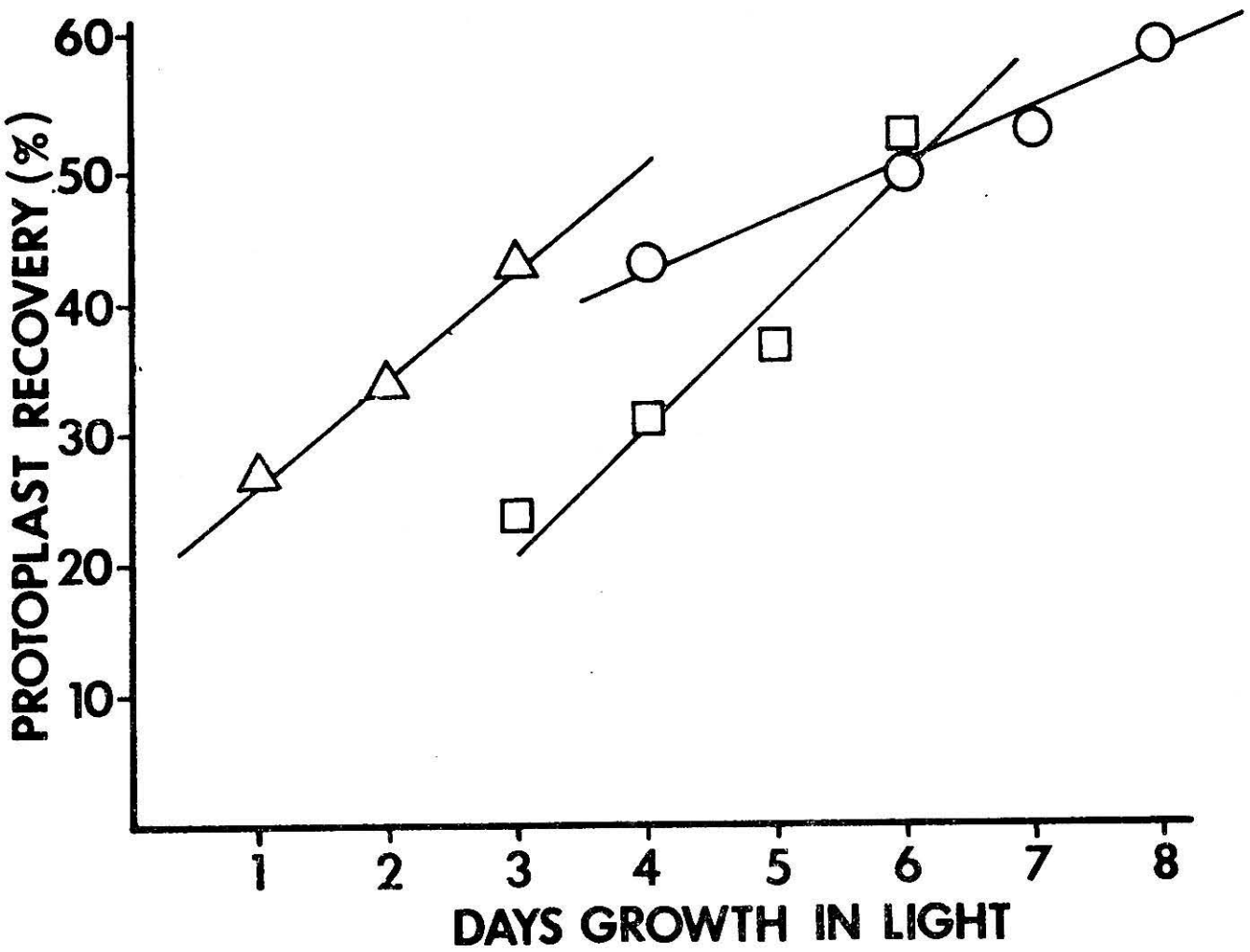

Fig. 10--Effect of illumination conditions on barley protoplast recovery after centrifugation. Etiolated barley plants were exposed to 8 hr light, 16 hr dark cycles. (△) = 4000 lux, 7 day dark grown; (2 identical centrifugation steps); (□) = same as (△) except 6 day dark grown; (○) = 6000 lux, 5 3/4 days dark grown, only one centrifugation.

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CHAPTER III

SCANNING ELECTRON MICROSCOPY OF BARLEY PROTOPLASTS

Summary

Scanning electron micrographs of barley protoplasts were compared using various preparatory techniques. Numerous features were observed which turned out to be artifactual characteristics of the processing procedure used in collecting and dehydrating the samples. The most successful technique gave protoplasts which presumably maintained their natural structural integrity, as judged by retention of sphericity and absence of holes in the plasma membrane. The relative numbers of fragmented protoplasts and cellular organelles was also greatly reduced.

1. Introduction

Protoplasts have been isolated enzymatically from many higher plants. A number of recent review articles (NICKELL and TORREY 1969, HESS 1972, COCKING 1973, HEYN et al. 1974, HOLL et al. 1974, JOHNSON and GRIERSON 1974, MELCHERS and LABIB 1974, MERRIL and STANBRO 1974, SMITH 1974, WIDDUS and AULT 1974) have pointed out the advantages of such protoplasts, e.g., they can be isolated in relatively homogeneous populations, and some can be cultured with high efficiency, or even regenerated into entire plants as in the case of carrot, petunia and tobacco. These advantages make them desirable for plant breeding, genetic and host-pathogen studies: (1) uptake of exogenous DNA, chromosomes, chloroplasts, nuclei, viruses and algae, (2) intra- and interspecies fusions, (3) virus replication and (4) fungal and bacterial effects. For these reasons, the importance of protoplast research for crop development has been widely recognized (see reviews above). We have been studying barley protoplasts in our laboratory because of barley's similarity to important cereal food crops.

Scanning electron microscopy (SEM) has been applied widely to animal cell cultures to demonstrate cell surface features (WESTBROOK et al. 1975, review by KESSEL and SHIH 1974), and more recently to plant cell cultures (HOMES 1974), but there have been no SEM reports of higher plant protoplasts. SEM has enormous potential for adding to our understanding, at the cellular or protoplast level, of important processes of current interest, e.g., (1) protoplast cell wall formation, subsequent division, and successive stages in plant regeneration, (2) mechanism of macromolecule, organelle, virus, or

alga uptake, (3) mechanism of protoplast fusion, and (4) process involved in attack by certain plant pathogens. We have developed a technique for the preparation and subsequent processing of barley protoplasts for SEM which is directly applicable in the above studies. We present results for variations of this technique on observed protoplast surface features, some of which were found to be artifacts of processing, an area for concern as pointed out recently (CLARK and GLAGOV 1976). Although only barley protoplast SEM are given here, viable tobacco protoplasts gave results which were indistinguishable, except tobacco protoplasts were somewhat larger.

2. Materials and Methods

2.1. Barley growth conditions

Barley (Hordeum vulgare var. Bonneville) was planted 2 to 3 cm deep in vermiculite in 10 cm tapered pots, which were connected by four cotton wicks to plastic reservoir jars containing 600 ml of modified Hoagland's nutrient (HUFFAKER et al. 1966) (diluted 1:2). Liquid levels in the reservoirs were maintained with distilled water. Plants were grown 5 3/4 days in darkness and then 7 to 20 days in light (produced by a combination of incandescent and fluorescent lamps, 760 ft-c.) at 25° and approximately 25% relative humidity.

2.2. Protoplast isolation and purification

Barley protoplasts were isolated from leaf slices (0.75 g) (approximately 1 mm^2) incubated (4 hr at room temperature, or 3 hr at 30°) in the protoplast release solution (6.25 ml): 4% cellulase R-10 and 1% macerozyme R-10 (Yakult Biochemicals); 0.01 M potassium

citrate, pH 5.6; and 0.45 M mannitol in modified Murashige and Skoog's (MS) medium (NAGATA and TAKEBE 1971). Protoplasts were purified by repeated dilution into fresh solutions of 0.5 M mannitol in modified MS and sedimentation onto 0.45 M sucrose "pads" (600 x g, 5 min) in an International clinical centrifuge. The maceration enzymes were routinely diluted about 1/50,000 by this procedure. Photomicrographs were taken using a Zeiss RA microscope using attached Nikon equipment.

2.3. Scanning electron microscopy

Protoplasts (about 4 x 10^6/ml) in 0.5 M mannitol were fixed by adding 8% glutaraldehyde dropwise to 2% final concentration and about 0.1 ml aliquots collected 30 min later on silver membrane filters (Flotronics #FM 13, 0.45µ). Protoplasts were collected by one of three techniques: (1) rapid vacuum aspiration with drying of some regions of the protoplast layer, (2) gentle vacuum aspiration with no drying of the protoplast layer, or (3) sedimentation (without aspiration) on the silver filter resting on a Whatman No. 4 filter paper "wick" with care to prevent drying as in (2). Specimens were dehydrated through a graded ethanol series: 10, 30, 50, 70, and 100% (10 min each), unless stated otherwise, rinsed in acetone, and dried by the CO_2 critical point procedure (ANDERSON 1951). Silver filters were mounted on specimen stubs, vacuum coated with carbon and gold-palladium, examined in an Hitachi SSM-2 at 20kV and recorded on Polaroid 55 P/N film. Specimen stubs were examined by SEM the day after processing.

3. Results and Discussion

In order to prepare very fragile barley protoplasts for examination by SEM, a number of precautions were taken. First, fixation was carried out in 0.5 M mannitol. This was shown to be isotonic for the protoplasts, while higher or lower concentrations caused shrinking or swelling, as observed in the light microscope. This was important as osmotic effects produce artifacts in or on cells (HOMES 1974, BRUNK et al. 1975). Second, pre-fixation was carried out before collecting protoplasts on silver filters since surface changes have been observed in cells that were post-fixed (de HARVEN et al. 1975). Third, silver filters were used since they provided optimum electrical grounding and minimized charging artifacts (de HARVEN et al. 1975). Even though all three of the preceding steps were incorporated into our procedure, difficulties were still anticipated since it was known that plant cells cultured in vitro collapsed easily (HOMES 1974). Protoplasts would be expected to be less stable since their cell walls have been removed.

Before SEM examination, protoplasts were routinely observed by light microscopy. Barley protoplasts prepared in mannitol typically had numerous chloroplasts rather uniformly distributed (non-polar protoplasts) beneath the surface of the plasma membrane, Fig. 1. A few less typical protoplasts had asymmetric distributions (polar protoplasts) with chloroplasts being grouped largely on one side. That this difference did not simply represent random protoplast orientation was obvious because of the proportion of each seen with various isolation procedures. Protoplasts isolated in mannitol were

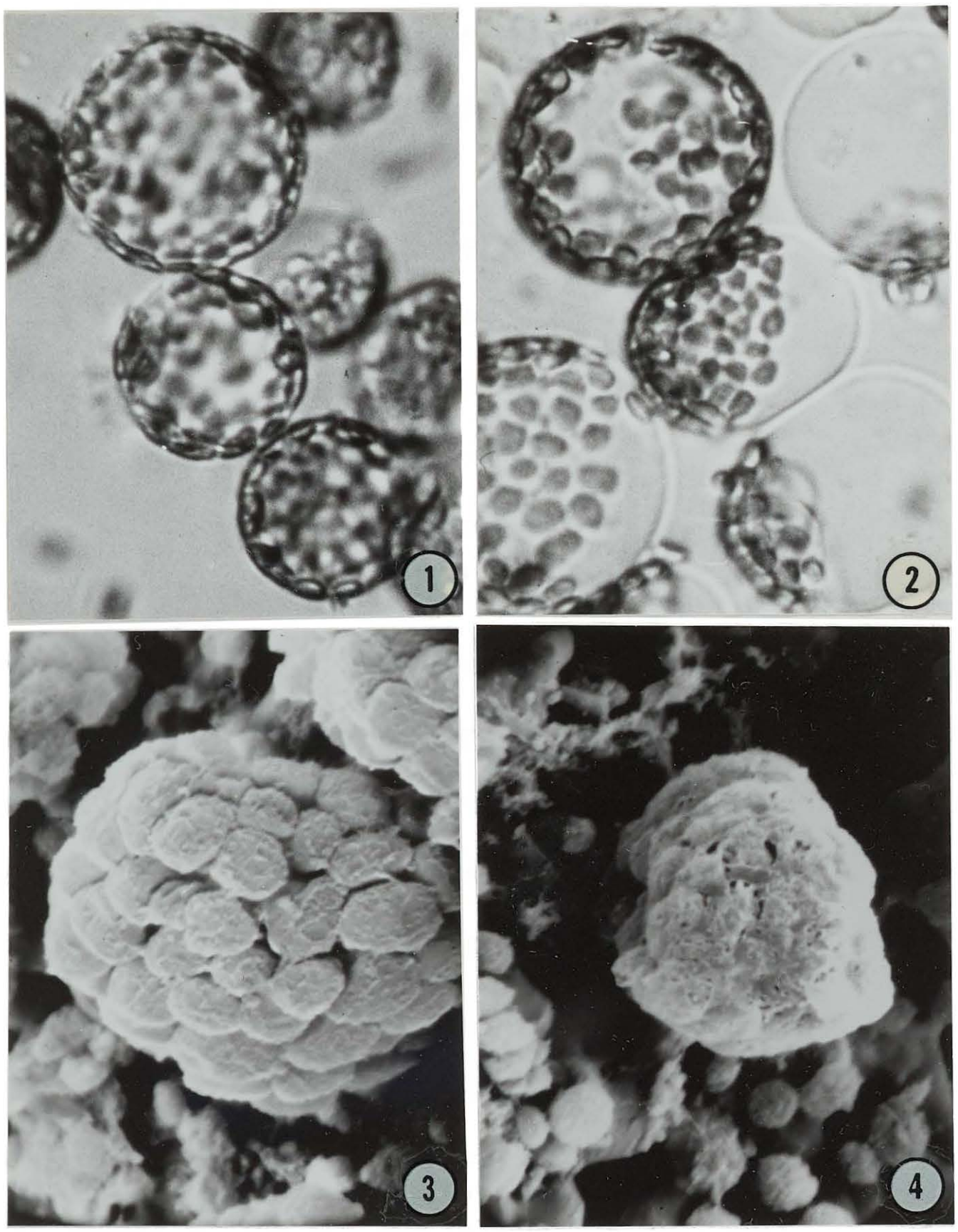


Fig. 1.--A light micrograph showing typical mannitol prepared barley protoplasts with chloroplasts rather uniformly distributed beneath the surface of the plasma membrane. X 1,600

Fig. 2.--A light micrograph showing typical "swollen" sucrose prepared barley protoplasts with chloroplasts largely grouped to one side. X 1,600

Fig. 3.--SEM of a typical barley protoplast collected by rapid aspiration, technique (1), showing the presence of numerous chloroplasts and resultant damage to the plasma membrane. X 2,400

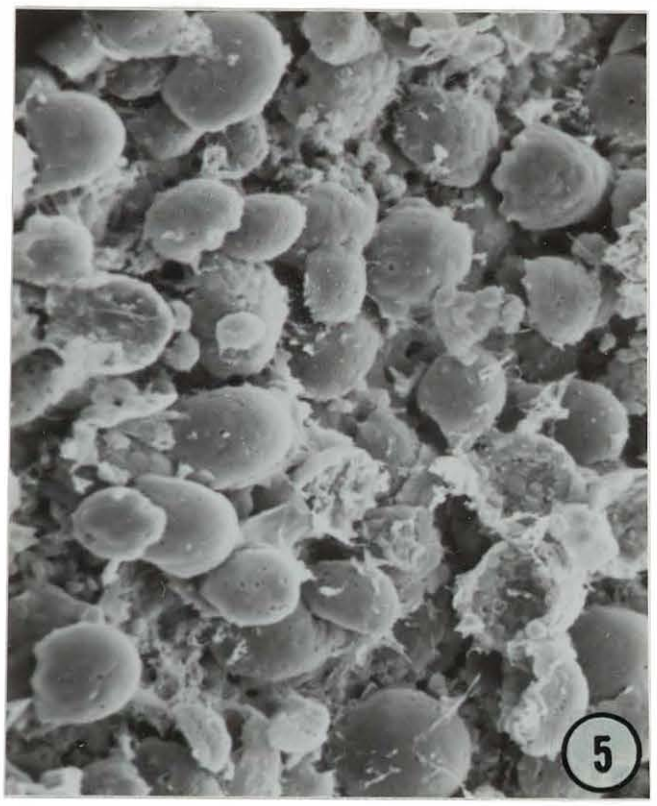
Fig. 4.--SEM of a typical barley protoplast collected as in Fig. 3 showing loss of sphericity and numerous holes. X 2,400

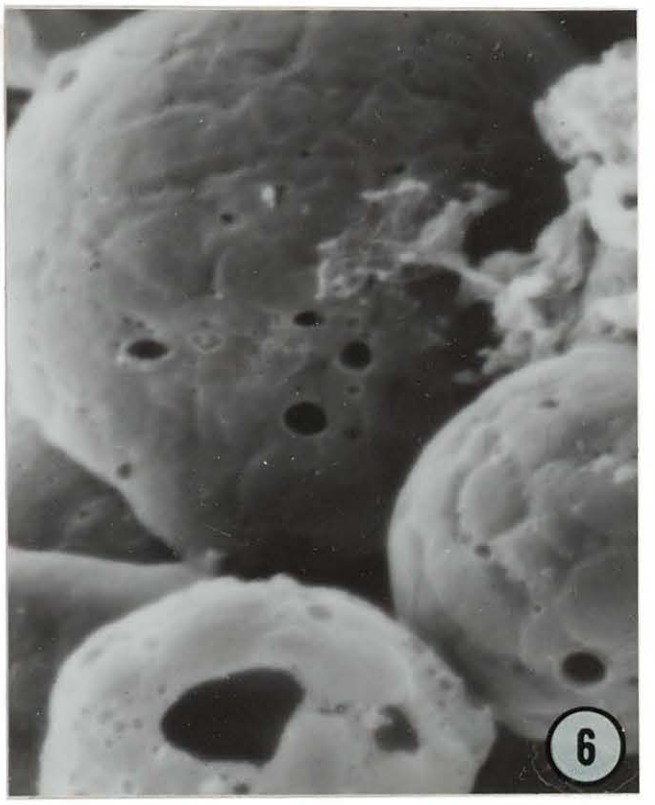
typically about 80% non-polar. In contrast, protoplasts isolated in sucrose were about 20% non-polar, Fig. 2, and were swollen. All SEM observations in this paper were made using mannitol prepared barley protoplasts.

