STUDIES ON ISOLATION AND FUSION OF PROTOPLASTS

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P R E F A C E

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far in part or full, for any other degree or diploma of any university.

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INTRODUCTION

Isolated living protoplasts (i.e. cells from which the rigid cell wall has been removed) constitute a very useful experimental system for studying the physiological, biochemical and genetical processes in plants.

The fact that the protoplasts are devoid of cell wall makes them a suitable material for studying the plasma membrane, absorption and uptake of various chemicals and macromolecules. It can also facilitate the extraction and isolation of various cell organelles and macromolecules. Moreover, the biosynthesis and deposition of the cell wall, which is one of the most important constituents of all plant cells, can be studied more elegantly by culturing protoplasts.

Current interest in studies on protoplasts is also because of the great potentialities they offer in producing parasexual-hybrids (i.e. the hybrids produced without the intervention of sex). The usual techniques so far employed for the production of hybrids were, grafting and cross-pollination. These techniques, however, are not always useful because of the problem of incompatibility in genetically divergent species. Many attempts have been made in the past to overcome this problem by employing test-tube fertilization and other techniques, such as stylar grafting and stump-pollination with varying degrees of success. The fusion of somatic protoplasts from two

different species offers an attractive alternative to
the usual method of sexual fusion and holds a promise in
the future for getting some new and useful hybrids. In
fact, the isolation and fusion of protoplasts from
haploid tissues would offer advantages comparable to
the bacterial systems.

As compared to animal cells, the work on somatic
hybridization of plant cells is rather scant, because of
the problems posed by the presence of a rigid cell wall.
Therefore, the first step in achieving success in fusion
is the isolation of intact protoplasts which can be
stabilized and grown _in vitro_.

Though the number of communications appearing on
this subject are increasing in the geometrical propor-
tion, yet the techniques of isolation, regeneration and
fusion of protoplasts need to be standardized, particu-
larly with regard to different species.

The present work was undertaken to standardize the
conditions for the isolation, regeneration and fusion
of protoplasts from various tissues of _Arabidopsis_, pea,
Petunia, _Datura_ and _Nicotiana_ with the ultimate aim
of generating a somatic hybrid in the test-tube. Attempts
were also made to utilise nutritional mutants of _Arabidopsis_
for bringing about fusion to facilitate the selection of

hybrids. It is hoped that the methods standardized would also be useful for species other than the ones used in our experiments.

ISOLATION OF PROTOPLASTS

Isolation of protoplasts is an important feat which
has been achieved in recent years. In the past, plant
protoplasts were isolated by mechanical means. The
earliest record of an attempt of isolation of plant
protoplasts is that of Klercker (1892). He peeled off
the epidermis of the leaf of Stratoites aloides and
plasmolysed the cells in a suitable plasmolyticum, so
that distinct shrinkage of protoplasts from the cell wall
was observed. He gave a cut on one end of the cell wall
with the help of a sharp microrazor, avoiding injury
to the protoplast which was gently squeezed out of the
cell. This was the only method used for the isolation
of protoplasts till 1960. The yield of protoplasts
obtained by this method was very poor. At the same time
this could be used preferentially for tissues which
showed distinct plasmolysis and good separation of
protoplasm from the cell wall. The advances made in
the field of enzymology encouraged Cocking (1960),
working at Nottingham, U.K., to utilize enzymes for the
isolation of protoplasts. This method was found to be
more convenient and profitable than the mechanical one
as the yield was higher. Cocking used the concentrated
enzyme callulase, extracted from Myrothecium verrucaria
(a fungus), for isolating protoplasts from actively
growing root tips. Takebe et al. (1968) introduced the

two-enzyme sequential method for the preparation of protoplasts. According to this technique, the enzyme pectinase (or macerozyme) was first used to separate the cells and then cellulase was used to degrade the typical cellulose cell walls. Later on these two enzymes were used in different proportion in mixture for isolating protoplasts. Although pectinase (or macerozyme) and cellulase are generally used, an important enzyme -- helicase isolated from the snail's gut-was employed by Bhojwani and Cocking (1972) for isolation of protoplasts from pollen tetrads. Other enzymes, recently introduced, include xylonase by Landova' and Landa (1975) for isolation of protoplasts from the leaves and petals of several members of the Asteraceae. Rhozyme, pectinol and meicelase are some of the other commerﬁcﬁl preparations used separately or in a mixture for the isolation of protoplasts (Bajaj, 1974). Various advantages were enumerated by Ruesink (1972) of using enzymatic rather than mechanical means for isolating protoplasts. These are, the ease of isolation of large number of protoplasts, less osmotic shrinkage and no breakage of cells. There can, however, be some deleterious effects of various impurities such as nucleases, lipases, peroxidases and phenolics if crude preparations of enzymes are used. Enzyme mixtures cleansed of toxic substances and impurities

by elution through Sephadex or Biogel, were, therefore, used by many workers. Harada (1973) combined the mechanical and enzymatic techniques to isolate protoplasts from the leaves of *Ipomea* and *Calysteolia*. He isolated the cells first by mechanical means and then transformed them to protoplasts by enzyme treatment. Since the introduction of enzymatic separation techniques, isolation of protoplasts has been reported from a wide variety of tissues of higher plants, such as roots (Cocking, 1960; Bawa & Torrey, 1971), root nodules (Davey *et al.*, 1973), coleoptiles (Ruesink & Thimann, 1965; Hall & Cocking, 1971), leaves (Takebe *et al.*, 1968; Power & Cocking, 1969, 1970; Otsuki & Takebe, 1969; Bourgin *et al.*, 1972; Ohyama & Nitsch, 1972; Evans *et al.*, 1972; Durand *et al.*, 1973; Kartha *et al.*, 1974b; Vasil & Vasil, 1974; Watts *et al.*, 1974), shoot apices (Gamborg *et al.*, 1975), petals (Potrykus, 1971a), pollen mother cells (Ito, 1973a), pollen tetrads and pollen grains (Bhojwani & Cocking, 1972; Bajaj & Cocking, 1972, 1973; Rajasekhar, 1973; Wakasa, 1973; Bajaj, 1974a,b), fruits (Raj & Herr, 1970; Gregory & Cocking, 1965), endosperm (Motoyoshi, 1972), aleurone layer (Taiz & Jones, 1971), potato tuber (Lorenzini, 1973), cladodes (Bui-Dang Ha & Mackenzi, 1973), callus of various tissues (Schenk & Hildebrandt, 1969a; Eriksson & Jonasson, 1969; Hellmann & Reinert, 1971; Holden & Hildebrandt, 1972; Butenko & Ivantsov, 1973;

Wallin & Eriksson, 1973) and single cell cultures
(Eriksson & Jonasson, 1969; Schenk & Hildebrandt, 1969a,b;
Kao et al., 1971; Grambow et al., 1972; Maretzki & Nickell,
1973; Gamborg et al., 1974).