Protoplasts collected by rapid aspiration, technique (1), and examined by SEM showed some resemblance to those seen by light microscopy, i.e., numerous chloroplasts were visible near the protoplast surface, but the plasma membrane in some cases was hardly distinguishable from chloroplast membranes, and appeared to be damaged in "weak" unsupported areas between chloroplasts, Fig.3. In other cases the membrane was clearly visible as were characteristic holes and an obvious loss in protoplast sphericity, Fig.4. That this technique was rather harsh was further evidenced by the large number of fragmented or broken protoplasts, free chloroplasts and cellular debris. These results were not entirely unexpected for these very fragile protoplasts.

Collection by more gentle aspiration with care to prevent air drying of the protoplast layer, technique (2), greatly decreased protoplast damage during the SEM processing procedure. A low magnification view using this technique showed a typical distribution of protoplasts, fragmented protoplasts, chloroplasts, membranes and cellular debris, Fig.5. Although some protoplasts were completely disrupted, others were rather intact but still contained holes, Fig.6.

Replacement of vacuum aspiration by more gentle settling of the protoplasts onto the silver filter using a paper "wick", technique (3), was found to be more satisfactory and gave largely intact protoplasts with only a few small holes, Fig.7. Irregular plasma membrane





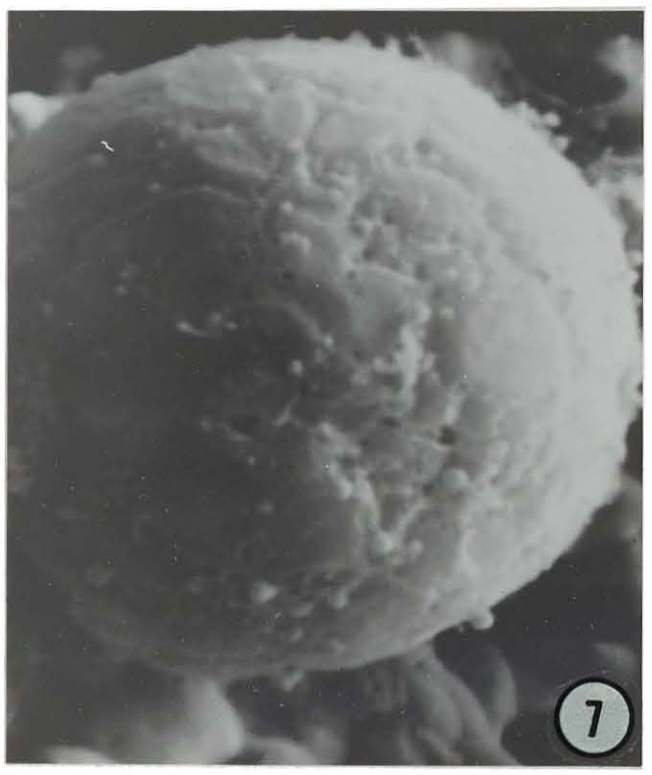


Fig. 5.--Low magnification SEM of barley protoplasts collected by gentle aspiration, technique (2), showing relative intactness of the protoplasts and still having numerous holes. X 480

Fig. 6.--SEM of typical barley protoplasts collected as in Fig. 5. Protoplasts were more intact than those shown in Fig. 3 and 4, but still showed numerous holes. X 2,400

Fig. 7.--SEM of a typical barley protoplast collected without aspiration using a paper "wick", technique (3), but placed directly into 100% ethanol rather than through the graded ethanol series. Irregular plasma membrane surfaces were observed, but protoplasts were generally intact with only a few small holes. X 3,300

Fig. 8.--SEM of a typical barley protoplast collected as in Fig. 7 but dehydrated through the graded ethanol series, showing retention of sphericity, absence of holes and resultant smooth plasma membrane surface. X 3,300

surfaces were characteristic of protoplasts that were dehydrated by direct treatment with 100% ethanol rather than a graded series. Protoplasts prepared by technique (3) and the usual graded ethanol dehydration series had smooth plasma membranes as well as the general absence of holes and retention of sphericity, Fig. 8. Protoplasts of the type shown in Fig. 8 showed deterioration after two months storage on specimen stubs at room temperature (without dessication). Such protoplasts were much more fragmented and had irregular membrane surfaces like those shown in Fig.7. For this reason, our SEM examinations were carried out on the day after processing.

Although barley protoplasts used in this particular study have never been regenerated, on the basis of vital staining data with Evan's blue (KANAI and EDWARDS 1973) and phenosafranine (WIDHOLM 1972), they would appear to be 95% viable. Much more convincing was the fact that micrographs obtained from tobacco protoplasts, which were capable of regeneration by SHEPARD and TOTTEN'S (1975) procedure, were indistinguishable from those shown except that tobacco protoplasts were somewhat larger.

In conclusion, barley (and tobacco) protoplasts were extremely sensitive to aspiration techniques generally used in collecting specimens for SEM. Replacement of aspiration with a filter paper "wick", placed directly under the silver membrane filter, allowed removal of extra-protoplast fixative medium in a reasonable length of time and greatly reduced protoplast damage. Dehydration through a graded ethanol series resulted in protoplasts with smooth plasma membrane surfaces with the general absence of holes.

Acknowledgments

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CHAPTER IV

UPTAKE OF BACTERIAL DNA BY BARLEY AND TOBACCO PROTOPLASTS

Summary

Protoplasts isolated enzymatically from barley and tobacco leaves took up ^{3}H-*B. subtilis* DNA, ^{125}I-*B. subtilis* DNA or ^{125}I-*M. luteus* DNA generally as a linear function of time (0-6 hr) and DNA concentration (0-200 µg/ml). There were no major differences in DNA uptake by barley or tobacco protoplasts using these radiolabeled DNAs. Up to 16 pg of exogenous DNA was taken up, an amount in excess of the endogenous DNA content of the host protoplasts, i.e., about 11 pg/protoplast. Poly-L-lysine and poly-L-ornithine (5 µg/ml) stimulated uptake 5- and 8-fold, respectively, but only at the expense of increased protoplast damage. Protoplasts were viable after uptake as shown by standard staining (for barley protoplasts) and culturing (for tobacco protoplasts) techniques.

DNA which became protoplast associated during uptake reactions was not exchangeable by a 10-fold excess of unlabeled DNA and was not released by DNase treatments. Nuclei, isolated after DNA uptake, contained 60-80% of the protoplast associated exogenous DNA. This nuclear associated DNA was not reduced by addition of large excesses of unlabeled DNA during the Triton X-100 isolation procedure.

Contaminating DNase activity was found in the commercial enzymes used to isolate protoplasts. Free protoplasts, extra-protoplast medium and especially ruptured protoplasts were shown to cause DNA depolymerization. In spite of these complications, about 20% of the exogenous DNA taken up by protoplasts after typical 4 hr uptake reactions was of average genome size (5-10 x 10^5 daltons), and therefore of potential significance to host gene expression.

Introduction

There is overwhelming evidence that higher plants take up foreign DNA, but the fate of such DNA is still a controversial issue (see recent reviews: Hess, 1972; Chaleff and Carlson, 1974; Heyn et al., 1974; Holl et al., 1974; Johnson and Grierson, 1974; Merril and Stanbro, 1974). The importance of such research for crop improvement has been widely recognized (Nickell and Torrey, 1969; Cocking, 1973; Melchers and Labib, 1973; Smith, 1974). Some of the genetic alterations suggested as future possibilities are: (1) the transfer of genes to confer disease, pest, or herbicide resistance and (2) the incorporation of genes for nitrogen fixation or improved nutritional quality. In addition, the study of gene expression in such nucleic acid uptake studies should add to our understanding of the processes of differentiation, morphogenesis and certain pathological conditions, i.e., tumor induction and virus infection.

Uptake of exogenous DNA has been reported for whole plants (Stroun, 1967; Ledoux and Huart, 1969; Ledoux et al., 1971; Ledoux and Huart, 1971a and b; Rebel, 1973; Ledoux and Huart, 1974; Ledoux, 1975), pollen grains (Hess, 1975), cells in culture (Bendich and Filner, 1971; Heyn and Schilperoort, 1973; Lurquin and Hotta, 1975; Owens, 1975), and plant cell protoplasts from higher plants (Ohyama et al., 1972; Hess et al., 1973;; Hoffman, 1973; Hoffmann and Hess, 1973; Ohyama et al.,1973; Gleba et al., 1974; Uchimiya and Murashige, 1975) as well as eukaryotic algae (Lurquin and Behki, 1975). The fate of the exogenous DNA after uptake has not been unequivocally characterized in any of these systems. Although integration and subsequent replication of exogenous DNA after

covalent attachment to host DNA have been reported by Ledoux and his colleagues (Stroun et al., 1967; Ledoux and Huart, 1969; Ledoux and Huart, 1971b) in experiments with whole plants, other workers have been unable to repeat various aspects of the work (Bendich and Filner, 1971; Hotta and Stern, 1971; Kleinhofs, 1975; Lurquin and Hotta, 1975). Contaminating bacterial DNA and techniques of DNA preparation have been suggested as alternative interpretations for the data (Kleinhofs et al., 1975).

DNA-mediated alterations in plants using either naked DNA or transducing bacteriophages carrying bacterial genes have been attempted, but progress has been limited by the unavailability of well-defined auxotrophic mutants. There are two reports for DNA-mediated alterations using naked DNA in whole plants: (1) correction of thiamine auxotrophs in Arabidopsis thaliana by bacterial DNA or calf thymus DNA (Ledoux and Huart, 1971b; Ledoux and Huart, 1974) and (2) transfer of the genes controlling anthocyanin biosynthesis and leaf shape in Petunia hybrida (Hess, 1972). These alterations have been transmitted through several generations and would imply a stable genetic addition to the recipient hosts. If such alterations can be unequivocally shown to be a consequence of the introduction of new DNA to host genetic material, then it seems that genetic transformation of plants will become a useful tool in modern agriculture. DNA-mediated alterations have also been reported using transducing bacteriophages, carrying specific bacterial genes, in cell cultures of sycamore (Johnson et al., 1973), tomato and A. thaliana (Doy et al., 1973). These cell cultures were able to grow on lactose or galactose as the sole carbon source only after bacteriophage uptake. However, the

interpretation of the results has been challenged by one of the principal investigators and confirmation awaits further investigation (Smith et al., 1975).

It has been difficult to determine the ultimate fate of DNA in the above whole plant DNA uptake experiments because of the complexity of the systems and the inconclusive results obtained, so we have chosen more simple, homogeneous protoplast systems for investigation. We have prepared protoplasts from two plants, i.e., barley and tobacco. The first is useful in defining DNA uptake because of its similarities to important cereal food crops. The second is useful because of the known ability of its protoplasts to regenerate cells and divide in culture (Shepard and Totten, 1975), a crucial test for determining protoplast viability after uptake. Aseptic protoplasts were obtained from both plants, thereby, eliminating the bacterial contamination known to complicate interpretation of results in whole plant studies (Kleinhofs et al., 1975). Two methods of barley protoplast preparation were used that gave protoplasts different in size and chloroplast distribution.

Two bacterial DNAs, Bacillus subtilis and Micrococcus luteus, were used in uptake experiments rather than more complex genetic containing materials such as, chromosomes, nuclei, chloroplasts, intact bacteria, or bacteriophages. These DNAs were convenient because of their very different guanine plus cytosine contents (Normore and Brown, 1970), and because of the biological activity of B. subtilis DNA, i.e., transforming activity (Bott and Wilson, 1967). B. subtilis DNA was labeled with either tritium or 125-iodine so that differences in relative uptakes could be compared. Such bacterial DNAs, with their

more simple structures and biochemical compositions, could readily be purified and their physical and biological properties determined before and after uptake. We have carried out such analyses and determined the efficiency, location, integrity and other fundamental characteristics of DNA uptake. Such information will be indispensable as suitable genetic markers become available and as selective systems allowing unmistakable recognition of biological expression of exogenous genetic information are defined.

Material and Methods

Plant growth conditions

Barley (Hordeum vulgare var. Bonneville) was planted 2 cm deep in vermiculite in 10 cm tapered pots connected by four cotton wicks to plastic reservoir jars containing 600 ml of modified Hoagland's nutrient (Huffaker et al., 1966) (diluted 1:2). Liquid level in the reservoir was maintained with distilled water as needed. Plants were grown for 5 3/4 days in darkness and then for 7 to 20 days in light (8 hr light per day, produced by a combination of incandescent and fluorescent lamps, 760 ft-c.) at 25° and approximately 20% relative humidity.

Tobacco (Nicotiana tabacum L. cv. Xanthi-nc) was grown according to Shepard and Totten (1975) except that a combination of incandescent and fluorescent lamps (580 ft-c.) was used. The temperature was maintained between 25 and 27°, and the plants were watered daily with Ortho 16-16-16 fertilizer (Chevron) (1.0 g/l).

Protoplast isolation and purification

Barley protoplasts were isolated from leaf slices (approximately 1 mm^2) incubated (4 hr at room temperature, or 3 hr at 30°) in the protoplast release solution (0.75 g leaf/6.25 ml): 4% cellulase R-10 and 1% macerozyme R-10 (Yakult Biochemicals); 0.01 M potassium citrate, pH 5.6; and either 0.3 M sucrose or 0.45 M mannitol in modified Murashige and Skogg's (MS) medium (Nagata and Takebe, 1971). The mannitol prepared protoplasts were purified by repeated dilution into fresh solutions of 0.5 M mannitol in MS and sedimentation onto 0.45 M sucrose "pads" (600 x g, 5 min) in an International clinical centrifuge. The maceration enzymes were routinely diluted about 1/50,000 by this procedure.

The sucrose prepared protoplasts were purified by centrifugation (400 x g, 10 min in an International IEC centrifuge) in sucrose (0.5 M, 100 ml) overlayered with mannitol (0.5 M, 1.0 ml) both containing MS nutrient using 100 ml Babcock bottles. Protoplasts were collected from the interface after each centrifugation step. Four such centrifugations were carried out so that the maceration enzymes were diluted approximately 1/200,000.

Tobacco protoplasts were isolated according to Shepard and Totten (1975) with a few modifications. Leaves were used when they were 20 to 24 cm in length; 5 g of leaf tissue was incubated in 100 ml of enzyme solution in 0.3 M sucrose for 8 hr without any shaking. The protoplasts obtained were purified as described (Shepard and Totten, 1975).

The number of the purified protoplasts was determined in a hemacytometer (average count of six different aliquots).

Photomicrographs were taken using a Zeiss RA microscope with attached Nikon equipment.

Preparation of bacterial ^{3}H-DNA and ^{125}I-DNA

Bacillus subtilis (strain 23) DNA was isolated by the method of Marmur (1961) omitting the isopropanol precipitation, and Micrococcus luteus DNA was obtained from Miles Laboratories.

^{3}H-B. subtilis DNA (5.0×10^{5} dpm/µg) was prepared according to M. D. Chilton's (personal communication) modification of Schachat and Hogness's (1973) nick translation labeling method using Worthington deoxyribonuclease (DNase I) (E.C. 3.1.4.5) and ^{3}H-TTP (New England Nuclear) except that the ^{3}H-B. subtilis DNA was not denatured. ^{125}I-B. subtilis DNA and ^{125}I-M. luteus DNA were prepared by the method of Commerford (1971) or by the minimal damage iodination conditions of Orosz and Wetmur (1974) using the following concentrations: 250 µg/ml native DNA, 0.1 M sodium acetate plus 0.04 M acetic acid buffer (pH 5.0), 1.2×10^{-4} M $TlCl_3$ (freshly prepared and adjusted to pH 5.0 with HCl), 2.0×10^{-5} M KI, and 100 µCi/ml carrier free $Na^{125}I$. The $Na^{125}I$ (Industrial Nuclear, Co.) was used within 7 days from the date of preparation. Reactions were carried out at 60° for 1 hr, and the final dialysis was in 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate). The specific activity of the preparations ranged from 0.2-2.2 $\times 10^{5}$ cpm/µg. The transforming activity of the B. subtilis DNA was not significantly affected after the iodination reaction when assayed according to Bott and Wilson (1967). The ^{125}I-B. subtilis DNA was sterilized (Marmur, 1961) for the transformation assay as well as for the culturing of tobacco protoplasts in DNA uptake experiments.

Density gradient centrifugation of labeled bacterial DNAs

Gradients were formed in a Beckman 50 Ti rotor in 1.25 g/ml CsCl solution ($n_{25°}$ = 1.400) containing ^{3}H-*B. subtilis* DNA (0.4 µg) or 125 I-*M. luteus* DNA (75 µg) by centrifuging at 30,000 rpm for 63 hr in 0.1 x SSC at 25° in a Beckman Model L3-40 ultracentrifuge. Unlabeled DNAs (60 µg) of known density were added for reference. After centrifugation, 0.1 ml fractions were collected by piercing the tube bottom, and CsCl density determined by refractometry (Bausch and Lomb, 25°) (Ifft et al., 1961). The relative positions of DNA peaks were determined by UV absorption and radioactivity. Fractions were diluted with 0.2 ml water and absorbance at 260 nm read in a Gilford recording spectrophotometer. Fractions were then counted in a well-type scintillation counter (Nuclear Chicago Autogamma) for ^{125}I or in 10 ml Aquasol or Biofluor (New England Nuclear) in a liquid scintillation counter (Packard Tricarb) for ^{3}H.

Protoplast uptake of labeled bacterial DNAs

The uptake reaction mixture consisted of washed protoplasts (0.63 - 9.4 x 10^{6}/ml) and one of the following DNAs: ^{125}I-*M. luteus* DNA, ^{125}I-*B. subtilis* DNA, or ^{3}H-*B. subtilis* DNA (0 - 200 µg/ml). Uptake was carried out in 10 x 35 mm plastic petri dishes (or if volumes exceeded 3 ml, in 20 x 60 mm plastic petri dishes) wrapped in parafilm and incubated at 27° (unless stated otherwise) in the dark for up to 6 hr. Polycations, whenever present, were poly-L-lysine HBr (Pilot Chemicals, Inc., MW 120,000) or poly-L-α-ornithine HBr (Sigma, MW 120,000).

After uptake, protoplasts were washed by one of two procedures in 12 ml conical centrifuge tubes. The first was that used for mannitol prepared barley protoplasts and was identical to the procedure described in the "Protoplast isolation and purification" section, except that it was repeated four times to efficiently remove radioactive DNA not associated with protoplasts. Approximately 60% of the protoplasts were recovered with only about 5% fragments present in the final protoplast band. Protoplast fragments were protoplasts with partially disrupted plasmalemmae, so they were no longer spherical but rather appeared upon microscopic examination to be chloroplast clumps. DNA uptake was normalized to pg DNA/protoplast on the basis of radioactivity associated with the protoplasts and hemacytometer counts. ^{125}I-DNA associated with protoplasts was determined in the well-type counter or ^{3}H-DNA associated with protoplasts was determined by adding sodium dodecyl sulfate to 1% to lyse the protoplasts and counting in 10 ml Biofluor in the liquid scintillation counter. The second washing procedure was that used for either sucrose prepared tobacco or barley protoplasts. Such protoplasts were washed using the following steps: (a) sedimentation (600 x g, 5 min) through a fresh solution of 0.5 M mannitol onto a 0.5 M sucrose "pad" and collection of the protoplast band from the interface, (b) transfer of the band to a new tube and addition of sucrose (0.8 M) to 0.53 M, and then, (c) floatation (600 x g, 5 min) of the protoplasts from the 0.53 M sucrose band through an overlayer of fresh 0.4 M sucrose to the interface formed with 1.0 ml of 0.5 M mannitol. The sucrose and mannitol solutions were prepared in modified MS (Nagata and Takebe, 1971) nutrient for washing the barley protoplasts and in Shepard and Totten's (1975) nutrient for washing the tobacco protoplasts. This

washing procedure for barley and tobacco protoplasts gave a final recovery of approximately 30% with about 10% fragments. Uptake was normalized to pg DNA/protoplast as described for the first washing procedure.

The viability of the protoplasts before and after the DNA uptake was determined by standard staining techniques for the barley protoplasts and by Shepard and Totten's (1975) culturing procedure for the tobacco protoplasts. Barley protoplasts were stained with either Evan's blue (Kanai and Edwards, 1973) (J. T. Baker) or phenosafranine (Widholm, 1972) (BDH Chemicals) solution prepared in 0.5 M mannitol in MS. Tobacco protoplasts were plated in Medium II (Shepard and Totten, 1975) at 2.0×10^4/ml and resultant colonies counted after 2 weeks of culture. For the conditions given, approximately 35% of the protoplasts formed colonies. Photomicrographs of tobacco colonies were taken as for the protoplasts.

Characterization of the bacterial DNA uptake

In order to better define the characteristics of DNA uptake, i.e., protoplast associated radioactivity after a brief incubation period of labeled ^{125}I-DNA or ^{3}H-DNA with protoplasts, the following experiments were carried out: (1) protoplasts were washed with an excess of unlabeled DNA or treated with DNase after various times of uptake, (2) nuclei were isolated from protoplasts after DNA uptake reactions, (3) DNA depolymerization before and after uptake was determined on Sepharose 4B columns and (4) protoplast release solutions (cellulase and macerozyme) and protoplasts were assayed for nuclease activity. Further details of these experiments are described below:

(1) A 10-fold excess of unlabeled DNA was added, after DNA uptake, to the first in the series of four washes of mannitol prepared barley protoplasts. The control (minus unlabeled DNA) contained an equal volume of 0.1 x SSC in the first of the four washes.

Sucrose prepared barley protoplasts were washed using the first step of the washing procedure (see "Protoplast uptake of labeled bacterial DNAs"), treated with either DNase I or buffer (control), and then washed by the standard procedure. DNase treatments were carried out under conditions which caused major depolymerization of ^{125}I-*M. luteus* DNA as determined by gel filtration (P-60 columns): 0.5 µg ^{125}I-*M. luteus* DNA, 3 mmoles $MgCl_2$, and 224 Kunitz units of DNase I (100 µg) per a one-ml reaction mixture of 0.5 M sucrose in MS. One ml samples were applied to P-60 columns (1 x 33 cm) and eluted with 0.1 M ammonium acetate at 17 ml/hr. The peak positions for ^{125}I-DNA or fragments were determined by UV absorption and radioactivity measurements for succeeding fractions (0.9 ml).

(2) Nuclei were isolated from protoplasts after the usual uptake and washing procedures, and the pg DNA/nucleus determined as for the protoplasts. Nuclei were isolated from tobacco protoplasts by the method of Mascarenhas et al. (1974) with a recovery of 35% of the nuclei. Nuclei were isolated from mannitol prepared barley protoplasts by treatment with 1% Triton X-100 (Beckman) in MS for 5 min at 22°, centrifugation (600 x g, 10 min, 2°) in a Sorvall SS-34 rotor in the Sorvall RC2-B centrifuge and subsequently resuspended in MS. The final recovery of the nuclei was 95%. Nuclei were stained with acetoorcein (Motoyoshi, 1971) for visualization in hemacytometer counts.

Nuclei were processed for thin sectioning according to a modification of Gardner et al. (1975). Nuclei were embedded in 3% H_2O-agar after fixation (10 hr) and then treated with osmium as described.

(3) The depolymerization of labeled DNA (a) by mixing with protoplasts, (b) by protoplasts after a 4 hr uptake, and (c) by extra-protoplast medium after a 4 hr uptake was determined on Sepharose 4B (Pharmacia) columns according to Lurquin and Behki (1975). Sepharose 4B columns (0.6 x 21 cm) were eluted with 2 M NaCl at 1.0 ml/hr. Location of DNA peaks was determined by collecting fractions (0.18 ml), diluting with water (0.5 ml), and reading their absorbance at 260 nm in a Beckman DB-G spectrophotometer. Fractions were then counted in 10 ml Aquasol in the liquid scintillation counter.

(4) The nuclease activity of (a) the protoplast release solution (minus protoplasts) and of (b) the protoplasts themselves were assayed using a standard B. subtilis transformation assay. (a) The protoplast release solution (minus leaf slices) was diluted to 1/60, 1/600, and 1/6,000 with a 2:1 mixture of 0.5 M mannitol in MS:0.45 M sucrose in MS, which is the proportion of mannitol:sucrose obtained for a typical purified protoplast suspension. Aliquots of the above dilutions were mixed with B. subtilis DNA (wild type) and assayed for ability to transform B. subtilis strain 169 trp_2 to tryptophan independence by the method of Bott and Wilson (1967) omitting the DNase I treatment. (b) Aliquots of protoplasts washed by the standard technique, and water ruptured protoplast pellets (pellets from washed protoplasts centrifuged at 600 x g, 5 min in an International clinical centrifuge) were incubated with B. subtilis DNA (wild type), filtered

consecutively through Whatman #2 and HA millipore (0.45 μ) filters with care to prevent drying, and assayed for transforming activity as described in (a) above. Extra-protoplast medium was obtained by filtering washed protoplast suspensions through Whatman #2 filter paper without any drying and, therefore, with minimal protoplast breakage as shown by microscopic examination. This medium was incubated with B. subtilis DNA, filtered through an HA millipore filter, and assayed for transforming activity as in (a).

Results

Characteristics of DNA uptake

Conditions for bacterial DNA uptake have been defined in terms of the effects of time, DNA concentration, temperature and polycations on the absolute amount of DNA associated with protoplasts, and the effects of such exogenous DNA on protoplast viability.

Time and DNA concentration effects on uptake. The kinetics of DNA uptake were carried out using tobacco and barley protoplasts prepared in sucrose and mannitol, respectively. Such protoplasts were incubated with either ^{125}I-B. subtilis DNA or ^{3}H-B. subtilis DNA for various periods of time and subsequently washed to remove unassociated DNA. Uptake was defined as amount of DNA remaining protoplast associated divided by the number of protoplasts recovered after the washing procedure, i.e., pg/protoplast, Fig. 1. DNA uptake was linear for barley and tobacco protoplasts. These results were typical of those obtained from many experiments. Occasionally, however, uptake proceeded only after a lag period, as will be seen later. Differences observed between tobacco and barley protoplasts or the two radiolabeled

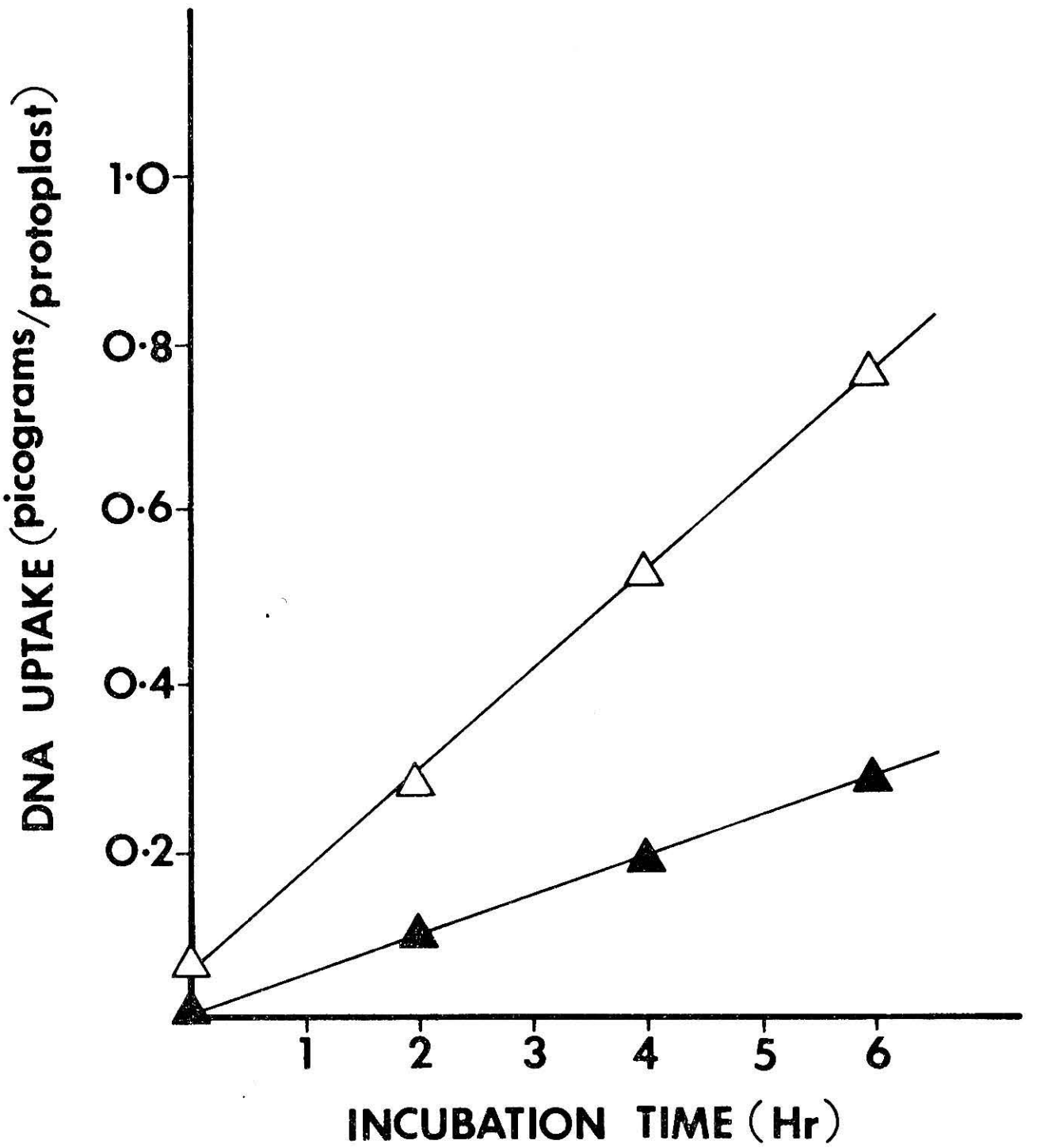

Fig. 1.--Kinetics of ^{3}H-DNA and ^{125}I-DNA uptake by barley and tobacco protoplasts. ^{125}I-B. subtilis DNA (9.0 µg/ml) was incubated with sucrose prepared tobacco protoplasts (1.5 x 10^{6}/ml) (▲), and ^{3}H-B. subtilis DNA (7.2 µg/ml) was incubated with mannitol prepared barley protoplasts (3.0 x 10^{6}/ml) (△). At indicated times aliquots were removed, protoplasts washed, counted in a hemacytometer, and their uptake determined on the basis of associated radioactivity.

DNAs were not considered to be significant since they were within the range obtained from one protoplast and DNA preparation to another. Higher protoplast concentrations generally gave greater uptake, but there was no obvious explanation for this phenomenon. Linear uptake kinetics have also been demonstrated in ammi protoplasts (Ohyama et al., 1972) and Chlamydomonos reinhardi cells (WT) (Lurquin and Behki, 1975). However, the absolute amount of DNA taken up was considerably less than we have found for tobacco and barley protoplasts. The washing procedure, used to remove DNA not associated with protoplasts after uptake, may account for this difference since it discriminated against fragmented protoplasts which sedimented through the sucrose "pad". This is a crucial point in light of the fact that nuclei isolated from Triton X-100 treated protoplasts very rapidly bind DNA (unpublished data). Although protoplasts may be stable when incubated in nutrient medium, they may not be stable during DNA uptake and subsequent washing procedures. As a consequence, one might be measuring DNA uptake by fragmented rather than intact protoplasts. It is not apparent from previous reports whether this has been taken into account (Ohyama et al., 1972; Lurquin and Behki, 1975). Furthermore, even if protoplast fragments are removed, uptake data must be normalized to pg/protoplast to correct for nonquantitative recoveries during all uptake and washing procedures.

The effect of increasing DNA concentrations on 4 hr uptake reactions was determined for barley and tobacco protoplasts. In these experiments, protoplast concentration and relative amounts of ^{125}I-B. subtilis DNA and unlabeled B. subtilis DNA were also varied, Fig. 2.

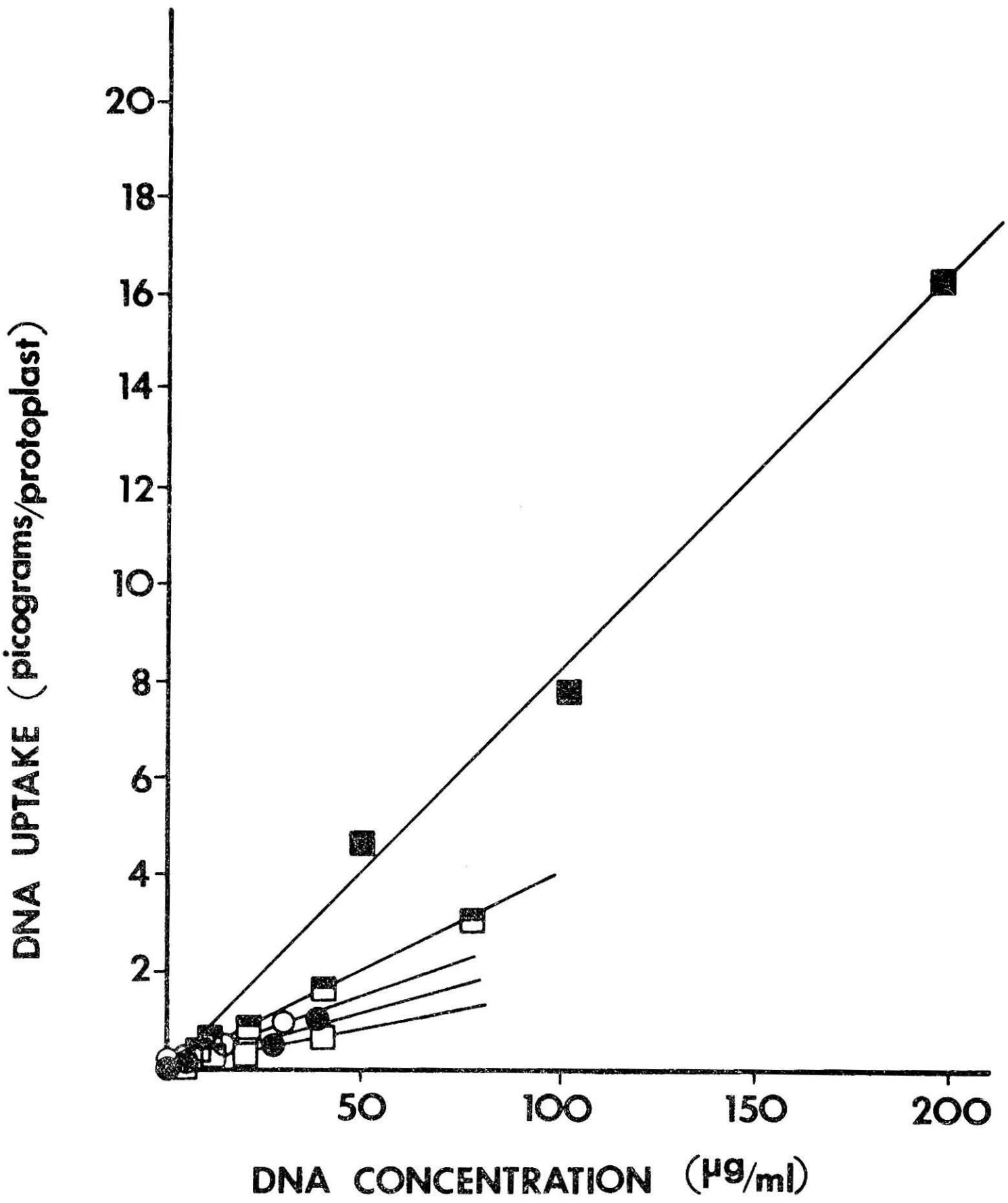

Fig. 2.--Effect of DNA concentration on uptake (4 hr) by barley and tobacco protoplasts. Variables in the uptake reactions include protoplast concentration and relative amounts of labeled and unlabeled B. subtilis DNA. Sucrose prepared tobacco protoplasts at 1.2 x 10^6/ml (●) or 1.5 x 10^6/ml (○) were incubated with ^{125}I-B. subtilis DNA at indicated concentrations. Sucrose prepared barley protoplasts at 1.3 x 10^6/ml were incubated with ^{125}I-B. subtilis DNA (□) or a mixture of unlabeled B. subtilis DNA and ^{125}I-B. subtilis DNA (1.2 µg/ml) (◨), or 2.6 x 10^6 protoplasts/ml with unlabeled B. subtilis DNA and ^{125}I-B. subtilis DNA (8.7 µg/ml) (■). DNA uptake was determined as in Fig. 1.