Though the protoplasts could be isolated easily,
their yield was reported to be dependant on many chemical
and physical factors, such as, the concentration of
osmoticum and the enzymes, volume and pH of the enzyme
mixture, temperature of the incubation mixture and length
of incubation, light intensity, speed of the shaker, age
of the plant material and the growth conditions. An
extensive study was needed for each species for determin-
ing the conditions for the reproducible isolation of a
large number of protoplasts. After isolation, protoplasts
were purified by the following procedures:

a) Floatation - Protoplasts were floated on sucrose
solution of high density (Gregory & Cocking, 1965; Chupeau
& Morel, 1970; Evans et al., 1972; Pilet et al., 1972).

b) Centrifugation - Protoplasts were sedimented
by low speed (ca. 100 x g) centrifugation for 1 min
(Nagata & Takebe, 1970; Motoyoshi, 1971).

c) Two-phase system - Intact protoplasts in the
filtrate were separated from other components at the
interface of an aqueous two-phase system, consisting of

dextran-polyethylene glycol, with sorbitol and sodium phosphate (Kanai & Edwards, 1973). It was not established, however, if this method had any deleterious effect on protoplast viability, and on their capacity for cell wall regeneration and cell division.

VIABILITY DETERMINATION

The isolation of protoplasts was considered significant only if it produced viable entities. To determine the viability of protoplasts, various methods have been used:

a) Observation of cyclosis as an indicator of active metabolism (Raj & Herr, 1970; Pelcher et al., 1974).

b) Evans blue dye exclusion by intact membranes (Glimelius et al., 1974; Kanai & Edwards, 1973).

c) Variation in the size of protoplasts with osmotic changes (Kanai & Edwards, 1973).

d) Indication of respiratory metabolism by oxygen uptake measured by an oxygen electrode (Taiz & Jones, 1971).

e) Indication of photosynthetic activity (Kanai & Edwards, 1973).

f) Use of fluorescein diacetate (FDA) as a protoplast viability stain (Larkin, 1976).

g) Macromolecular synthesis by protoplasts (Sakai & Takebe, 1970; Blaschek et al., 1974 and Fuchs & Galston, 1976).

CULTURE OF PROTOPLASTS

Protoplasts have been widely used for somatic hybridization. The criterion for employing the proto-plasts for such studies depended on their reproducibility in aseptic cultures at a very high percentage. Protoplasts like all cells have also been found to have the potency to develop into complete plants. Various techniques have been employed to maintain continuous culture of protoplasts in synthetic media.

Techniques

Most workers have chosen to work on species whose conditions for culture are well established and the cells of which have differentiated into plants. Following techniques have been employed for the culture of protoplasts:

1. **Plating** - Protoplasts suspended in liquid medium (composition discussed later) are mixed gently and quickly with an equal volume of the same medium prepared in agar and maintained at 45C in a molten state. About 5 ml aliquots of the above are then poured into tight-lidded Falcon plastic petri-dishes which are sealed with Parafilm to prevent desiccation. Concentration of protoplasts varied between $5 \times 10^3 - 1 \times 10^5$ protoplasts/ml in the medium. The dishes are then kept inverted at 28C with continuous illumination at 2,300 lux. This method, first standardized by Nagata and Takebe (1971), is a modification

of the plating technique devised by Bergmann (1960).
This has now been used successfully, with or without
further modifications, for culturing protoplasts by
various workers. Plating technique has an advantage in
that the protoplasts remain fixed in a particular position
and the whole sequence of division, growth and develop-
ment of a plant can be easily observed and the plating
efficiency (i.e. the percentage of protoplasts forming
colonies) can be estimated. Once small colonies are
formed, they can be transferred to agar media for further
growth and differentiation.

Power et al. (1976) used a slight modification in the
plating technique for culturing leaf protoplasts of Petunia.
They added 8 ml of protoplast suspension at twice the final
concentration to a dish containing 8 ml of the same medium
which was previously solidified with 0.5% agar. In this
way they could maintain a high protoplast density in the
liquid layer without reducing the plating efficiency. By
using this technique, Power et al., 1976 could obtain
complete plants of Petunia from protoplasts. In this
technique, however, due to the high density of protoplasts,
coalescence between the adjacent protoplasts could occur
eventually resulting in a chimeral tissue rather than
forming colonies from single cells. To eliminate this
difficulty, Raveh et al. (1973) and Raveh and Galun (1975)
used nondividing, X-irradiated protoplasts as feeder

(nurse cells) to support the division of viable protoplasts
plated at densities as low as 5-50/ml. Kao & Michayluk
(1975) have reported success in obtaining sustained
division of cells and formation of tissue from a single
protoplast of _Vicia hajastana_ cultured in just 4 ml of a
nutrient medium with a complex composition. This is
remarkable and these new techniques utilising less number
of protoplasts for culture may be of great help in
obtaining plants from various species.

Light intensity and protoplasts density are two
critical factors for plating efficiency. These factors
need to be carefully controlled. As already discussed,
density of protoplasts can be modified according to the
different cultural conditions. As regards the light
intensity, it has been reported by Enzmann-Becker (1973)
that the plating efficiency of tobacco protoplasts could
be enhanced considerably, if they were incubated for the
first two days at low light intensity (300 lux) and then
transferred to a high light intensity (3000 lux).

2. _Suspension and drop cultures_ - Protoplasts can
also be cultured in liquid media in 25-50 ml Erlenmeyer
flasks with or without shaking (25-50 rpm) and incubated
at 35-28C in continuous light (500-2000 lux). Aeration
of the cultures is quite sufficient even without shaking
if only a small volume ca. 2 ml of the protoplast suspension

is incubated in a 25 ml Erlenmeyer flask (Vasil & Vasil, 1974) or when ficoll is added to the medium which causes them to float on the surface (Eriksson & Jonasson, 1969).

Even though, there is limited growth of the protoplasts in liquid media and the cell aggregates have to be transferred ultimately to an agar-solidified nutrient medium for further growth, it has certain advantages. Better control of the growing millieu of cells can be maintained as most of the cells are surrounded by the medium. Also the cells would be more uniform physiologically. However, the ease of culturing in liquid media varies with the source of protoplasts. For example, tobacco is very tolerant and grows well whereas Petunia is not. Kao et al., (1971) developed a modification of the suspension culture technique, called the liquid droplet method which was later successfully utilized by other workers. Small aliquots of protoplasts (containing 1 x 10^4 or 1 x 10^5 protoplasts/ml) were placed in 50 μl drops in plastic petri dishes, sealed with parafilm and incubated at 25-30C either at low light intensity (100-500 lux) or in dark. Enough aeration was provided for the small size of the drops. Fresh nutrient medium was added to the same cultures after cell wall regeneration and initiation of cell division. Ultimately cell suspension cultures were obtained.

3. **Microculture chambers** - This technique, developed
by Jones et al. (1960), has been employed to observe the
growth of individual protoplasts at high resolution under
the microscope. Vasil and Vasil (1973, 1974) employed this
technique to study the mesophyll protoplasts of tobacco
and Durand et al. (1973) to study protoplasts of Petunia.
It is particularly useful in observing the fusion of
protoplasts and the development of fused protoplasts
where no markers are available for the identification of
the fused products. This technique essentially consists
of placing a droplet of protoplast suspension of ca. 30 μl
(containing several protoplasts) on a microscope slide
which is endlosed on top by a cover glass placed on
either side of the drop. The culture is sealed with
paraffin oil to prevent desiccation. After the protoplasts
undergo a few divisions and develop colonies consisting
of 75 or more cells, they are eventually transferred to
fresh agar nutrient medium for further development.