Uptake was seen to be a linear function of the DNA concentration at least up to 200 µg/ml. The highest concentrations resulted in an amount of exogenous DNA uptake (16 pg/protoplast) comparable with the endogenous DNA content of the cells (11 pg/protoplast) (Heyn and Schilperoort, 1973; Sciaky, 1973). Differences observed between barley and tobacco, and among various ratios of labeled and unlabeled DNAs do not follow a consistent pattern and are probably not significant. However, larger concentrations of protoplasts favor higher uptake (compare open and closed squares) as mentioned above.

These results are different from those of Ohyama et al. (1972) in which uptake was linear only up to 10 µg/ml and where the extent of ammi protoplast uptake (0.015 pg/protoplast assuming a 100% protoplast recovery) was much less than that obtained for barley and tobacco. They did observe an 8-fold difference among ammi, carrot and soybean protoplasts, but this is within the variation observed in Fig. 2.

Differences in methods of protoplast preparation may contribute to differences observed in DNA uptake. Barley protoplasts prepared in mannitol were morphologically very different from those prepared in sucrose both with respect to size and chloroplast distribution. Mannitol prepared protoplasts were generally smaller with a rather uniform (non-polar) distribution of the chloroplasts, while sucrose prepared protoplasts were swollen, for the most part, and had an uneven (polar) chloroplast distribution, Fig. 3a and 3b, respectively. Since no consistent difference in DNA uptake was observed, the importance of sucrose and mannitol in this regard remains obscure.

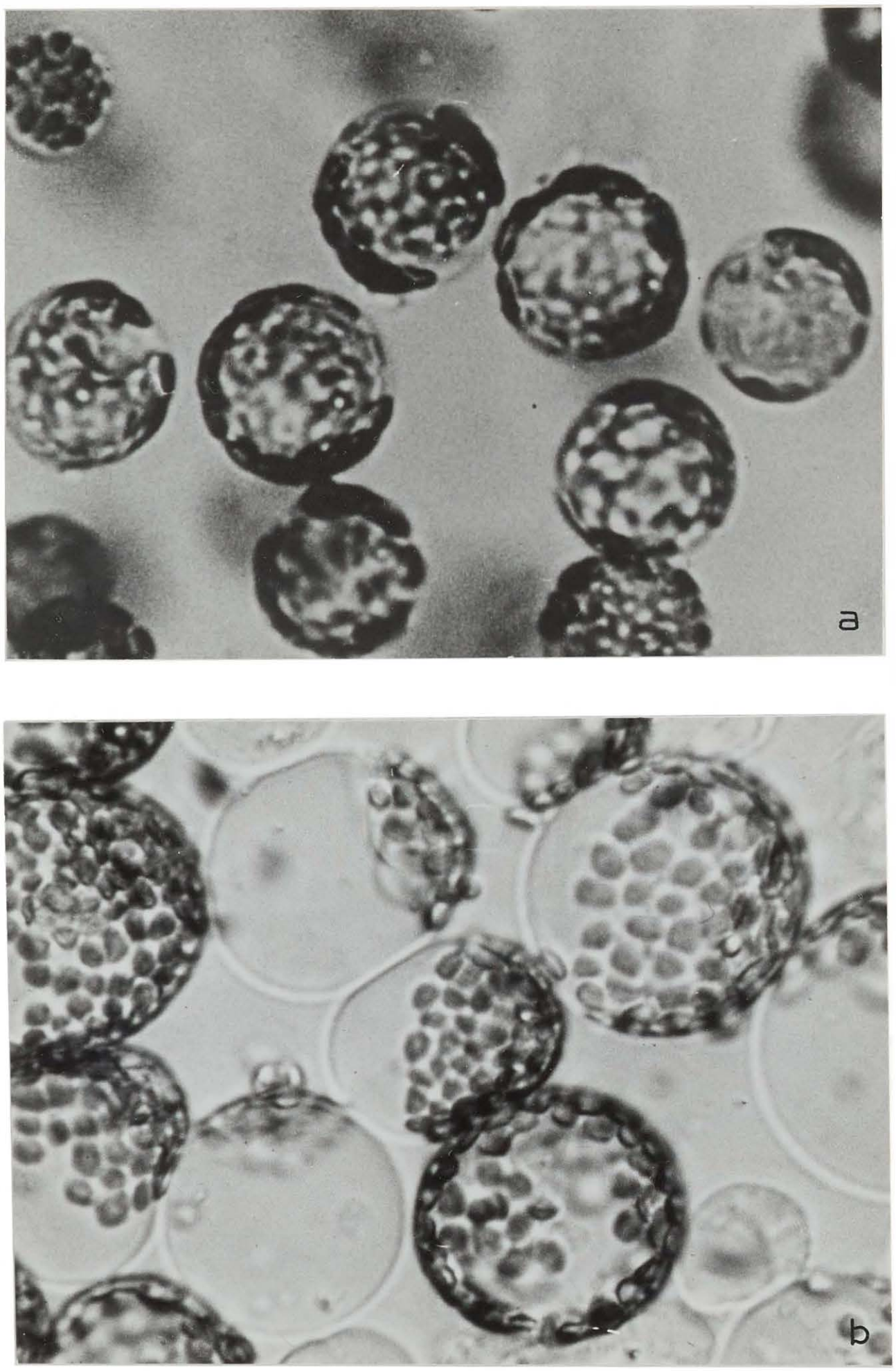

Fig. 3.--(a) Mannitol prepared barley protoplasts. X 1,500. (b) Sucrose prepared barley protoplasts. X 1,500.

Temperature and polycation effects on uptake. In order to demonstrate the effects of temperature on DNA uptake, barley protoplasts were incubated at 3° and 27° with ^{125}I-DNA for various periods of time and subsequently washed as described previously, Fig. 4. Linear uptake kinetics were observed at both temperatures although that observed at 3° was significantly lower. These results are consistent with those of Ohyama et al. (1972) who found a four-fold difference in uptake at 0° and 28° for ammi protoplasts, and Uchimiya and Murashige (1975) who found low uptake at 0° for tobacco protoplasts and suggested that uptake is an energy requiring process.

Polycations such as, DEAE-dextran, poly-L-lysine and poly-L-ornithine have been used to stimulate DNA uptake in plant cells and protoplasts (Ohyama et al., 1972; Hoffmann, 1973; Gleba et al., 1974; Lurquin and Behki, 1975; Uchiyama and Murashige, 1975). The mechanism for this stimulation has not been clearly defined, but it has been reported that polycations cause aggregation of ammi protoplasts (Ohyama et al., 1972) and C. reinhardi cells (Lurquin and Behki, 1975), protect DNA from nuclease action (Hoffmann, 1973; Holl et al., 1974), reduce DNA induction of DNase activity (Heyn and Schilperoort, 1973) and reduce re-utilization of donor DNA after uptake in tobacco cells (Heyn and Schilperoort, 1973). Most of these properties could be exploited in uptake experiments, were it not for the fact that at least one of these polycations, DEAE-dextran, is toxic to protoplasts. Membrane disruption and protoplast destruction have been observed in ammi (Ohyama et al., 1973) and in tobacco (Gleba et al., 1974), respectively, with DEAE-dextran concentrations of 5 µg/ml or more. Hoffmann (1973) and Holl (1974) also stated that protoplasts were

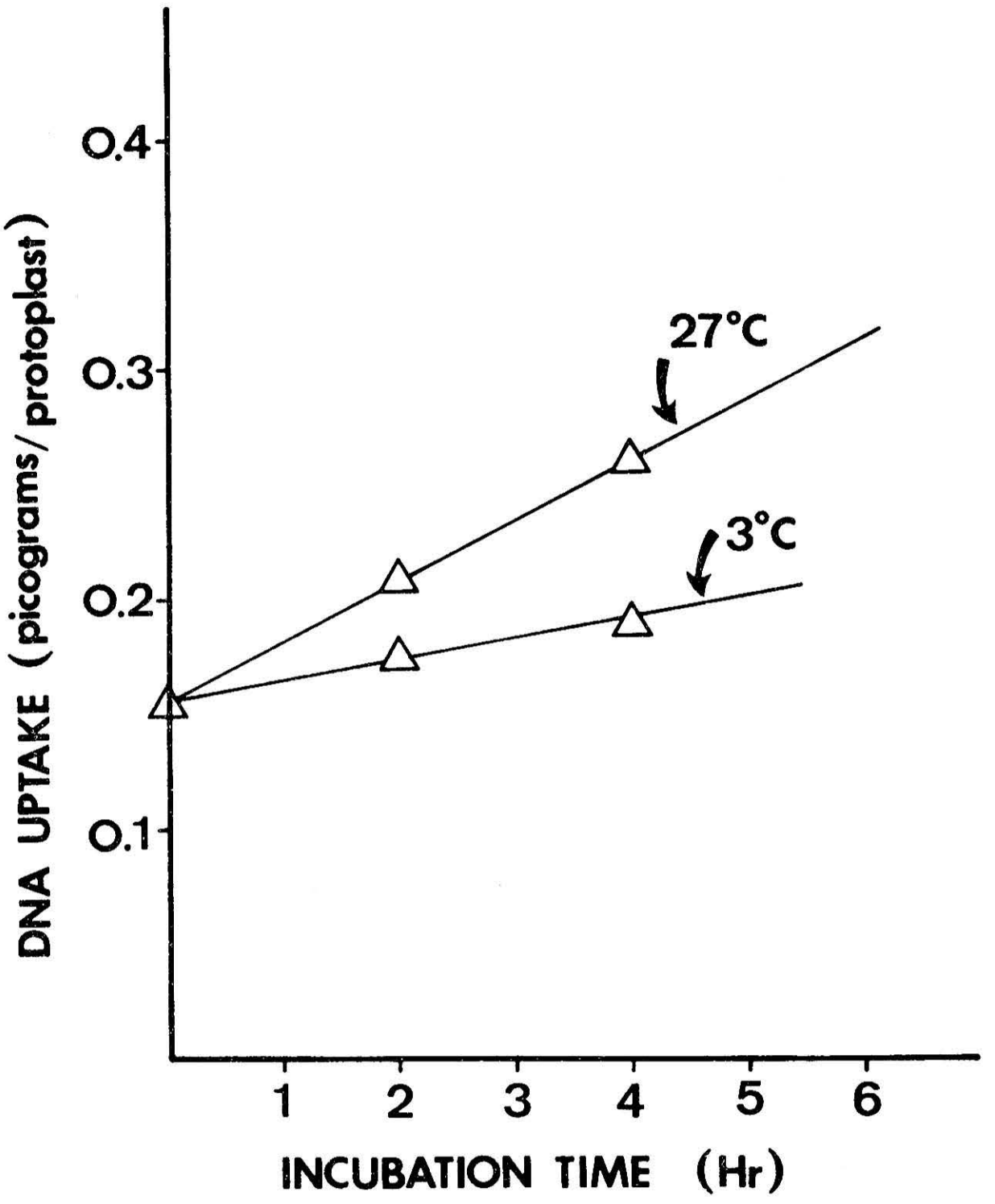

Fig. 4.--Effect of temperature on ^{125}I-DNA uptake by barley protoplasts. Mannitol prepared protoplasts (3.5 x 10^6/ml) were incubated with ^{125}I-M. luteus DNA (7.5 µg/ml) for the indicated times and temperatures, and DNA uptake determined as in Fig. 1.

destroyed by the levels of DEAE-dextran required to protect exogenous DNA from DNase I. For these reasons, only poly-L-lysine and poly-L-ornithine were used in our uptake studies. Such studies were carried out by incubating barley protoplasts for 4 hr with ^{125}I-DNA in the presence and absence of polycations. Protoplasts were found to aggregate upon addition of the polycations at a concentration of 5 µg/ml. At the end of the incubation period, protoplasts were washed and counted. The percentage of fragmented protoplasts was also determined. The data is represented in terms of DNA uptake and protoplast fragmentation for the control (minus polycations), poly-L-lysine and poly-L-ornithine, Fig. 5. About a 5-fold and 8-fold stimulation in uptake occurred for poly-L-lysine and poly-L-ornithine, respectively. Increased fragmentation was also observed in the presence of these polycations, e.g., up to twice as many protoplast fragments for poly-L-ornithine.

Stimulation of DNA uptake was in the range of that reported for ammi protoplasts (6 to 7-fold) (Ohyama et al., 1972), C. reinhardi cells (10 to 15-fold) (Lurquin and Behki, 1975) and tobacco protoplasts (2-fold) (Uchimiya and Murashige, 1975). The number of protoplast fragments observed for the control (33%) was considerably higher than usual (10%) probably due to the fact that these protoplasts had been stored overnight (2°) before the uptake reaction. The increase in damage caused by the polycations was not unexpected in light of the effect of DEAE-dextran described above. Lurquin and Behki (1975) also found disruption of the C. reinhardi mutant (CW_{15}) with poly-L-lysine and poly-L-ornithine. Ohyama et al. (1973) found that poly-L-ornithine

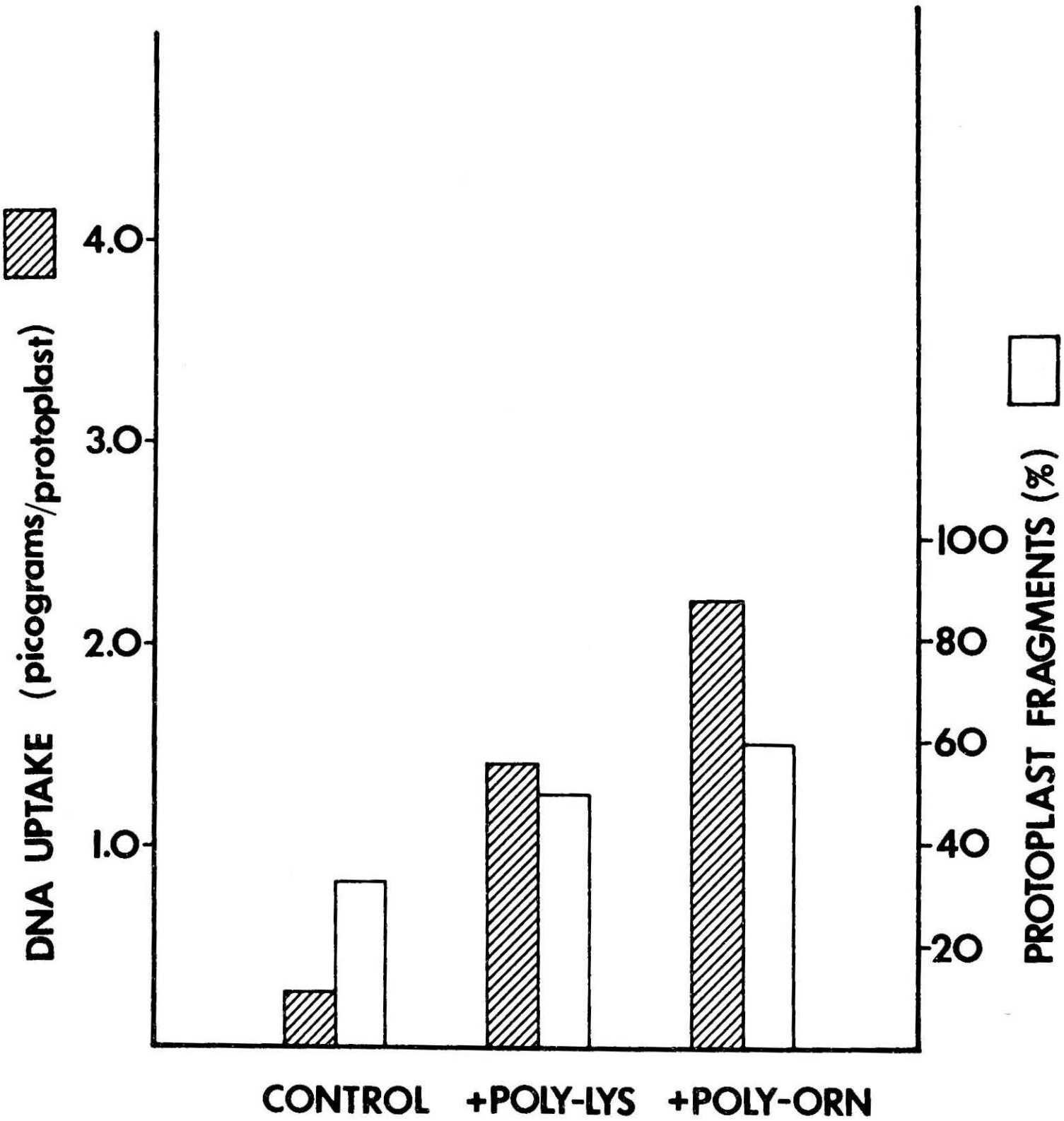

Fig. 5.--Effect of polycations on ^{125}I-DNA uptake (4 hr) and fragmentation of barley protoplasts. Sucrose prepared protoplasts (4.0 x 10^6/ml) were incubated with ^{125}I-*M. luteus* DNA (3.5 µg/ml) either without polycations (control) or with polycations (poly-L-lysine or poly-L-ornithine at 5 µg/ml), and DNA uptake determined as in Fig. 1. The percentage of protoplast fragments was determined after the protoplasts had been washed after DNA uptake.

at a concentration greater than 1 µg/ml in uptake reactions caused death to ammi and soybean protoplasts after 2 days in culture. These results discouraged the use of such polycations, even though the stimulation with poly-L-ornithine (8-fold) seemed to outweigh the increased fragmentation (2-fold). Culturing studies have not yet been carried out on tobacco protoplasts in DNA uptake experiments to determine the relative merit of polycations in this system.