Medium Composition

Generally, the basic nutritional requirements for the
growth of protoplasts are more or less similar to those
of the culture cells with slight modifications to meet
the special requirements of protoplasts. The nutrient
medium for the culture of protoplasts principally consisted
of the following compounds:

1. **Inorganic salts** - Various workers (Arnold & Eriksson, 1976; Constabel, 1975; Dorion et al., 1975 and Uchimiya & Murashige, 1976) have used media which varied widely in total salt concentrations Gamborg et al. (1976) have stated the requirement of both nitrate and ammonium as source of inorganic nitrogen. Excess of ammonium (>8 mM) according to them could be deleterious. They suggested that an organic acid, such as succinate, can help the utilization of ammonium and lessen the toxic effects. They also found that 1-3 mM concentration of phosphate, sulphate and magnasium and 20-25 mM concentration of potassium and nitrate was sufficient for the growth of protoplasts. According to Constabel (1975), a higher concentration of calcium ions in the medium is beneficial. He found that the supplementation of the medium with 5-10 mM $CaCl_2.2H_2O$ alone or in combination with $CaH_4(PO_4)_2H_2O$ for the first period after isolation improved the conditions for protoplast culture. The micronutrients recommended by Gamborg et al. (1975) included, iodide, boric acid, salts of maganese, zinc, molybdenum, copper, cobalt and iron. Iron is supplied usually in the chelated form.

2. **Osmotic stabilizer** - Mannitol or sorbitol at a concentration varying between 0.4M-0.7M has been used either separately or in icombination to maintain the osmolarity (Gamborg, 1976). Mannitol does not enter the metabolism of cells whereas sorbitol is used up as carbon

source. Sucrose has also been used as an osmoticum,
but since this is utilised by the plant cells, it is
added in the medium in combination with mannitol
(Murashige & Skoog, 1962). Kameya and Takahashi (1972)
utilized mineral salts as osmotic stabilizers. These
were, however, not found to be more suitable than the
non-ionic compounds.

3. **Carbon source** - Glucose or sucrose at a concen-
tration of 2-4% is the most widely used carbon source.
Plant cells grow quite well on both glucose and sucrose,
but sometimes sucrose alone was not found to be satis-
factory for the growth of protoplasts (Gamborg, 1976).
In some media, 1-3mM ribose or some other pentose was used
to supplement the other carbon sources (Kao et al., 1973;
Wallin & Eriksson, 1973).

4. **Vitamins** - Vitamins which are used in routine
plant cell culture media, have been found to be beneficial
for protoplast culture also (Gamborg, 1976). Of these,
thiamine is absolutely essential, while pyridoxine,
nicotinic acid and myo-inositol improve growth. Additional
vitamins are required when protoplasts are to be grown
at very low densities (Kao & Michayluk, 1975).

5. **Growth regulators** - These were found to be essential
for the growth and division of protoplasts (Gamborg, 1976).

Auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA) have been used frequently at concentrations varying between 10^{-7} to 5×10^{-5}M. These were found suitable for inducing cell divisions (Gamborg, 1976). Indoleacetic acid (IAA), which also induced cell division was found to get degraded by enzymes released by cells (Cocking, 1960). This was unstable to autoclaving, whereas 2,4-D and NAA were found to be stable. Cytokinins, such as kinetin, benzyladenine, zeatin and isopentenyl adenosine, were also needed along with the auxins for proper growth (Gamborg, 1976).

6. Organic supplements - These include protein hydrolyzates (e.g. casein hydrolyzate), yeast or malt extracts and coconut milk. The latter at a concentration of 1-5% (v/v) improves the growth of protoplasts considerably (Kao & Michayluk, 1975). Protoplasts media generally contain one or more amino acids. Another approach has been to add 0,01 to 0.25% of vitamin-free casamino acids or casein hydrolyzate (Constabel, 1975).

All the above mentioned compounds were included in the various protoplast media, such as media of Murashige and Skoog (1962), modified Murashige and Skoog commonly referred to as Nagata and Takebe (1971), Frearson et al., (1973), Gamborg et al. (1968), Ohyama and Nitsch (1972) and Vasil and Vasil (1974). The choice of the media for culturing protoplasts is rather emperical. A rationalised

approach is still required to be worked out in this direction.

Physical Factors

As has already been mentioned, light intensity and protoplast density are two important factors which determine the growth of protoplasts *in vitro*. In addition, the pH and the osmolarity of the medium and temperature are also critical for successful growth of the protoplast. Protoplasts have been shown to grow well at a pH of 5-6 (Gamborg et al., 1975) and temperature between 22-28C (Gamborg, 1976).

Wall Regeneration

As soon as the cell wall degrading enzymes are washed and the protoplasts are placed in suitable medium the wall regeneration starts. The first step is a noticeable increase in the size of protoplasts and rearrangement of the protoplasmic contents. The presence of newly synthesized wall can be demonstrated clearly by plasmolysis of the cells. Nagata and Takebe (1970) demonstrated the cell wall synthesis by staining with a fluorescent dye, calcofluor white. This method was found to be more sensitive than any other conventional wall staining procedures or observation through polarizing microscope, as it required no prior fixation of the material. However, objections have been raised by Burgess and Fleming (1974a)

about the wall material which was seen on the plasmalemma
after a few hours of culture. According to them, the
observed newly synthesized material was a remnant of the
previous wall material which remained due to incomplete
enzyme digestion of the cell wall. Wall regeneration
can be best demonstrated by electron microscopic, surface
replica and freeze etching studies. According to Fowke
and Williamsons cited in Vasil (1976) cellulose micro-
fibrils were observed within one hour of culture in
protoplasts from soybean and Vicia cell culture by using
the replica techniques.

Division and Growth

Nuclear division was reported by some workers to take
place after the protoplasts had regenerated the wall
(Kao et al., 1970, 1971; Nagata & Takebe, 1970). Wall
regeneration, however, was not a necessity and some others
(Reinert & Hellmann, 1971; Fowke et al., 1974b; Meyer, 1974;
Meyer & Abel, 1975) observed nuclear divisions without
cell wall formation. Even nuclear divisions were often not
followed by cytokinesis thus resulting in the formation
of multi-nucleate protoplasts (Eriksson & Jonasson, 1969;
Motoyoshi, 1971; Kao et al., 1973; Reinert & Hellmann, 1973;
Fowke et al., 1975a). After wall regeneration, the
reconstituted cells were reported to undergo a considerable
increase in size and the first division took place within

a week resulting in the formation of an aggregate of cells within two weeks of culture. As the divisions continued, the number of chloroplasts per cell was found to decrease and the cells appeared to be more vacuolated. After three to four weeks of culture, pale green colonies (0.3-1 mm in diameter) were observed. Further growth of these colonies was controlled by the auxin cytokinin ratio in the fresh medium which was specific for each species. Following this technique, Takebe et al. (1971) and Nagata and Takebe (1971) regenerated complete plants of N. tabacum. Later on, plants were regenerated by other workers too. Embryoids, but not plantlets were regenerated from protoplasts isolated from roots of carrot (Kameya & Uchimiya, 1972), and leaves of Antirrhinum majus (Poirier-hamon et al., 1974). Besides, cells clusters or calli were regenerated from protoplasts of many species, like soybean (Kao et al., 1970), Haplopappus gracilis (Kao et al., 1971) and Pisum sativum (Constabel et al., 1973).

FUSION OF PROTOPLASTS

Though the isolation and regeneration of protoplasts has been achieved in a variety of tissues, yet the rate of progress in accomplishing somatic hybridization of such protoplasts has been rather slow in plant cells as compared to animal cells. In fact, the technique of somtic cell

hybridization was first initiated in animal cells by
Barski, Sorieul and Cornefort (1960) of the Cancer
Institute. Plant cells, owing to their totipotent
nature, have certain advantages over animal systems as
they can be induced to regenerate into whole plants
on a defined medium, which is not possible in animal
systems.

Two types of fusions, spontaneous and induced,
have been defined by Power et al.(1970). Spontaneous
fusion occurs between two or more adjacent protoplasts
during the enzymatic isolation of protoplasts. It has
been shown with the help of electron micrographs that
spontaneous fusion of protoplasts results from the
removal of constrictions on the plasmodesmata, permitting
their expansion rather than breakage. This results in
the mixing of cytoplasms from the adjacent protoplasts.
Spontaneous fusion is strictly intraspecific. But most
plant protoplasts when completely separated from each
other do not fuse spontaneously. An exception to this
has been reported in freshly isolated protoplasts from
pollen mother cells where protoplasts fuse just by
colliding with each other (Ito & Maeda, 1973).

Unlike in spontaneous fusion, induced fusion need
not necessarily involve fusion from the same plant species.
It is brought about by applying an inducer. In animals,

inactivated "Sendai" virus has been used to induce cell
fusion. However, in plants the inducing agents are such
which bring the protoplasts in close contact with each
other over a large area of their membranes and finally
cause them to fuse. Nickell and Heinz (1973) felt that
current interest in the fusion of isolated protoplasts
is high because of the potentiality of this technique
in bringing about fusion of somatic cells from distantly
related species to produce new plants.

The techniques for protoplast fusion are now well
standardized. As will be discussed in the following few
pages, many inducers have been used to bring about fusion —
each having its own merits and demerits.

Influence of Various Fusing Agents

Inorganic salts - Power, Cummins and Cocking (1970)
reported the first success in intra-and inter-specific
fusion between protoplasts obtained from root tips of
maize and oat seedlings using 0.25M sodium nitrate as
inducer. Sodium nitrate has also been used for inter-
specific fusion of flower petal protoplasts of Torenia
fournieri and T. bailloni (Potrykus, 1971a). However,
an interesting report came from the Brookhaven laboratory
where Carlson et al. (1972) reported the successful
production of somatic hybrid of Nicotiana glauca and
N. langsdorffii using protoplasts as the starting material.
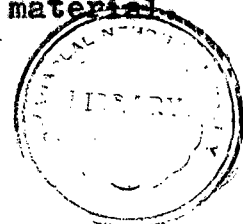
They isolated the protoplasts from the leaf mesophyll
of these two species and fused them using 0.25M NaNO$_3$.
About 25% of the protoplasts were found to fuse. These
fused protoplasts regenerated on a medium without added
hormones on which neither of the parental strains could
grown. The number of calli obtained from the fused
protoplasts was 33 and only 3 of these could be regenerated
into hybrid plants. The parasexual hybrids (or the hybrids
produced by the fusion of somatic cells) exhibited charact-
eristics (morphological, karyological and biochemical)
intermediate of those possessed by the parents. The
hybrids were found to produce flowers and fertile seed
capsules which were identical to those of naturally
occurring amphiploid of N. glauca and N. langsdorffii.
This work, if confirmed, can certainly claim to be a
milestone in achieving the goal on somatic hybridization.
But there have been certain objections raised with
regard to this communication. It has been reported by
Melchers and Labib (1974) that such a high degree of
fusion (25%) in tobacco mesophyll protoplasts cannot be
obtained by using NaNO$_3$ as the inducer. Also the fact,
that Carlson et al. (1972) found, that out of 2 x 10^7
protoplasts of N. glauca and N. langsdorffii, not a
single one could regenerate on Nagata and Takebe medium,
has been refuted by Chupeau et al. (1974) who succeeded
in getting divisions, in protoplasts of both species and

growing them in Murashige and Skoog medium.

Giles (1972) found that the intraspecificially fused protoplasts of soybean obtained by using 0.8M $NaNO_3$ were capable of cell wall regeneration and showed limited mitotic activity over a period of several weeks. He also observed cell wall regeneration in soybean x crabgrass (Digitaria) protoplast aggregates, which were also obtained by treatment with 0.8M $NaNO_3$. Only 1% interspecific fusion was achieved in contrast to 70% intraspecific fusion. The interspecific protoplast aggregates were not observed to undergo mitosis. Later on, Giles (1974) also obtained complementation by protoplast fusion with the help of $NaNO_3$ in chlorophyll deficient mutant strains of maize (wd) with a wild type (wt). While in one experiment, 20% fusion was obtained, generally 5% protoplasts were involved in fusion. Sodium nitrate was found to be useful in inducing fusion but its effect was generally limited to protoplasts having fairly similar osmotic characteristics. It was also shown to have a poor effect on protoplast viability and induced comparatively low frequency of fusion (Potrykus, 1973b; Burgess & Fleming, 1974b; and Melchers & Labib, 1974). Kameya and Takahashi (1972) tried to induce interspecific fusion between the root cell protoplasts of Brassica chinensis and leaf cell protoplasts of B. oleracea with the help of various inorganic salts. They concluded that fusion effect of $NaNO_3$ was due to

sodium rather than nitrate ions. They also observed that
sodium was more effective than potassium in bringing about
fusion and that calcium was ineffective. Keller and
Melchers (1973) induced a high frequency of fusion (20-50%)
between isolated tobacco leaf protoplasts in the presence
of 0.05M $CaCl_2$ at high pH (10.5) and high temperature
(37C). This method did not have any irreversible deleter-
ious effects on the viability of protoplasts as demonstrated
by the active regeneration and growth of the protoplasts.
Using this method, Melchers and Labib (1974) fused the
protoplasts of haploids produced by "anther culture " of
two light sensitive varieties of N. tabacum. The result
was a double heteroxygote hybrid of the two recessive
genes for light sensitivity which complemented to give
normal green leaf colour and resistance to high light
intensity.

Influence of gelatin and dextran sulphate - Kameya
(1973) reported that gelatin or high molecular weight
gelatin derivatives, at a concentration of 2-5% induced
aggregation pf protoplasts of Allium fistulosum, Brassica
chinensis and Daucus carota. He also obtained hybrid plants
by the aggregation and fusion of protoplasts of green leaf
and albino callus of haploid tobacco using dextran sulphate
and gelatin.

Influence of lectins - Glimelius et al. (1974) used
lectin isolated from jackbean meal concanavalin A (Con A)

to agglutinate protoplasts isolated from cell suspension
cultures of Daucus carota. They observed that Con A was
as effective in agglutinating plant protoplasts as in the
case of animal cells. They also found that the agglutina-
tion process was dependant on Con A concentration, proto-
plaôt density, treatment time, the temperature and the
membrane condition.