Protoplast viability after uptake. The ultimate test to demonstrate protoplast viability, before and after DNA uptake, is to show that the protoplasts can reform cell walls and then divide in culture. Since conditions for culturing barley protoplasts are not known, viability tests were limited to staining, before and after uptake, with either Evan's blue (Kanai and Edwards, 1973) or phenosafranine (Widholm, 1972). Microscopic examination revealed that Evan's blue stained only protoplasts that were clearly seen to be fragmented while phenosafranine stained all fragments plus about 5% of those which did not appear to be fragmented. According to Widholm (1972), cells which take up the stain are classified as "dead". In typical DNA uptake reactions (1.0×10^6 protoplasts/ml plus 5 µg/ml ^{125}I-*M. luteus* DNA for 3 hr) only 5 to 7% of the intact protoplasts took up the stain. Therefore, DNA uptake did not greatly alter barley protoplast viability. These results are consistent with those obtained with tobacco protoplast cultures. Colony formation was observed using protoplasts which had been incubated for up to 4 hr in the presence or absence (control) of ^{125}I-*B. subtilis* DNA (8.4 µg/ml). The percentage of colonies formed from treated protoplasts relative to

the control is shown in Fig. 6. No significant difference was observed. Such colonies were also microscopically indistinguishable, Fig. 7a and 7b. Similarly, DNA uptake concentrations up to 40 µg/ml showed no significant effect on colony formation after a 2.5 hr uptake period, Fig. 8. Fig. 8 also shows that protoplasts could be plated directly into unlabeled DNA in the plating medium in concentrations of at least 20 µg/ml without loss in viability. These results are in agreement with Lurquin and Behki (1975) who found that DNA concentrations of up to 100 µg/ml did not inhibit growth of C. reinhardi cells (WT) or protoplasts (CW_{15}), and Bendich and Filner (1971) who found that addition of 25 µg/ml DNA to tobacco cells in culture did not affect cell growth. In contrast, Ohyama et al. (1973) observed ammi and soybean protoplast death after 2 days in culture when DNA uptake concentrations exceeded 5 µg/ml. Gleba et al. (1974) also observed tobacco protoplast destruction at DNA concentrations greater than 4 µg/ml in incubation reactions. The reasons for these differences are not readily apparent.

Nature of DNA associated with protoplasts

In experiments described above, uptake was defined as radioactivity which remained protoplast associated following incubation with exogenous ^{125}I-DNA or ^{3}H-DNA. The nature of this association was further characterized by using an unlabeled DNA wash, by DNase treatments and by determining the amount of exogenous DNA which became nuclear associated during the uptake period.

Unlabeled DNA wash and DNase treatment. If radiolabeled DNA survives the extensive washing procedure after uptake but remains

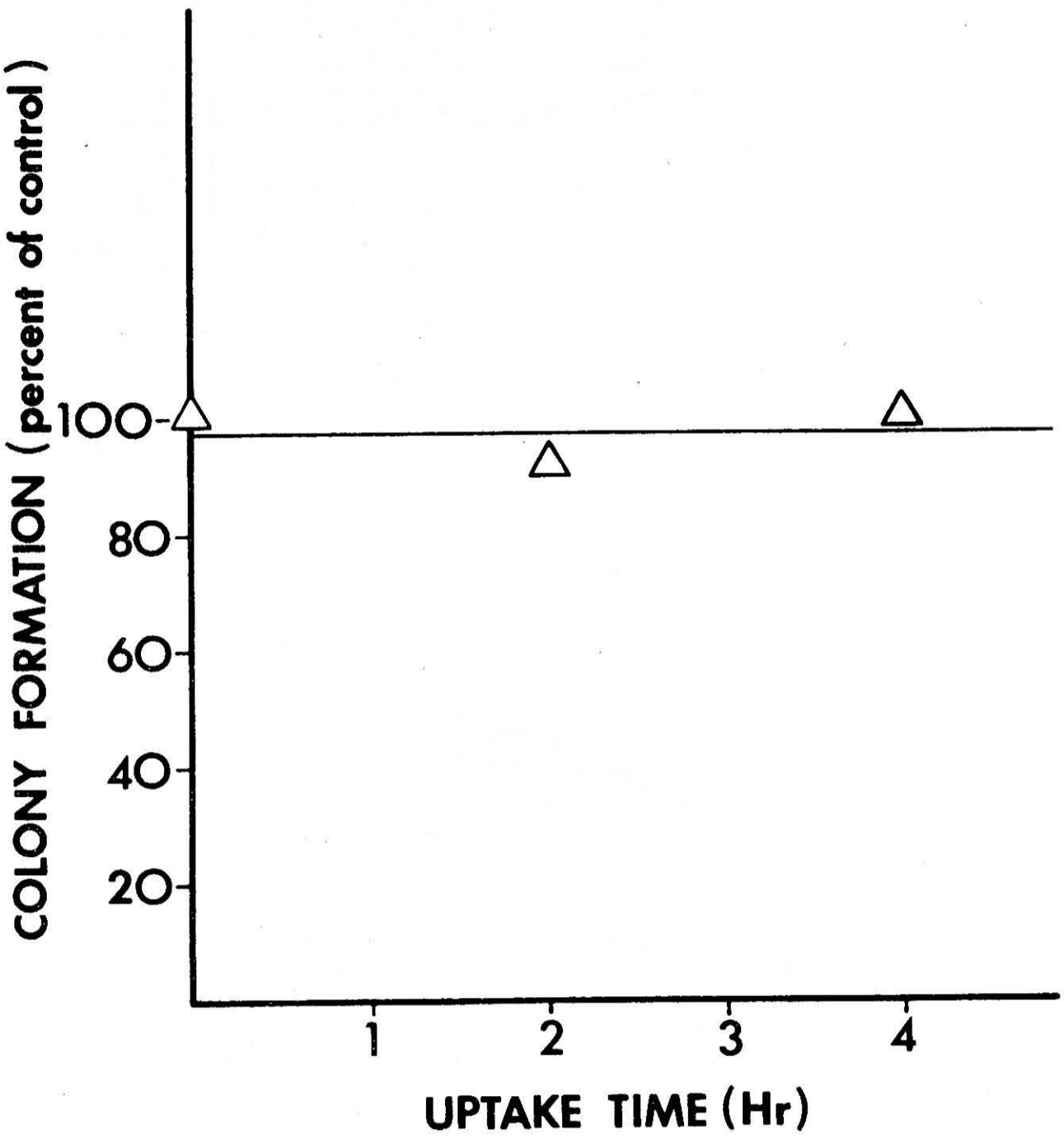

Fig. 6.--Effect of ^{125}I-DNA uptake (0-4 hr) on tobacco protoplast colony formation. Sucrose prepared protoplasts (1.0 x 10^6/ml) were incubated with ^{125}I-*B. subtilis* DNA (8.4 µg/ml) for the indicated times and subsequently plated. Control = colony formation for protoplasts without added DNA.

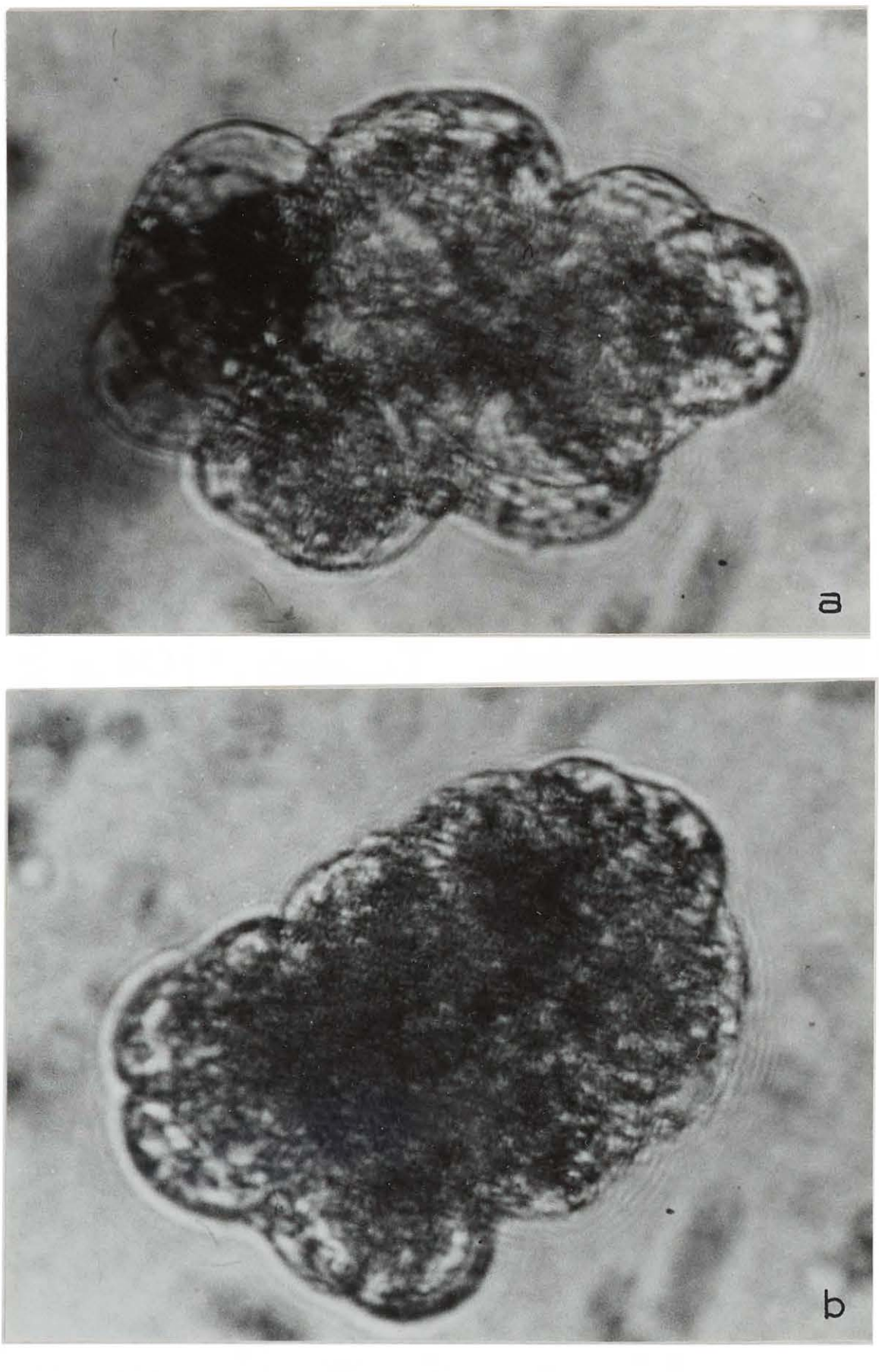

Fig. 7.--(a) A typical colony formed from a protoplast for the control (minus DNA, without prior incubation) after 2 weeks in culture. X 900. (b) A typical colony formed from a protoplast after DNA uptake (8.4 µg/ml ^{125}I-*B. subtilis* DNA per 1.0 x 10^6 protoplasts/ml for 4 hr) after 2 weeks in culture. X 900.

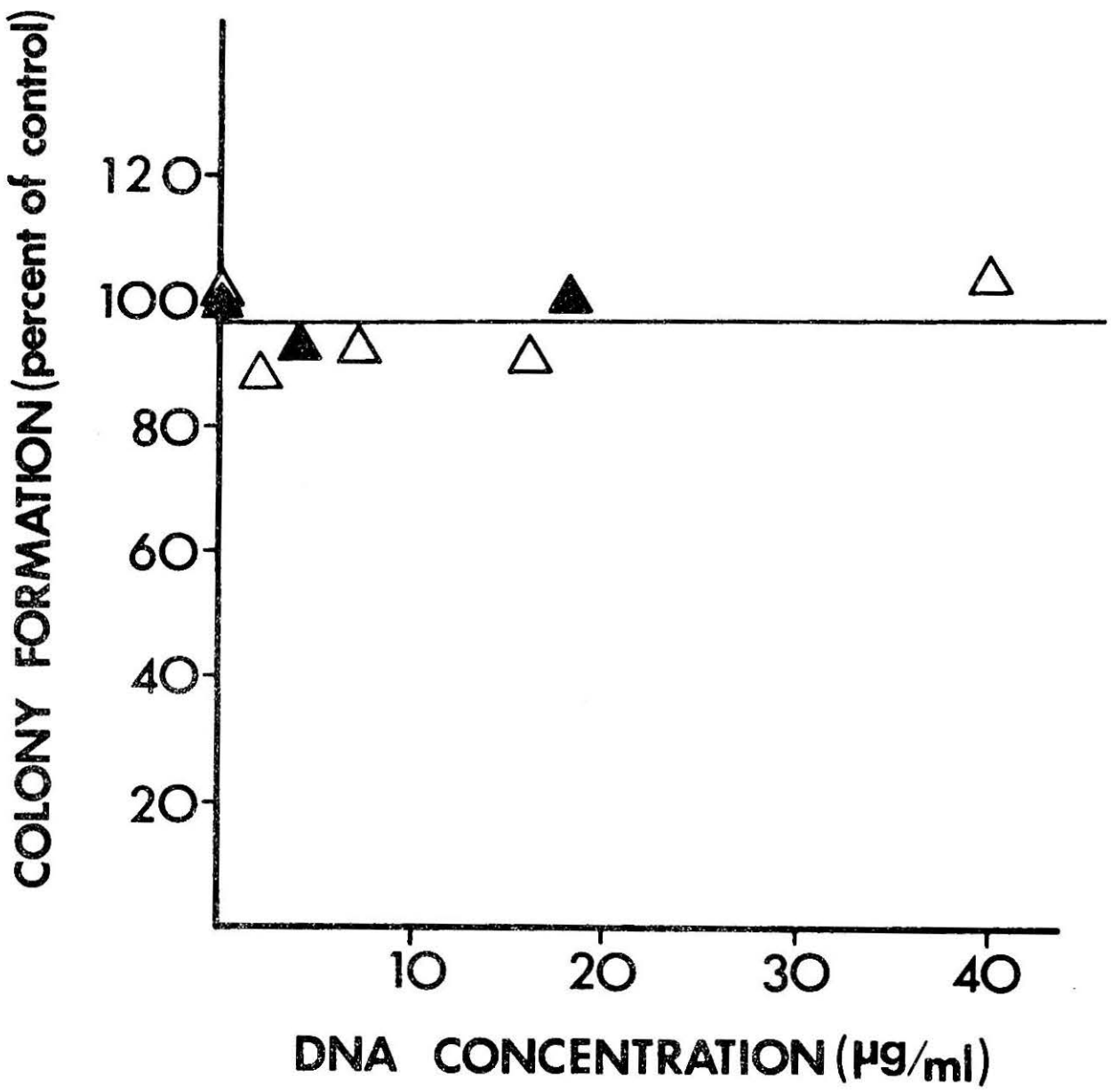

Fig. 8.--Effect of DNA concentration on tobacco protoplast colony formation. Protoplasts prepared in sucrose and plated in medium containing unlabeled B. subtilis DNA at the indicated concentrations (▲); protoplasts prepared in sucrose (2.8×10^6/ml) incubated with ^{125}I-B. subtilis DNA (2.5 hr) at indicated concentrations and subsequently plated in medium without added DNA (△). Control = colony formation for protoplasts without added DNA.

rather loosely bound to the protoplast membrane, one might expect loss of protoplast associated radioactivity when large excesses of unlabeled DNA are added to washing medium or when such protoplasts are treated with DNase. That neither of these treatments had any effect on counts remaining protoplast associated is shown in typical kinetic experiments, Fig. 9. Lurquin and Behki (1975) also found that DNA taken up by C. reinhardi cells (WT) was not exchangeable with excess C. reinhardi (2-fold) unlabeled donor DNA.

The DNase treatments were carried out under conditions which gave major depolymerization of the ^{125}I-M. luteus DNA. This was shown in gel filtration studies of uptake reactions from which protoplasts were omitted, in the presence and absence of DNase, Fig. 9 insert. The fact that the DNase treatments had no effect on uptake indicates that the DNA is somehow tightly sequestered with the protoplasts presumably with no loose "tails" that would be susceptible to DNase attack.

These results are in contrast with those for C. reinhardi (Lurquin and Behki, 1975) and tobacco cells (Owens, personal communication) in which approximately 90% of the associated radioactivity was DNase sensitive. This might be expected if cell walls prevent DNA penetration of the membrane.