Influence of polyethylene glycol (PEG) and other
inducers - Kao and Michayluk (1974) for the first time
obtained a high frequency (10%) of fusion resulting in
heterokaryons of Vicia hajastana and Pisum sativum by
high molecular weight PEB (MW 6000-7,500) treatment. It
was observed that very few heteroplasmic fusions occurred
during the period when protoplasts were incubated in PEG
solution. However, many heterokaryons resulted soon
after the dilution of PEG in the fusion mixture. The same
phenomenon was also observed in PEG-treated protoplasts
from suspension-culture cells of Glycine max and from
leaves of Hordeum vulgare. Some of the Vicia-pea hetero-
karyons divided once. More than 10% of the soybean-barley
hybrids divided after 7 days. According to Kao and
Michayluk (1973) PEG concentration was very important for
heterokaryon formation and they found no significant differ-
ence in fusion by using PEG 1540 (MW 1300-1600) and PEG
6000 (MW 6000-7,500) in equal amounts (w.v.). They also
observed that prolonged incubation of protoplasts in PEG

solutions reduced the fusion frequency and sometimes proved harmful for the survival of protoplasts.

Later, Constabel and Kao (1974) obtained tight agglutinations of protoplasts isolated from pea leaves and *Vicia* cell cultures by the use of PEG 6000. Addition of calcium salts in PEG solution was found to enhance the agglutination. They also reported that pretreatment of protoplasts with 0.1g/l lysozyme apparently promoted the formation of heterokaryocytes despite the lower degree of agglutination. Pretreatment of protoplasts with 0.01g/l lysozyme, 0.1g/l papain, 0.01% glutaraldehyde and 0.01g/l lysolecithin was found to reduce agglutination, from which it was concluded that the integrity of the plasmalemma and electrostatic charges on the outer surface of the membrane are necessary for PEG-induced agglutination and ultimately fusion.

Constabel *et al.* (1976) found that the products of all interspecific and intergeneric fusions underwent cell division. The capacity to divide proved the compatibility of the combinations of protoplasts tested. However, they warned that this compatibility of protoplasts from distantly related species should not be extrapolated to the assumption that differentiation process at later stages would generally remain unimpaired. PEG-induced

fusion has certain advantages since it is nonspecific and, therefore, useful for both inter -and intra-specific fusion. It also induces a high fusion frequency (100%), Vasil et al. (1975). There are a few disadvantages of using PEG for fusion. It results in the formation of large clumps by the fusion of many protoplasts. Such clumps do not have any practical importance. However, by carefully modifying the fusion conditions, like molecular weight and concentration of PEG, pH, temperature of the mixture etc., it is possible to obtain fusions between 2-3 protoplasts. Another disadvantage is that sometimes PEG causes desiccation of protoplasts resulting in their shrinkage.

In addition to the above mentioned, many more fusion inducers have been used by various workers with limited success. Keller et al. (1973) observed the frequency of protoplast fusion to be 0.1-1% in the presence of several possible inducing agents which included "Sendai" virus, high temperature, deplasmolyzing osmotic shock, sodium nitrate, lysolecithin, ATP, ADP, cyclic-AMP and poly-L-ornithine. Differential nuclear staining with modified carbol-fuchsin permitted the identification of hetero-karyotic protoplasts in suspension cultures of Glycine max and V. hajastana. Binding (1974) observed a high proportion of hybrid protoplasts in hybrid protoplasts after

treatment with 85% sea water; $0.2M$ $Ca(NO_3)_2$; $0.2M$ $CaCl_2$;
$0.9M$ $NaNO_3$; and 0.05% lysozyme in $0.6M$ mannitol. He
concluded that weak deplasmolysis, alkaline pH and prolonged
contact favoured fusion. In addition to above, many other
chimicals have been used by workers. These include, poly-
L-ornithine, poly-D⊖lysine, poly-L-lysine, cytocholasin B
and protamine sulphate (Grout & Coutts, 1974), lysozyme
(Potrykus, 1971b), glycerol and dimethyl sulfoxide (Ahkong
et al., 1975a) with varying degrees of success.

Immunological Method

Hartmann et al. (1973) used a novel method for
agglutinating protoplasts from suspension cultures of
various species. They found that the antibody prepared
against Vicia protoplasts agglutinated both Vicia and
soybean protoplasts as well as a mixture of the two.
Soybean and bromegrass antibody was found to cross-react
and agglutinate with Vicia protoplasts. They also
demonstrated by carbol-fuchsin staining of nuclei that
Vicia and soybean protoplasts were mixed randomly in the
aggregates. Protoplasts were found to be viable and
undergo division after the antibody treatment. This
method, however, has not gained much popularity since
this method is useful in aggregating rather than fusing
protoplasts.

MATERIALS AND METHODS

Materials used were leaves and haploid callus of
Datura innoxia Mill., _Nicotiana tabacum_ Linn., and
leaves of _D. metel_ Linn. and _N. rustica_ Linn., belonging
to the family Solanaceae. Selection of these materials
was based on the fact that both _Datura_ and tobacco have
been reported to be very suitable for the production of
haploid callus from anthers, and these haploid cells as
well as somatic cells divide very easily on simple
inorganic medium (_see_ Sunderland, 1971) thus providing
an added advantage for studies on somatic cell hybridization.

In most of our work, however, the haploid and
diploid callus obtained from various strains of _Arabidopsis
thaliana_ (L.) Heynh., has been employed. The advantages
of using _Arabidopsis_, as an experimental material, are
well known -- it is easy to grow these plants under
sterile cultures, it has a short life cycle (30-40 days),
and a small chromosome number (n=5). Further, many
mutants, shown to be adaptable to cultural conditions
have been isolated in _Arabidopsis_, which could be utilized
for obtaining somatic cell hybrids.

The methods standardized for the above materials
were also employed to isolate protoplasts from leaves of
wheat, radish, cabbage, potato and _Petunia_. Protoplasts
were also successfully isolated from the haploid callus

of *Petunia*.

ISOLATION OF PROTOPLASTS

Materials

Leaves of *Nicotiana* and *Datura* - Leaves of *Nicotiana* were collected from potted plants and those of *Datura* from wildly growing plants. Approximately 1 gm leaves, whose lower epidermis had been peeled off, were treated with 20 ml of a mixture of pectinase and cellulase of the composition as shown below:

Chemical	Concentration
Pectinase (NBC)[1]	0.5% (w/v)
Cellulase (NBC)	5.0% (")
Sucrose (B.D.H.[2], ANALAR)	20% (")
OR	
Mannitol[3]	0.6M

The pH of the mixture was adjusted to 5.6 with N/10 HCl. After the initial vacuum infiltration for 5-10 min, leaf pieces were incubated in this enzyme mixture for 5 hr at 36C (either in a water-bath or in a BOD chamber). Known volume (10 ml) of the suspension was drawn out from the enzyme mixture with a 'Pasteur' pipette

1. NBC - Nutritional Biochemical Corporation, Cleveland, Ohio.
2. B.D.H. - British Drug House.
3. Obtained from - Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan.

and distributed equally in two centrifuge tubes, to each of which an equal volume (5 ml) of 20% sucrose solution was added. Centrifugation was carried out at about 800 rpm for 5 min. Thereafter, different fractions containing protoplasts were collected (see Flow-Sheet No. I). Alternatively, the protoplasts were collected by the floatation method by which a layer of protoplasts free of cells and debris was obtained in 20% sucrose.

Pea - Pea seeds were washed and germinated on germination paper, soaked in water. The radicles from 4-day-old seedlings were employed for experimental work. Seedlings were maintained in water-culture. The radicles were dipped in water by inserting through holes bored in aluminium foil used as cover for the containers. Some of the seedlings in water culture were kept in darkness, whereas the rest of the cultures were maintained in light. Protoplasts were isolated from the radicles, the dark grown etiolated shoots as well as from green leaves. The lower epidermis of the green leaves was peeled off before treating with the protoplast isolation mixture which comprised the following: macerozyme 1% (w/v), cellulase 'Onozuka' 5.0% (both obtained from Yakult Biochemicals Co. Ltd., Japan) and mannitol 0.5M.

The pH of the mixture was adjusted to 5.8 and the incubation carried out at 25C in a water-bath shaker without

Flow Sheet NO.1
Isolation & Collection Of Protoplasts

Leaf pieces with their lower epidermis removed

Leaf

suspension

Leaf pieces vacuum infiltrated with the enzyme mixture : 0·5% pectinase + 5% cellulase } 20% sucrose pH 5·6

5 ml. of enzyme mixture containing protoplasts + 5 ml of 20% sucrose.

Centrifuge at 800 rpm for 5 min

1ml

Fr_1

observed on a slide

To remaining 1ml of mixture add 1ml of 20% sucrose and centrifuge for 2 min at 800 rpm

observed on a slide

Fr_2

Fr_3

Fr_3

Fr_2

observed on a slide

any prior vacuum infiltration or shaking. The protoplasts were collected by centrifugation and washed thrice by removing the supernatant and adding fresh 0.5M mannitol after which they were suspended in mannitol solution only.

Arabidopsis - Seeds of Arabidopsis were sterilized for 7 min in a solution containing equal parts of ethyl alcohol and 20% (volume) of H_2O_2. They were then grown on Arabidopsis medium prepared according to Griffing (1956). The composition of the medium is shown on page-39. After giving a cold treatment at 4C for 48 hr, seeds were transferred to the culture room at 25C under fluorescent light. Plants varying in age from 25 days to 60 days (the age of the plant counted from the day of germination onwards) were employed for different experiments. The protoplasts were isolated from leaves, shoots, roots, flower buds and mature flowers. These parts were clipped and put in the enzyme mixture containing : macerozyme 0.5% (w/v), cellulase 'Onozuka' 2% (w/v) and mannitol 0.4M-0.5M. The pH of the mixture was adjusted to 5.6 and the incubation carried out at 25C in a water-bath shaker at low speed.

Callus - Haploid callus was obtained following the technique of another culture (Guha and Maheshwari, 1966). Pollen grains of Nicotiana at the uninucleate stage were cultured on White's medium (WM), comprising the following

34

(mg/liter), MgSO$_4$ 360, Na$_2$SO$_4$ 200, K$_1$NO$_3$ 80, KCl 65, NaH$_2$PO$_4$
165, Ca(NO$_3$)$_2$.4H$_2$O 260, MnSO$_4$.4H$_2$O 3.0, ZnSO$_4$.7H$_2$O 0.5,
H$_3$BO$_3$ 0.5, CuSO$_4$.5H$_2$O 0.025, Na$_2$MoO$_4$.2H$_2$O 0.025, CoCl$_2$
0.025, ferric citrate 10.0, glycine 7.5, niacin 1.25, thia-
mine hydrochloride 0.25, calcium pantothenate 0.25 and
pyridoxine hydrochloride 15. Pollen grains of Datura and
Petunia were cultured on WM supplemented with coconut milk
(15%). Hapolid callus of Arabidopsis was obtained by
culturing the anthers on WM supplemented with kinetin
5 ppm, 2,4-D 2 ppm, and casein hydrolyzate (CH) 100 ppm.
The callus was subcultured on WM supplemented with kinetin
2 ppm and 2,4-D 2 ppm. Approximately 500 mg of callus
cells were plasmolysed in 20 ml of either 0.7M mannitol
or 20% sucrose containing different concentrations of
pectinase (0.2%, 0.5%, 2%) and cellulase (2%, 5%) with
or without dextran sulphate. The pH of the enzyme
mixture was maintained between 5.4-5.8 and the callus
was incubated at 30C for 4-6 hr. The suspension was
examined for locating the isolated protoplasts.

Diploid callus was obtained either directly from
the seeds or by subculturing leaves and shoots on White's
medium supplemented with kinetin (10^{-5}M) + 2,4-D (2.4 x
10^{-6}M). Further subculturing was done on White's medium
supplemented with kinetin 2 ppm + 2,4-D 2 ppm + CH 100 ppm.
Haploid callus of Arabidopsis and P$_y$ and T$_z$ mutants of

Arabidopsis was obtained by culturing the anthers on
Arabidopsis medium supplemented with vitamins + kinetin
2 ppm + 2,4-D 2 ppm + indoleacetic acid (IAA) 6 ppm +
CH 100 ppm. Further subculturing was done on
Arabidopsis medium supplemented with vitamins + kinetin
2 ppm + 2,4-D 2 ppm. For isolating the protoplasts,
about 500 mg of one-month-old subculture of callus was
incubated in the enzyme mixture containing : macerozyme
0.5%-1% (w/v), cellulase 'Onozuka' 5.0% (w/v) mannitol
0.4M, or sucrose 20% (w/v). The pH of the mixture was
adjusted to 5.6. The incubation was carried out in a
water-bath shaker shaking at low speed.

RADIOISOTOPE INCORPORATION STUDIES

Isolated protoplasts were suspended in Murashinge
and Skoog's liquid medium (1962), such that the final
concentration of protoplasts was 4×10^4 protoplasts/ml.
The suspension was distributed equally in four tubes
and ^{3}H-leucine (2.5 uc/ml) or ^{3}H-uridine (2 uc/ml) was
added to three tubes. In the fourth tube which served
as the control, protoplasts were first killed with 1 ml
of 10% trichloroacetic acid (TCA) and the label added
afterwards. The incubation was stopped at different
intervals by chilling the protoplasts. The radioactive
precursors were removed with a pasteur pipette and the
protoplasts washed thoroughly with precooled autoclaved

Murashige and Skoog's medium after which they were
suspended in 1 ml sterile water. An equal volume of
10% TCA was added to it. The TCA precipitate was
collected and washed on a glass-fibre filter with
100 ml 5% TCA. The filter after drying was put in
toluene scintillation mixture and counts recorded in
liquid scintillation counter.