The fact that DNA uptake in these two experiments was not linear but proceeded only after a brief lag period was probably of little consequence in the interpretation of the results, and represented one of the variables occasionally seen from one protoplast preparation to another as described above.

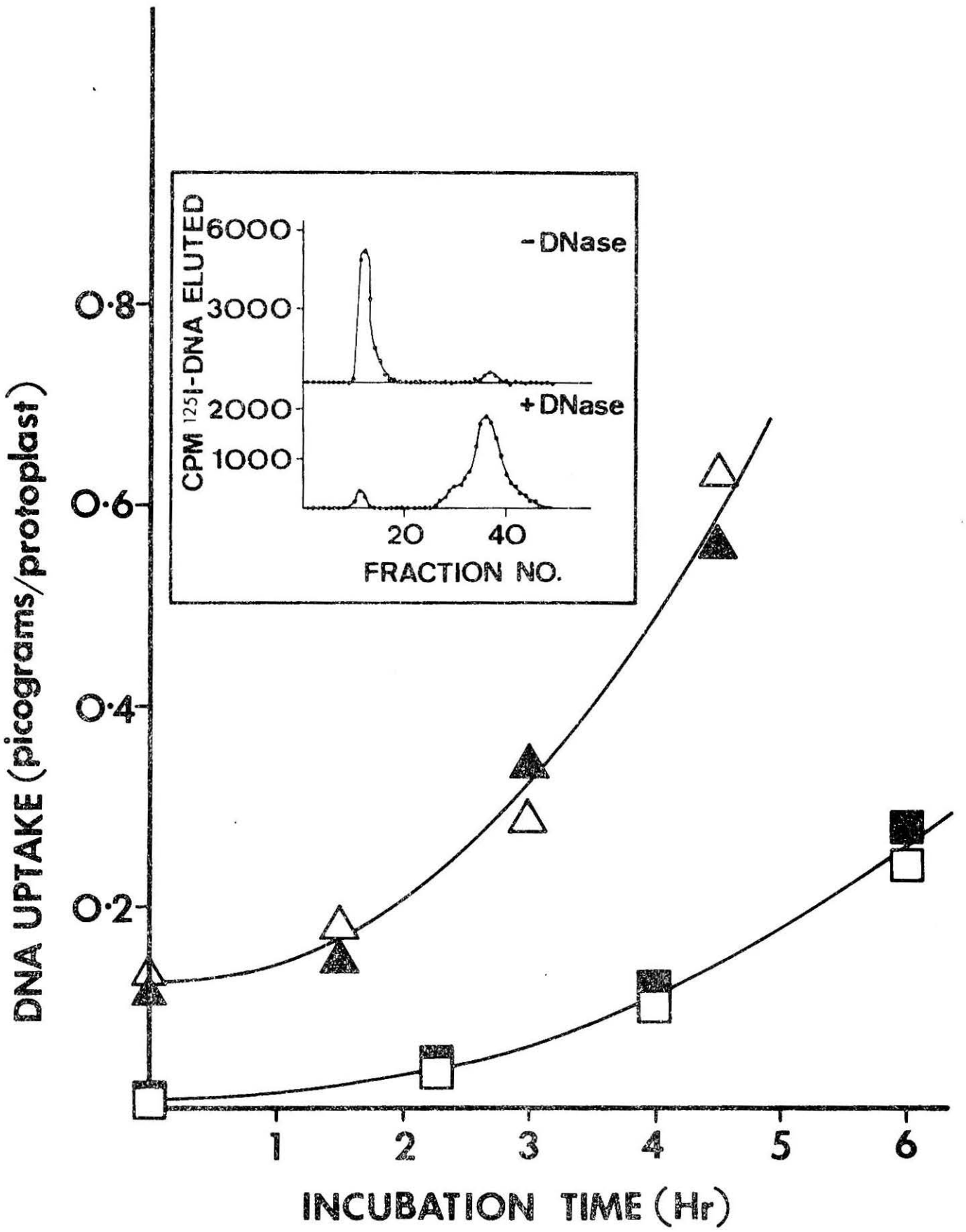

Fig. 9.--Effect of an unlabeled DNA wash or DNase on ^{125}I-DNA uptake by barley protoplasts. Mannitol prepared protoplasts (2.2 x 10^6/ml) were incubated with ^{125}I-M. luteus DNA (6.0 µg/ml) for the indicated times, and then washed using a 10-fold excess of unlabeled M. luteus DNA (□ DNA wash, ■ control = no DNA in wash). Sucrose prepared protoplasts (9.4 x 10^6/ml) were incubated with ^{125}I-M. luteus DNA (19 µg/ml) for the indicated times, washed once, and then treated with DNase (△ DNase treatment, ▲ control = no DNase treatment) using conditions shown for the insert. Insert shows gel filtration (P-60) controls for ^{125}I-M. luteus DNA (3.5 µg) (minus DNase) and for ^{125}I-M. luteus DNA (0.5 µg) treated with DNase (100 µg/ml) for 5 min at 37° C under DNA uptake conditions.

Nuclear associated exogenous DNA. Although protoplast associated radioactivity was shown to resist exchange by large excesses of unlabeled DNA and to resist extensive DNase treatments, no conclusive proof had been obtained that exogenous DNA had in fact penetrated the protoplast membrane. Such information might be available if one could show DNA association with sub-protoplast components. The most likely candidate is, of course, the nucleus. The presence of nuclear associated exogenous DNA was shown by removing barley protoplasts at various times during typical uptake reactions, washing as usual to remove unassociated DNA, determining the radioactivity and number of intact protoplasts, treating with Triton X-100 to solubilize protoplast and chloroplast membranes (Mascarenhas et al., 1974), and then isolating the nuclei by centrifugation and determining their number and radioactivity. The results were expressed in terms of pg/protoplast and pg/nucleus at various times during the uptake reaction, Fig. 10. From 60-75% of the radioactivity was nuclear associated. Similar data were obtained for tobacco protoplasts with 60-80% of the associated radioactivity in the nuclear fraction (not shown). The barley nuclei were quantitatively recovered (about 95% on the basis of protoplast number) after such Triton X-100 treatments and representative preparations are shown in Fig. 11a and 11b as viewed in the light and electron microscopes, respectively. As judged by electron microscopy, most of the nuclei retained their structural integrity and had negligible cytoplasmic contamination. In addition, the nuclei were bounded by only a single membrane layer. This is consistent with results of other workers who have shown the outer membrane to be removed by comparable Triton X treatments (D'Alessio and Trim, 1968).

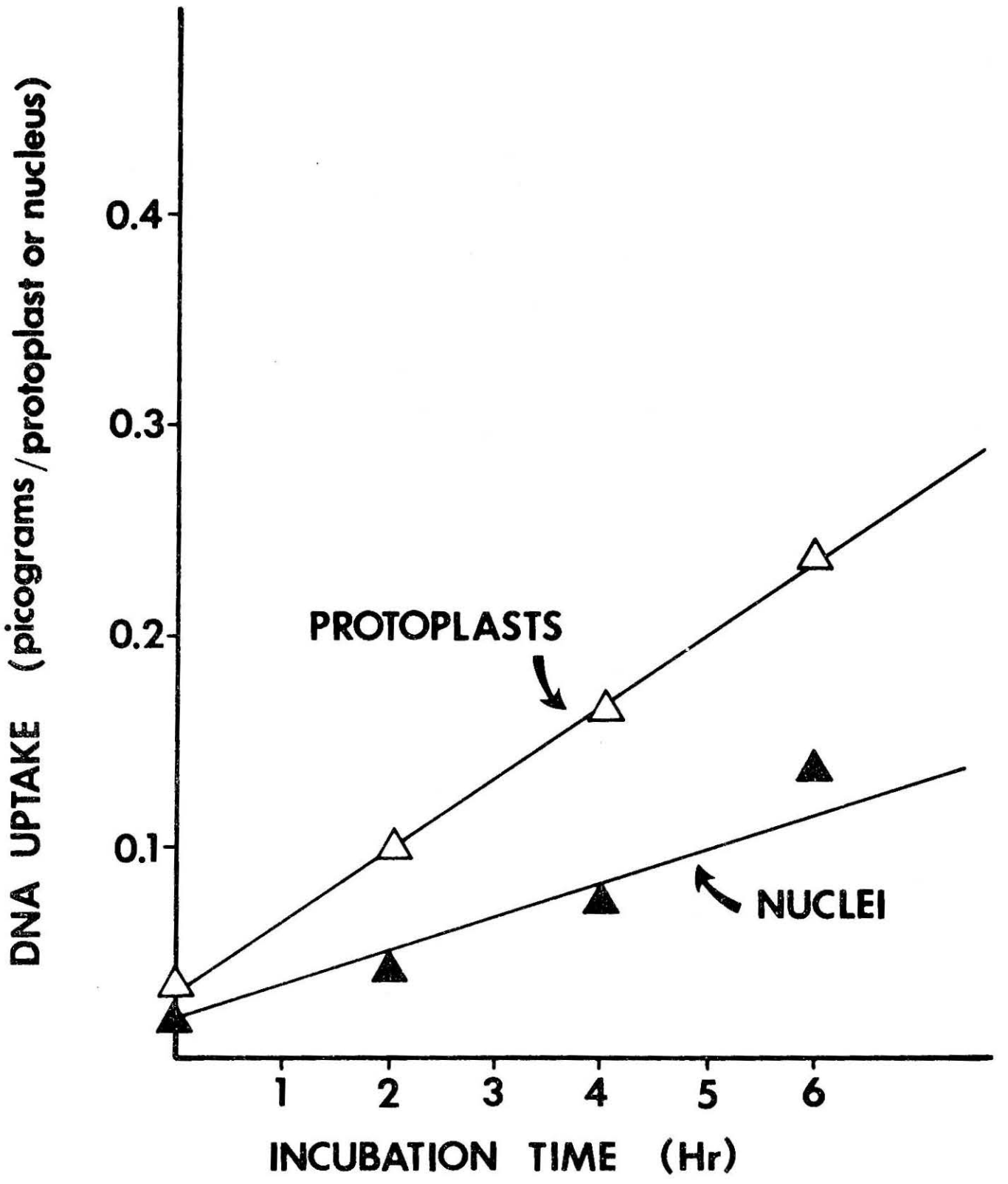

Fig. 10.--Kinetics of ^{125}I-DNA uptake by barley protoplasts and by the nuclei isolated from these protoplasts after uptake. ^{125}I-*M. luteus* DNA (4.5 µg/ml) was incubated with mannitol prepared protoplasts (1.8×10^6/ml) and DNA uptake determined as in Fig. 1. The protoplasts were then treated with Triton X-100 and resultant nuclei were washed, counted in a hemacytometer, and their radioactive uptake determined.

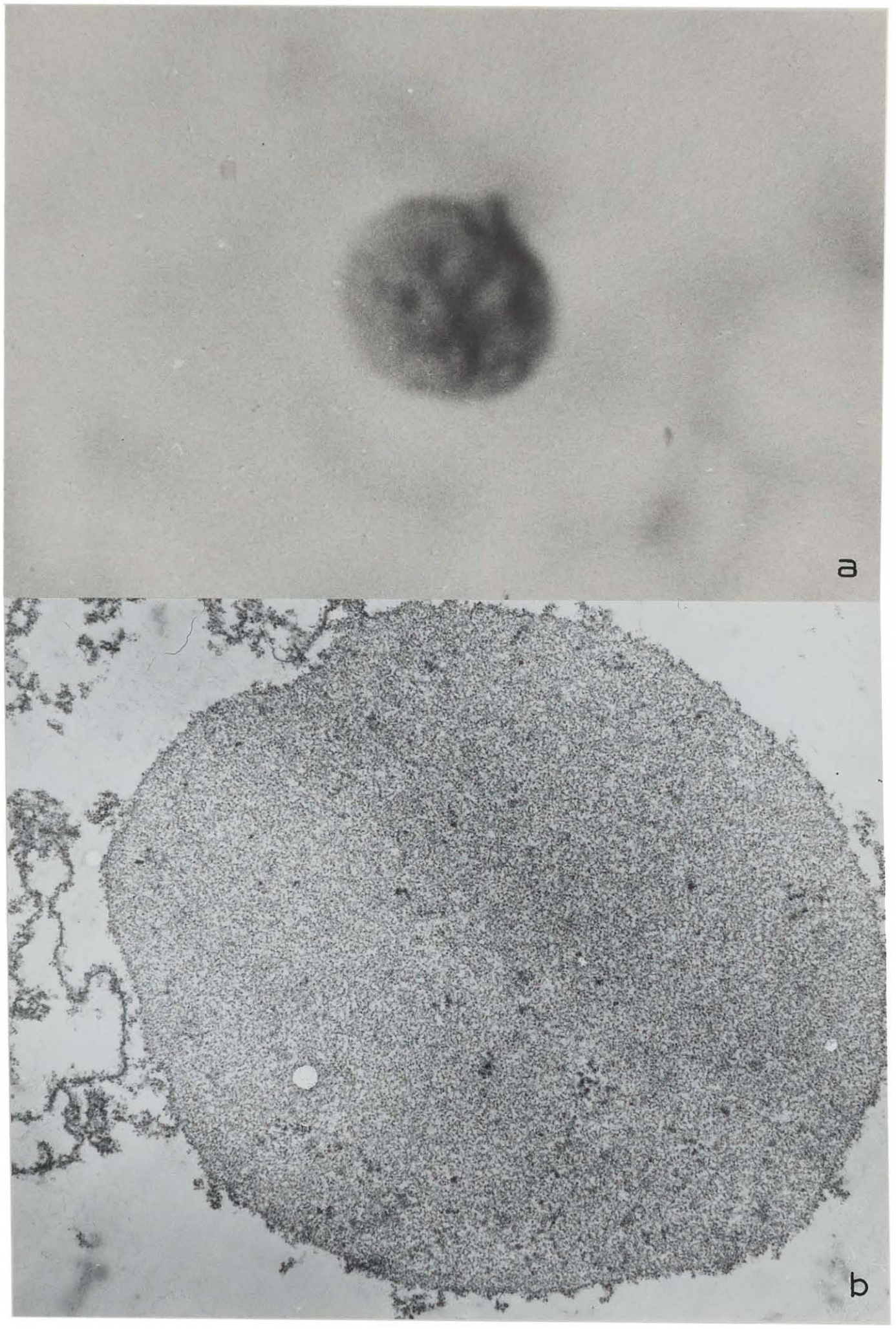

Fig. 11.--(a) A typical barley nucleus isolated by the 1% Triton X-100 treatment and stained with aceto-orcein. X 7,300. (b) An electron micrograph of a typical barley nucleus isolated by the Triton X-100 treatment. X 18,000

If indeed this membrane was removed, then the labeled donor DNA must have at least penetrated the outer and, perhaps, even the inner membrane. An alternative explanation would be that the exogenous DNA was bound to the outer membrane which no longer surrounds but remains attached to the nucleus. In either case, the results are consistent with a significant amount of exogenous DNA having penetrated the protoplast and becoming nuclear associated.

In order to show that this nuclear associated radioactivity was not an artifact of the isolation procedure, nuclei were isolated in the presence and absence of large excesses of unlabeled M. luteus DNA (450-fold greater than DNA uptake) at various times during DNA uptake (2.2×10^6 mannitol prepared barley protoplasts/ml plus 6.0 µg/ml ^{125}I-M. luteus DNA for up to 6 hr). There was no signficant reduction in the amount of nuclear associated DNA found. Had the ^{125}I-DNA become nuclear associated during the Triton X disruption of the protoplasts, it should have been diluted out by large excesses of unlabeled DNA added in control experiments.

The percentage of nuclear associated DNA found in barley and tobacco protoplast uptake reactions is in agreement with that found for petunia protoplasts by Hoffmann (1973) (85%). A lower value (25%) was obtained for tobacco protoplasts by Uchimiya and Murashige (1975). This lower value can probably be explained by the differences used in the protoplast preparations and DNA uptake conditions.

Depolymerization of DNA during uptake

The extent to which exogenous DNA was fragmented during and after uptake was analyzed by isopycnic centrifugation, gel filtration

and loss of biological activity, i.e., transforming activity of exogenous B. subtilis DNA.

CsCl density-gradient centrifugation. This technique is potentially useful in detecting fragmentation because of increasing band widths accompanying such processes (Lurquin and Behki, 1975), and in detecting reincorporation, ^{125}I-nucleotides from heavy exogenous DNA (1.737 g/cm^3) into light endogenous DNA (1.702 g/cm^3). Density-gradient analysis of ^{125}I-M. luteus DNA before uptake is shown in Fig. 12. The ^{125}I-M. luteus DNA banded at a buoyant density of 1.737 g/cm^3, 0.006 g/cm^3 higher than unlabeled M. luteus DNA (1.731 g/cm^3) (Lurquin and Behki, 1975), because of 2-3% iodination of cytosine residues (Orosz and Wetmur, 1974). DNA iodinated to this extent has normal reassociation kinetics (Anderson and Folk, 1976). The ^{3}H-B. subtilis DNA had a broad band in CsCl gradients (Fig. 13) as expected for DNA prepared by the nick translation method without subsequent ligation repair. Such DNA has a single-strand molecular weight of 2.5-5.0 x 10^5 daltons (Chilton, personal communication). For this reason, our nicked, double-stranded DNA would be expected to show size heterogeneity. Its density (1.715 g/cm^3) was higher than the known density for B. subtilis DNA (1.703 g/cm^3) (Ledoux et al., 1971) due to the incorporation of the tritium.