CULTURE OF PROTOPLASTS

Isolated protoplasts were passed through a steel
mesh (pore size 100 μ) after which these and the fused
protoplasts were washed with Murashige and Skoog (1962)
medium which contained the following (mg/l): NH_4NO_3 825,
KNO_3 950, $CaCl_2.2H_2O$ 220, $MgSO_4.7H_2O$ 1213, KH_2PO_4 680,
H_3BO_3 6.2, $MnSO_4.4H_2O$ 22.3, $ZnSO_4.4H_2O$ 8.6, KI 0.83,
$Na_2MoO_4.2H_2O$ 0.25, $CaSO_4.5H_2O$ 0.025, $CoSO_4.7H_2O$ 0.030,
thiamine hydrochloride 1, naphthaleneacetic acid 3,6-
benzylamino purine 1, mesoinositol 100, $FeSO_4.7H_2O$ 27.8
and Na_2EDTA 37.3. It also contained 10g/l sugar, 12 g/l
agar and 0.5M mannitol. The pH was adjusted to 5.8.
Sometimes, the protoplasts were washed with modified
Murashige and Skoog medium, commonly referred to as
Nagata and Takebe (1971) medium, and comprising the
following (g/l) : KH_2PO_4 0.027, KNO_3 0.101, $MgSO_4$ 0.246,
$CaCl_2$ 1.47, KI 0.166, $CuSO_4$ 0.249, 2,4-D 1 ppm, kinetin
1 ppm, mannitol 0.5M. Centrifugation was followed at a
very low speed ca. 500 rpm and the supernatant was removed

with the help of a pasteur pipette. After three washings, the protoplasts were suspended in liquid or solidified Nagata and Takebe or Gamborg B_5 medium containing the following (mg/l) : $NaH_2PO_4.H_2O$ 150, KNO_3 3000, $(NH_4)_2SO_4$ 134, $MgSO_4.7H_2O$ 500, $CaCl_2.2H_2O$ 150, iron (sequestrene 330 Fe') 28, nicotinic acid 1, thiamine.HCl 10, pyridoxine. HCl 1, m-inositol 100, $MnSO_4.H_2O$ 10, H_3BO_3 3 and $ZnSO_4.7H_2O$ 2 and containing the following (ug/l) : $Na_2MoO_4.2H_2O$ 250, $CuSO_4$ 25, $CoCl_2.6H_2O$ 25, KI 750 and also sucrose (20 g/l) and 2,4-D (2 mg/l). The pH of the medium was adjusted to 5.5. Modifications of auxin and kinetin concentration in these media were also used. The concentrated proto-plast suspension was then mixed with molten medium kept at 45C and poured in small petri-plates which were sealed with parafilm. Alternately, the concentrated protoplast suspension was diluted with liquid medium and poured in petri-plates which were sealed and kept in dark for 48 hr, after which they were transferred under fluorescent light (ca. 2,000 lux). The protoplasts were plated at a density varying from 5×10^4 - 2.5×10^5 protoplasts/ml. However, instead of 0.7M mannitol, 0.5M mannitol was added to the medium. Routine studies were made by drawing out some suspension from the petri-plates and observing under the microscope.

STUDIES ON FUSION

After isolation, protoplasts were washed with 0.5M mannitol. They were then allowed to fuse in a solution containing any of the following chemicals : nitrates of Na^+, K^+, Ca^{++} or Mg^{++}, high molecular weight polyethylene glycol, gelatin, or concanavalin A or in $CaCl_2$ solution at high pH and temperature. Cell fusion was observed under the microscope after staining the protoplasts either after or before fixing them. Cell aggregations were also studied by spectrophotometer. The initial optical density of the samples and the controls was taken at 720 nm. Then the chemical for inducing fusion was added in the sample and the change in O.D. recorded after different intervals of time. A decrease in O.D. indicated aggregation of protoplasts.

FIXATION AND STAINING

The protoplast suspension was centrifuged at ca. 700 rpm after addition of an equal volume of the fixative whose composition is given below:

Acetic alcohol (1 part glacial   :   75% (v/v)
acetic acid and 3 parts ethanol)

in

Mannitol or sorbitol   :   0.2M

Protoplasts were stained with a drop of modified carbol fuchsin stain (Miller et al., 1971) which had the following composition (per 100 ml):

Carbol fuchsin (Carr & Walker, 1961) : 2-10 ml

Acetic acid (45%) : 90-98 ml

Sorbitol : 1.8 gm

INVESTIGATIONS ON MUTATION

Seeds of A. thaliana were treated with 16 mM ethylmethane sulfonate for 16 hr, washed, surface sterilized and placed on a minimal medium. The plants which were growing poorly on the minimal medium were suspected to be mutants. These were picked up and transferred to a medium rich in amino acids where they grew well. As no seed setting was obtained in these, their callus was obtained by the technique mentioned earlier. Attempts were made to find out which amino acids the mutant could not synthesize by eliminating one amino acid in an experimental set, each time.

COMPOSITION OF ARABIDOPSIS MEDIUM (AM)

Major Salts	g/liter
KH_2PO_4	0.272
KNO_3	0.607
$Ca(NO_3)_2.4H_2O$	0.945
$MgSO_4$	0.241

Dissolved the first three chemicals in 500 ml of H_2O to which was added $MgSO_4$ dissolved in another 500 ml of H_2O.

Minor Salts	mg/liter
$Na_2B_4O_7 \cdot 10H_2O$	4.35
$MnSO_4 \cdot H_2O$	1.516
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.078
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0184
Iron	mg/liter
$FeSO_4 \cdot 7H_2O$	10.00
Disodium Edetate (EDTA, Disodium salt)	17.14

Dissolved 1.714 gm EDTA in 85 ml of distilled water. To this minor salts were added and pH was adjusted to 4.6. Air was drawn through solution until the colour of the solution became dark red.

To prepare the basal medium, added 100 ml of the major salt solution to 900 ml distilled water. Added 1 ml minor salt solution per liter of medium. Adjusted the pH to 6.0. Ten grams of sucrose, and molten Nobel Agar to make the final concentration 0.8%, were added to the medium which was dispersed in tubes and autoclaved at 15 psi for 15 minutes.

RESULTS

INVESTIGATIONS ON FINDING SUITABLE CONDITIONS FOR THE

ISOLATION OF PROTOPLASTS FROM DIFFERENT PLANTS

Materials

1. **Nicotiana and Datura** - Protoplasts were isolated

as described earlier (see Materials and Methods). A

drop of the mixture, observed in a cavity slide, was found

to contain a number of protoplasts which were large and

spherical in Nicotiana (Figs. 1-2) but comparatively

small in Datura. Protoplasts of Datura were less green

than those of Nicotiana. The size of protoplasts of

Datura leaves varied between 20-30 μm in diameter and

Nicotiana leaves from 30-50 μm in diameter. Chloroplasts,

which were quite characteristic in both the species were

seen to change their positions under the influence of

light from the microscope. Such movements of the contents

of the protoplasts indicated that they were alive. Starch

grains, cytoplasmic threads and vacuoles were also observed

in the protoplasts. In addition, a curious phenomenon

was observed in Nicotiana protoplasts where protoplasts

often showed conical protuberances which could have

developed for the purpose of fusion (Fig. 1). Occasionally

spontaneous fusions between protoplasts were observed

(Fig. 3).

For maintenance of shape of the protoplasts, osmotic

stabilizers such as mannitol and 20% sucrose were used.

Figs. 1-2.　Protoplasts isolated from leaves of Nicotiana

　　　　　　　tabacum.　Note the protuberance in one of

　　　　　　　the protoplasts in Fig. 1. X 800.