Although quantitative recoveries were obtained when various amounts of ^{3}H-B. subtilis DNA were used in density-gradient experiments, ^{125}I-M. luteus DNA recoveries were extremely variable, i.e., about 40% for 75 µg (Fig. 12) and about 10% for less than 5 µg. Silanation (Anderson and Folk, 1976) of the centrifuge tubes did not improve these results. This ruled out CsCl gradient analysis after uptake

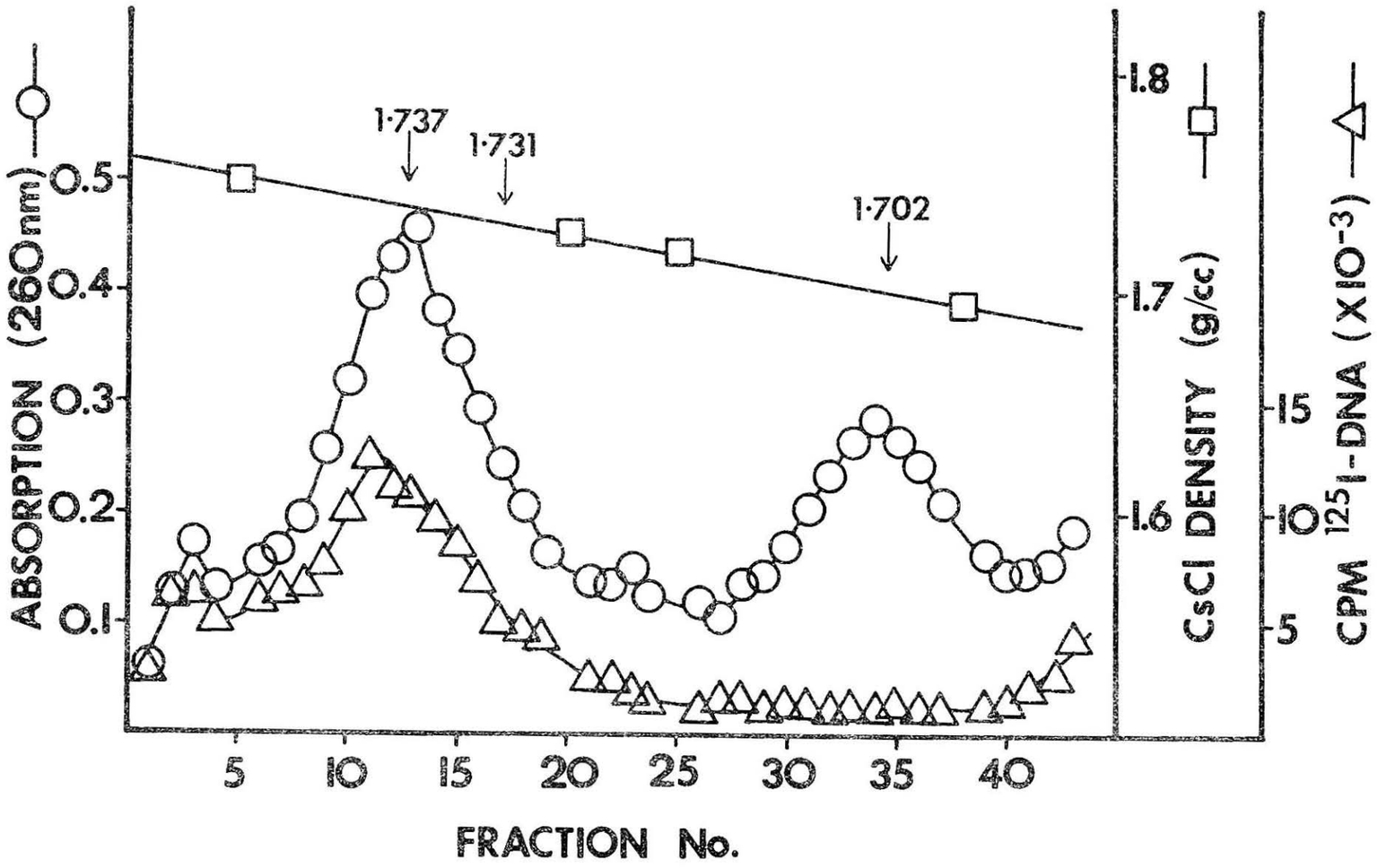

Fig. 12.--CsCl density-gradient centrifugation of ^{125}I-*M. luteus* DNA. Density marker: barley DNA (d = 1.702).

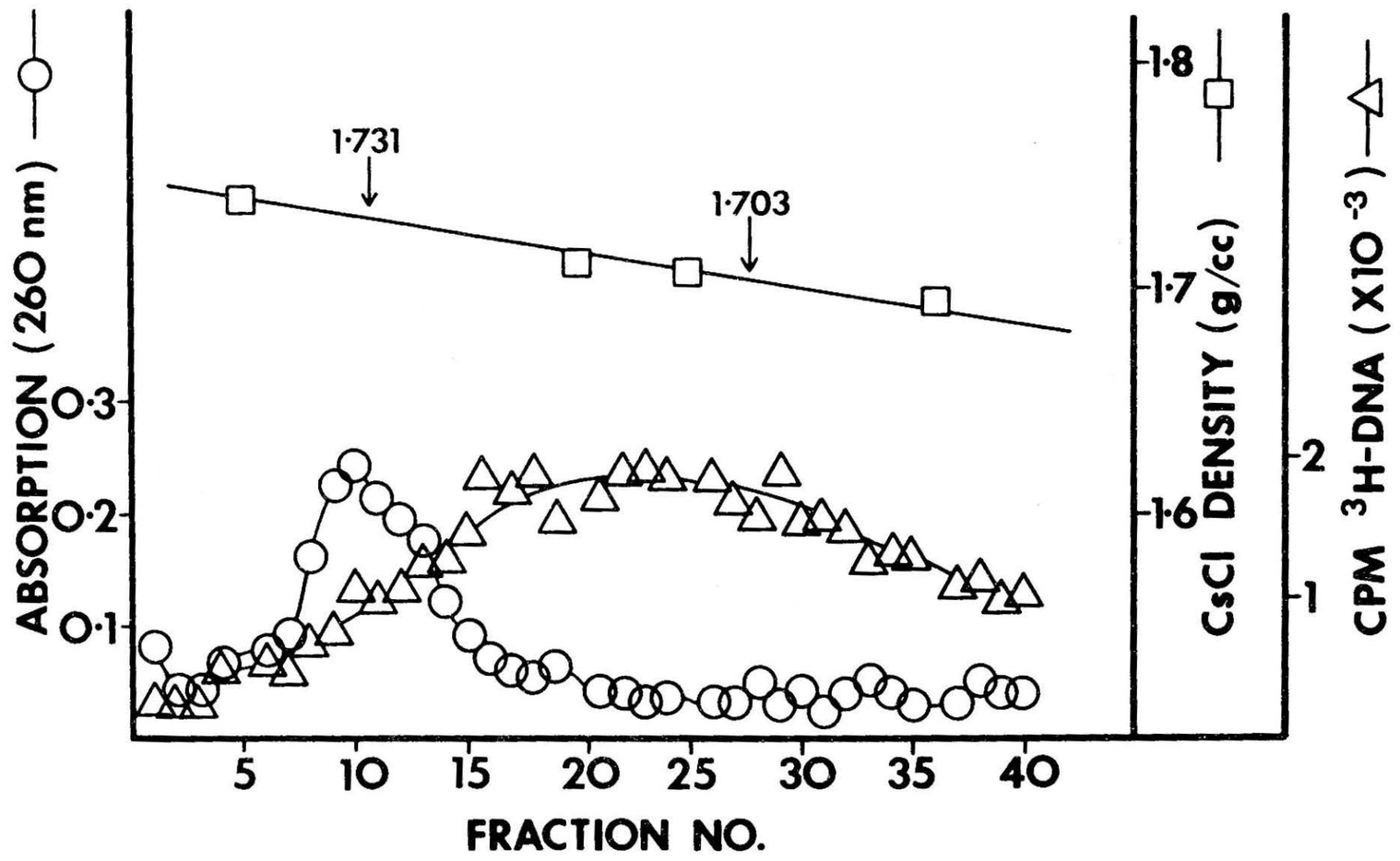

Fig. 13.--CsCl density-gradient centrifugation of ^{3}H-*B. subtilis* DNA. Density marker: *M. luteus* DNA (d = 1.731).

because of relatively small amounts of exogenous ^{125}I-DNAs recovered after incorporation by the protoplasts. Although this technique may otherwise have detected reincorporation, as explained above, it is not sensitive enough to detect significant amounts of integrated foreign DNA (Lurquin and Behki, 1975). Since ^{3}H-B. subtilis DNA had a rather disperse molecular distribution in CsCl gradients and a density close to that of barley and tobacco DNAs, centrifugation studies after DNA uptake using this technique were also futile.

Gel filtration. Another technique useful for detecting fragmentation during DNA uptake involves Sepharose 4B chromatography. It has the disadvantage of not discriminating among DNA fragments greater than about 2×10^6 daltons, but works well with lower molecular weight species (Miller et al., 1974; Lurquin et al., 1975). In these experiments, DNA fragmentation during uptake was detected by a shift in the elution of the radioactive DNA from the excluded toward the included position. The technique involved exposing protoplasts for a given period to ^{3}H-B. subtilis DNA, and subsequently isolating protoplasts, treating with detergent (containing marker unlabeled B. subtilis DNA) to solubilize membranes, and adding to gel filtration columns. Results are given for the total reaction (^{3}H-B. subtilis DNA, detergent, pronase, unlabeled B. subtilis DNA) at zero time (control), for protoplast associated and for extra-protoplast associated DNA at 4 hr, Fig. 14 a, b and c, respectively. The unlabeled marker B. subtilis DNA [circles, MW approximately 52×10^6 daltons since it was prepared by Marmur's method (Morrison and Guild, 1972)] was excluded from the gel, while the zero time control ^{3}H-B. subtilis DNA (triangles), prepared by the nick translation method and known to be much more

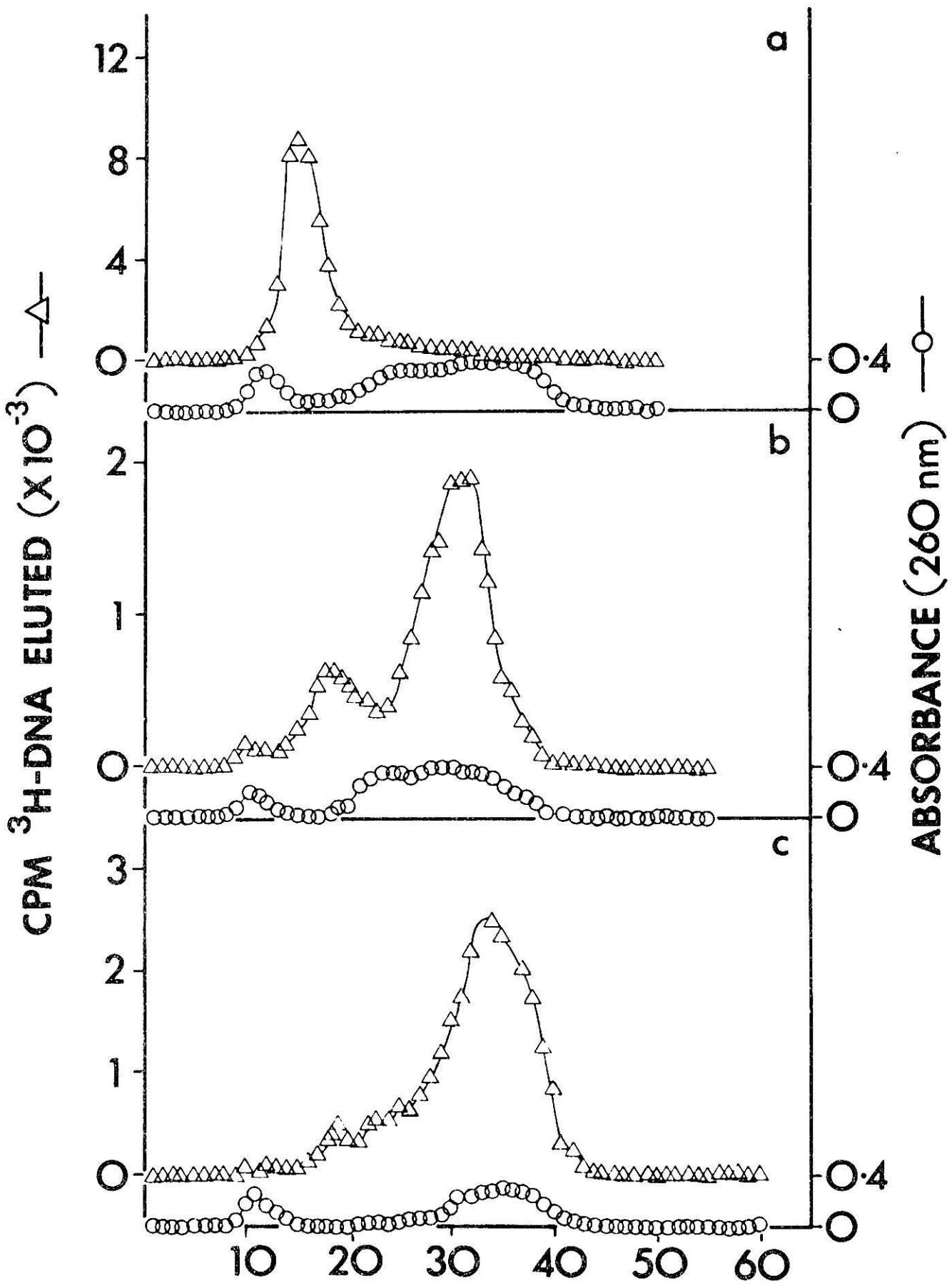

Fig. 14.--Molecular sieving of ^{3}H-DNA on Sepharose 4B columns before and after uptake (4 hr) by mannitol prepared barley protoplasts. (a) ^{3}H-B. subtilis DNA (3.2 µg) mixed with protoplasts (1.0 x 10^{6}, (b) ^{3}H-B. subtilis DNA (11 µg/ml per 3.1 x 10^{6} protoplasts/ml) after uptake for 4 hr by protoplasts (1.0 x 10^{6} to column), (c) ^{3}H-B. subtilis DNA in extra-protoplast suspension after uptake as in (b) (diluted 1:10). Samples were treated by the Lurquin and Behki (1975) procedure before addition to columns. The void volume was determined using high molecular weight B. subtilis DNA (40 µg) eluting in Fraction 11.

fragmented, was slightly retained and had a molecular weight of about 1-2 x 10^6 daltons [calculated according to Miller et al. (1974)]. This seems to be in agreement with the single-strand molecular weight for ^{3}H-B. subtilis DNA (Chilton, personal communication), as mentioned above. The 260 nm absorbing material eluting after fraction 15 was due to absorption of detergent and pronase used in the isolation of DNA from protoplasts and not fragmented B. subtilis DNA marker. After a 4 hr uptake (Fig. 14b), most of the protoplast associated DNA moves in the included peak, but a small amount (20%) was partially excluded and had a molecular weight of about 5-10 x 10^5 daltons [calculated according to Miller et al. (1974)]. The exogenous DNA in the extra-protoplast suspension, after 4 hr incubation, was predominantly low molecular weight (Miller et al., 1974; Lurquin et al., 1975).

Sepharose 4B chromatographic analysis of ^{125}I-M. luteus DNA before and after uptake was carried out using Triton X-100, rather than sodium sarcosylate and omitting the pronase treatment. Although similar results were obtained, they are inconclusive due to low recoveries (less than 50%) (see Discussion).

Lurquin and Behki (1975) have reported similar depolymerization of DNA incubated in the culture medium with algal protoplasts (C. reinhardi, CW_{15}), but little depolymerization occurred for DNA incubated in the culture medium with the cells (WT) or for DNA actually bound to the algal protoplasts or cells.

Depolymerization obtained during DNA uptake by barley and tobacco protoplasts was perhaps expected since DNases have been isolated and purified from germinating barley (Liao, 1976) and tobacco

suspension cultures (Oleson et al., 1974). At least one of the barley DNases has been shown to resemble pancreatic DNase, while the tobacco DNase is a sugar-unspecific nuclease degrading DNA to oligonucleotides and 5'-mononucleotides (Liao, 1976; Oleson et al., 1974). DNase levels are known to be elevated in excised leaves of several plants, and DNases solubilized by high salt (0.3 - 0.5 M) buffers (Wilson, 1975). These two facts would indicate that DNase levels might be elevated in protoplasts and/or extra-protoplast suspension medium.

B. subtilis DNA transforming activity. Transformation assays can be used to show fragmentation during DNA uptake since the resultant number of transformants obtained from B. subtilis transforming DNA gives an indication of the average molecular weight of the DNA involved (Morrison and Guild, 1972). ^{125}I-B. subtilis DNA (10 days after iodination) before uptake into plant protoplasts showed transforming activity comparable to that for unlabeled B. subtilis DNA, i.e., in its ability to transform B. subtilis strain 168 trp_2 to tryptophan independence. The ^{125}I-B. subtilis DNA must, therefore, have a molecular weight of at least 18×10^6 daltons (Morrison and Guild, 1972). Since both B. subtilis and M. luteus DNA were prepared by Marmur's technique, their molecular weights could be as high as 52×10^6 daltons (Morrison and Guild, 1972). Since good transforming activity was obtained from such preparations, little fragmentation occurred during the iodination and short term storage (10 days, -20°). ^{125}I-B. subtilis DNA stored for periods of one year retained about 10% of the transforming activity relative to unlabeled B. subtilis DNA, and therefore had a molecular weight of about 7×10^6 daltons (Morrison and Guild, 1972). We were concerned about breakdown of

^{125}I-DNAs since ^{125}I decay is known to cause single and double strand breaks in ^{125}I-iododeoxyuridine labeled DNA (Krisch and Sauri, 1975). The ^{125}I-DNAs used in our uptake studies were at most 2 months old.