Fig. 3.　Spontaneous fusion between protoplasts of

　　　　　N. tabacum. X 800.

1

2

3
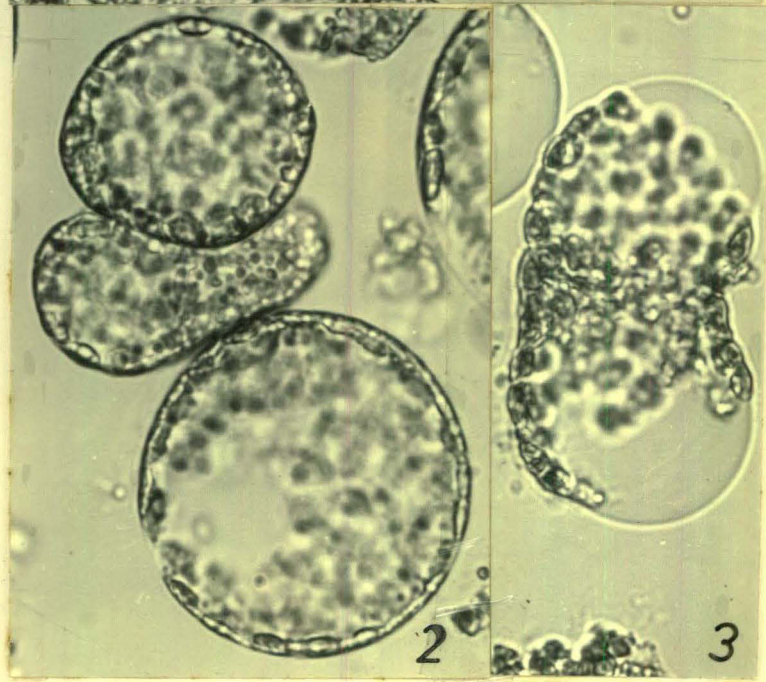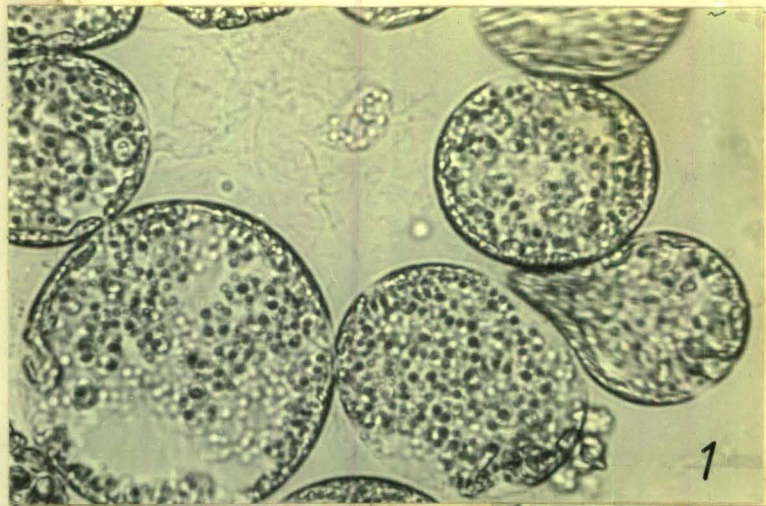
In 0.6M mannitol protoplasts maintained a regular shape for 48 hr whereas in 20% sucrose, they started losing their shape after 20 hr. The yield of protoplasts was 1×10^4 protoplasts/ml/g in <u>Datura</u> and 1×10^5 protoplasts/ ml/g in <u>Nicotiana</u>. In order to find out the method to get high yield of pure protoplasts after isolation in the enzyme mixtures, protoplasts were repeatedly centrifuged and fractions Fr_1, Fr_2 and Fr_3 (<u>see</u> Materials and Methods and Flow Sheet No. 1) were collected and the number of protoplasts in each fraction counted. It was seen that 52.5% protoplasts were lost in fraction 1 (Fr_1) during the first washing and 13.3% protoplasts were lost in fraction 2 (Fr_2), after the second washing. The sedimented fraction 3 (Fr_3) contained only 34.17% protoplasts which could be used for culturing or fusion. Another procedure, the floatation method, was employed for the recovery of pure protoplasts (<u>see</u> Materials and Methods). By this method, a green band of chlorophyllous protoplasts, free of debris was obtained in 20% sucrose.

2. <u>Pea</u> - The enzyme mixture employed for the isolation of protoplasts from radicles, etiolated shoots, leaves and apical buds of pea had the same composition as for <u>Nicotiana</u> and <u>Datura</u> (<u>see</u> Materials and Methods). The number of protoplasts, isolated after 4 hr was, 6×10^4/ml from both radicles and shoots, and 3.75×10^5/ml from both leaves

and apical buds. The size of the leaf protoplasts varied
from 20-50 µm in diameter and they were densely filled
with chloroplasts (Fig. 4). Pea radicle protoplasts
were 20-60 µm in diameter with hardly any chloroplasts.
The cytoplasm was thinly distributed on the periphery
of the protoplast leaving a large vacuole in the centre,
(Figs. 5-6). Protoplasts from etiolated shoots of pea
were 40-60 µm in diameter, lacking chloroplasts. Cyto-
plasm was found to be sparsely distributed at the
periphery leaving a central vacuole (Fig. 7). Pea apical
bud protoplasts were 20-50 µm in diameter and contained
a good number of chloroplasts. The vacuole was quite
characteristic (Fig. 56). Protoplasts isolated from
various parts of pea were further utilized in intraspecific
and intergeneric fusion experiments.

3. <u>Arabidopsis thaliana</u> - Since this material was
employed for most of the experiments, a detail investigation
was undertaken to study the chemical and physical parameters
suitable for the isolation of protoplasts.

Influence of Chemical Factors

1. <u>Osmoticum</u> - In most of the experiments, mannitol
was used as the osmotic stabilizer. To about 1 gm leaf
material of <u>Arabidopsis</u>, 10 ml of an enzyme mixture
containing 0.5% macerozyme, 5% cellulase and 0.3M, 0.4M or

Fig. 4. Protoplast from pea leaves showing dense
chloroplasts x 320.

Fig. 5-6: Protoplasts from pea radicles showing large
vacuoles in the center.
Fig. 5 - x 320
Fig. 6 - x 800.

Fig. 7. Protoplasts isolated from etiolated shoots
of pea x 320.

4

5

6

7
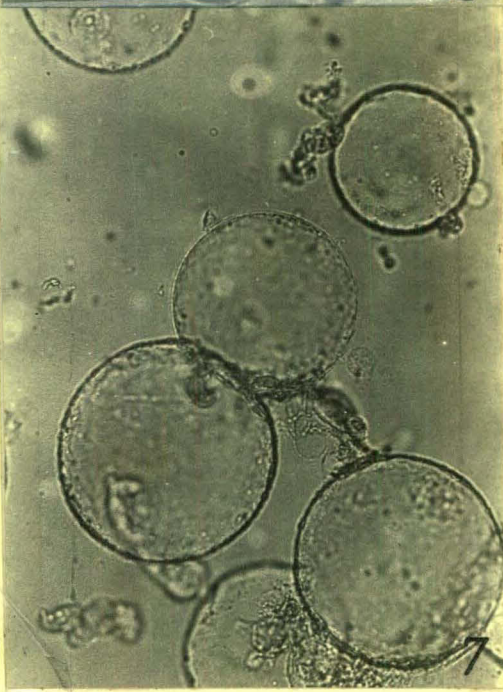