Fragmentation experienced during the uptake of transforming DNA can also be correlated with loss of transforming activity. In an attempt to localize the possible source of DNA fragmentation activity (DNase), an investigation of DNase activity of protoplast releasing enzymes, extra-protoplast suspensions and protoplasts was carried out.

Loss of transforming activity resulting from zero and 2 hr incubations of B. subtilis DNA with various dilutions of the protoplast releasing enzyme mixture is shown in Fig. 15. Very little difference was seen between the zero time experiments and the control (minus protoplast release enzymes), but a 6,000-fold dilution was required to eliminate significant loss during 2 hr incubations. Since this assay discriminates against DNA fragments smaller than 18 x 10^6 daltons, (Morrison and Guild, 1972), as discussed above, it is much more sensitive than the TCA-solubilization assay often used to monitor DNase activity. The protoplast releasing enzymes were routinely diluted 1/50,000 or 1/200,000 by our protoplast purification procedures (Materials and Methods), so this source of DNase activity should have been eliminated unless such DNases were selectively taken up during protoplast isolation and released during uptake. An alternative explanation could be the presence of endogenous DNases since they have been found in cell cultures that have not been treated with protoplast releasing enzymes (Bendich and Filner, 1971; Heyn and Schilperoort, 1973; and Lurquin and Behki, 1975).

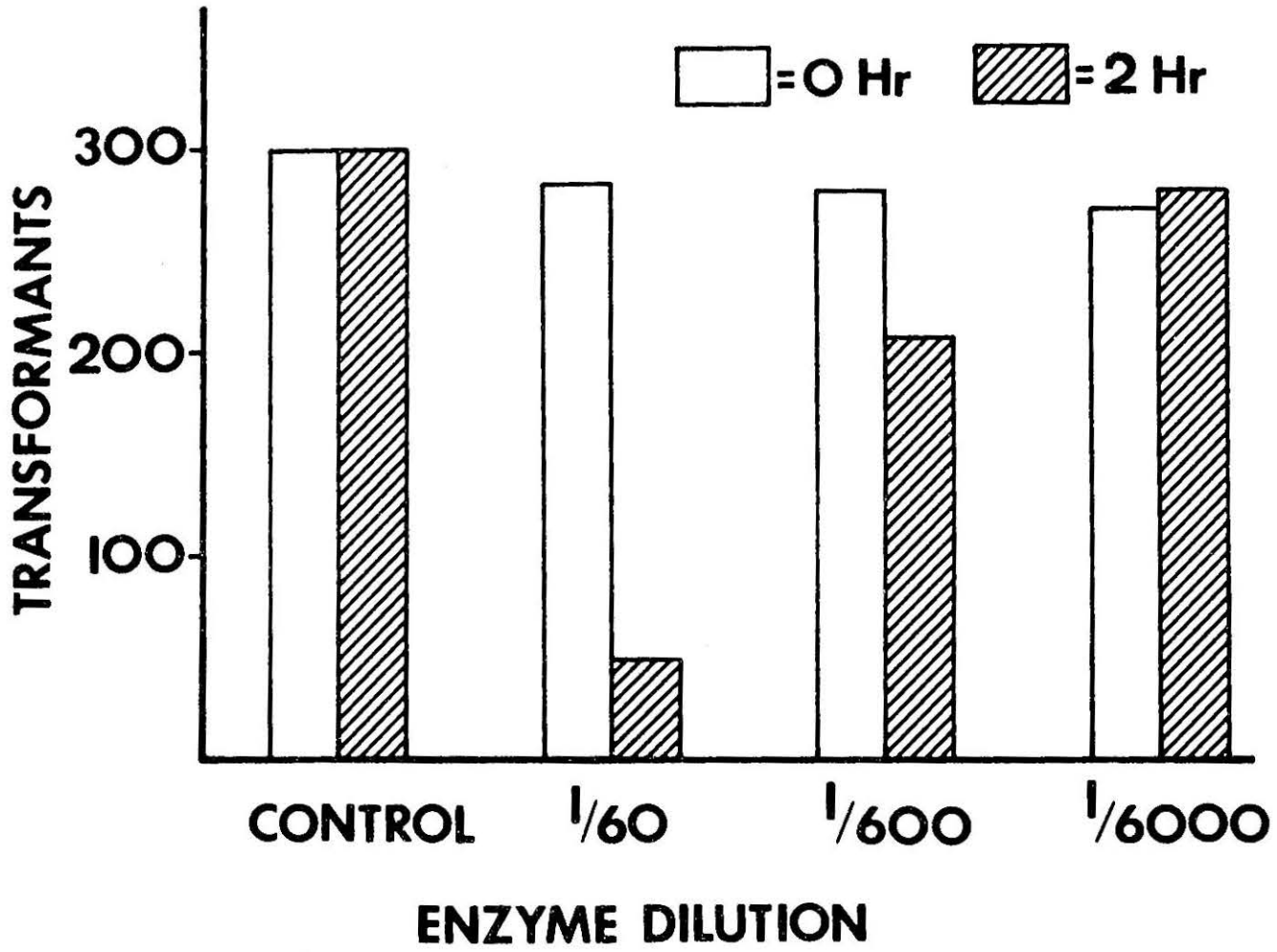

Fig. 15.--Effect of dilution of protoplast releasing enzymes on DNA transforming activity. Enzyme solutions (in the absence of protoplasts) were diluted as indicated, and aliquots (1.0 ml) were either mixed with B. subtilis DNA (7.0 µg/ml) (0 hr) and assayed immediately for transforming activity or incubated for 2 hr with B. subtilis DNA (7.0 µg/ml) and then assayed for transforming activity.

DNase activity of various components of the uptake system was also determined by the loss of B. subtilis DNA transforming activity. In such experiments, transforming DNA was incubated for various hydrolysis periods directly with protoplasts, with medium in which protoplasts had been suspended for 1 or 2 hr, or with ruptured protoplasts, Fig. 16. Protoplasts incubated with unlabeled B. subtilis DNA under uptake conditions showed considerable DNase activity, as did the extra-protoplast medium extracted from protoplasts after 1 or 2 hr incubations. Protoplasts ruptured by the addition of water showed even more DNase activity as no transformants resulted. Similar results were obtained for tobacco protoplasts, which demonstrated a slightly higher level of DNase activity (not shown).

Other workers have also found DNase activity in DNA uptake experiments. The DNase activity in the culture medium of C. reinhardi cells (CW_{15}) has already been described (Lurquin and Behki, 1975). DNase activity for tobacco cell cultures, either excreted into the medium (Bendich and Filner, 1971) or induced by the addition of DNA (Heyn and Schilperoort, 1973), has been reported. These experiments with barley and tobacco protoplasts are the first clear demonstration of DNase activity in higher plant protoplasts. Soybean (Holl et al., 1974) and petunia (Hoffmann and Hess, 1973) protoplasts have been reported to have little DNase activity, but neither was assayed by a method comparable in sensitivity to the transforming activity used in our studies.

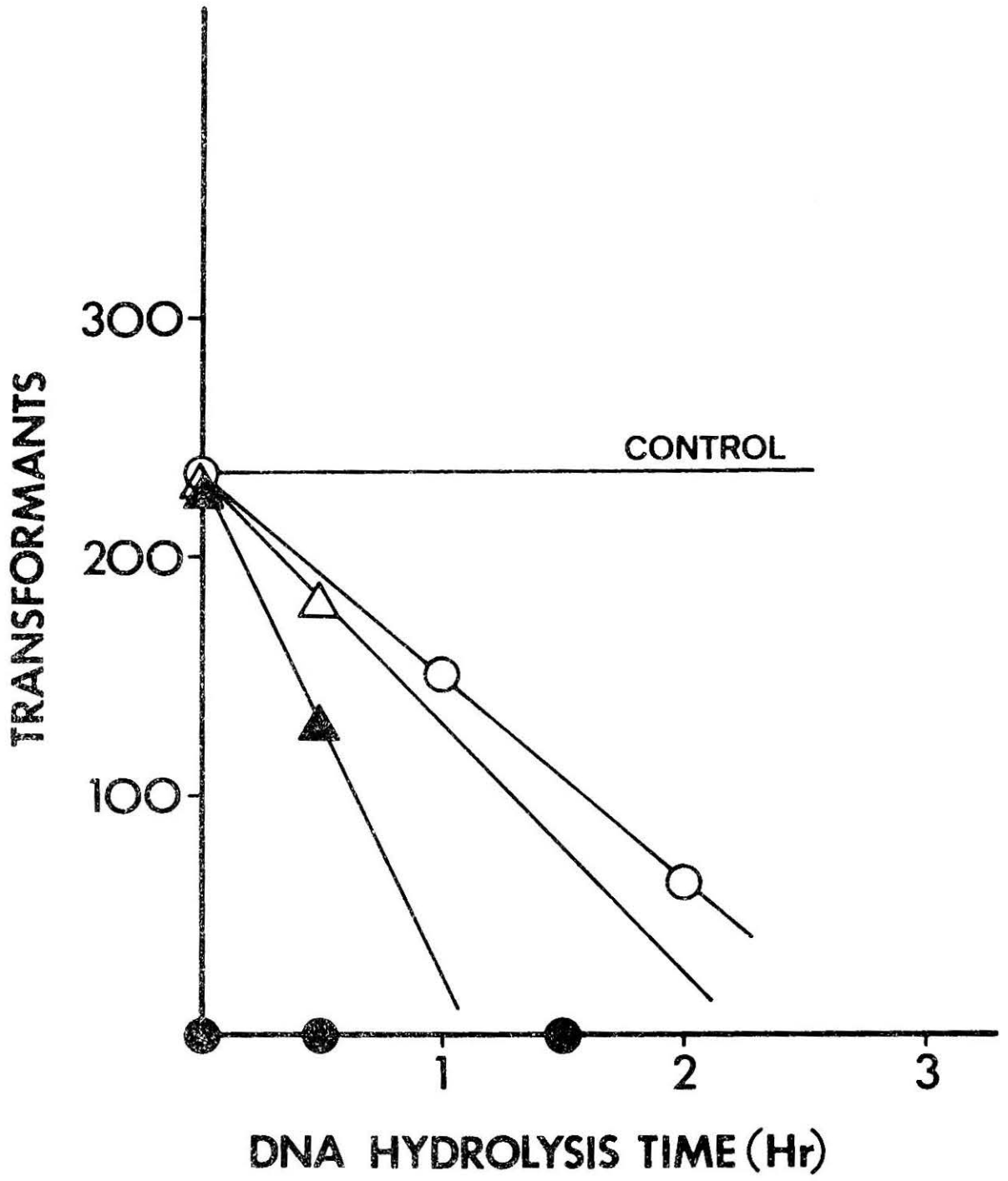

Fig. 16.--Kinetics of transforming DNA hydrolysis by protoplast suspensions, extra-protoplast medium, and ruptured protoplasts. B. subtilis DNA (transforming DNA) (7.0 µg/ml) was incubated with (a) protoplast suspensions (1.0 x 10^6/ml) (○), (b) medium in which protoplasts (1.0 x 10^6/ml) have been suspended for 1 hr (△) or 2 hr (▲), or (c) protoplast (1.0 x 10^6/ml) pellets ruptured by the addition of water (1.0 ml) (●). Control = transformants resulting without protoplasts.

Discussion

The recent excitement involving the use of plasmids for cloning prokaryotic and eukaryotic DNA in bacteria (Marx, 1976) has as yet stimulated very little activity in trying to increase genetic pools available to plant breeders. Even less activity has resulted in characterizing the nature of macromolecular uptake in eukaryotic cells. It is one thing to mass produce genes, but quite another to integrate them into the chromosomal material of eukaryotic cells. The present paper has dealt with a systematic study involving the uptake of bacterial DNAs by living plant protoplasts.

Both barley and tobacco protoplasts were used in these studies because the former is related to important food crops and the latter is readily regenerated into intact plants. Comparisons of DNA uptake data obtained from DNAs labeled with either ^{125}I or ^{3}H, or DNAs having different guanine plus cytosine contents, or DNAs of different size distributions did not reveal any consistent differences. Although the extent of DNA uptake varied considerably from one experiment to another, such variations were within the range observed for protoplasts isolated from plants grown under identical conditions but at various times during a 2 year period.

DNA uptake in the above experiments usually increased linearly with time and DNA concentration. However, saturating exogenous DNA concentrations were not observed even at 200 µg/ml. At this concentration, excessive protoplast fragmentation occurred making experiments at higher DNA concentrations unreliable. The maximum exogenous DNA uptake observed (16 pg/protoplast) was comparable to the endogenous

DNA content of tobacco and barley (11 pg/cell) (Heyn and Schilperoort, 1973; Sciaky, 1973). This represents considerably more DNA uptake than has been observed by others, although comparisons are difficult to make because recoveries are not usually given. Assuming 100% recoveries, we have calculated the following values: A. thaliana, 700 pg/seed (Ledoux and Huart, 1971a); tobacco cells, 0.017 pg/nucleus (Bendich and Filner, 1971); C. reinhardi cells, 0.18 pg/cell (-DNase) or 0.02 pg/cell (+DNase) (Lurquin and Behki, 1975); petunia protoplasts, 0.001 pg/protoplast (Hoffmann, 1973); and ammi protoplasts, 0.015 pg/protoplast (Ohyama et al., 1972).

The fact that DNA uptake had negligible effect on protoplast viability was conclusively demonstrated by regenerating tobacco protoplasts which had taken up genomic quantities of exogenous bacterial DNA. Vital stain data from barley protoplasts, although less convincing, were consistent with these results.

A major problem in interpreting DNA uptake data reported in the literature is the fact that recoveries and viability data are seldom given. This is a critical point for at least two reasons. First, if protoplast recoveries vary with experimental conditions, i.e., time, DNA concentration, etc., then data cannot be directly compared, and may be misinterpreted unless such differences are taken into account. Second, we have found that nuclei bind DNA much more rapidly, and in greater amounts, than protoplasts under identical conditions. As a consequence, DNA uptake by protoplast fragments may give rise to anomalous results. We resolved these problems by using a washing procedure which not only discriminated against fragments,

but also allowed representative sampling for protoplast counting and fragment determination by microscopic analysis, and viability tests.

The fate of DNA taken up by protoplasts, or cells, is of major concern. Generally, uptake is defined as protoplast associated DNA after a given uptake period. That most of this DNA penetrates barley and tobacco protoplasts was demonstrated by finding exogenous DNA associated with nuclei, isolated from protoplasts after uptake. This is not surprising in light of the rapid binding of DNA by nuclei described above. Consistent with this interpretation was the fact that large excesses of unlabeled DNA, or DNase treatment failed to reduce protoplast associated DNA. An alternative explanation might be that the few fragments contaminating protoplasts after the washing procedure selectively bound most of the DNA.

Another important concern was the extent to which exogenous DNA remains intact during uptake. Analyses in this area proved to be more difficult than expected because of the specific binding of ^{125}I-DNA to Sepharose, Sephadex, BioGel, Millipore filters, and polyallomer and nitrocellulose centrifuge tubes. Standard techniques such as high salt, 8M urea, large excesses of carrier DNA, and silanation, failed to improve yields significantly. However, by using B. subtilis transforming DNA, we were able to show significant amounts of nuclease activity associated with protoplast releasing enzymes, isolated protoplasts, extra-protoplast medium and especially fragmented protoplasts. Consistent with these findings was the depolymerization of ^{3}H-B. subtilis DNA during uptake reactions. Exogenous DNA re-isolated from protoplasts after uptake was 80% degraded. However, the remaining 20%

was of average gene size and, therefore, of potential significance in the genetic expression of the cell.

It is important to realize that optimum conditions for DNA uptake by eukaryotic cells have not been completely defined. The biological state of the recipient host may be of utmost importance, not only in determining extent of DNA uptake, but also the DNA's subsequent survival from endogenous DNases. Once within the cell membrane at what point in cell growth can exogenous DNA best be incorporated with endogenous genetic material? Closely related is the form in which exogenous DNA can best be presented to the host, i.e., naked, complexed with polycations, histones or other proteins, chromosomes, or packaged in lipoprotein, bacteriophage, algae, or cellular organelles. The possiblity of controlling DNase levels to aid in this incorporation by using DNase inhibitors, such as EDTA known to inhibit tobacco DNase (Oleson et al., 1974), or suitable hormone treatments such as kinetin additions known to reduce RNase levels (Lázár et al., 1973), raises further questions.

These and related questions await further investigation and will be answered by performing experiments like those reported in this paper. We have shown that large amounts of two types of bacterial DNA can be taken up by two kinds of protoplasts, i.e., barley and tobacco, that more than half of the DNA was nuclear associated and that at least a fraction of the DNA was gene sized after uptake by the protoplasts.

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ISOLATION, PURIFICATION, SCANNING ELECTRON MICROSCOPY AND BACTERIAL DNA UPTAKE OF PLANT PROTOPLASTS

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ABSTRACT

Protoplasts were isolated from tobacco and barley leaves in sucrose or mannitol using commercially available cellulases and macerozymes. Barley growth and protoplast isolation and purification conditions were optimized so that protoplasts were obtained in high yields free of unwanted debris and organelles.

A technique for processing barley and tobacco protoplasts for examination by scanning electron microscopy was developed in which protoplasts seem to have maintained their structural integrity.

Barley and tobacco protoplasts took up ^{3}H-*B. subtilis* DNA, ^{125}I-*B. subtilis* DNA or ^{125}I-*M. luteus* DNA as a linear function of time (0-6 hr) and DNA concentration (0-200 µg/ml). Up to 16 pg of exogenous DNA was taken up per protoplast of which approximately one half became nuclear associated. Protoplasts were viable after the uptake as shown by standard staining and culturing techniques. Approximately 20% of the DNA taken up after typical 4 hr uptake reactions was of average gene size (5-10 x 10^5 daltons), and therefore of potential significance to host gene expression.

COMMITTEE APPROVAL: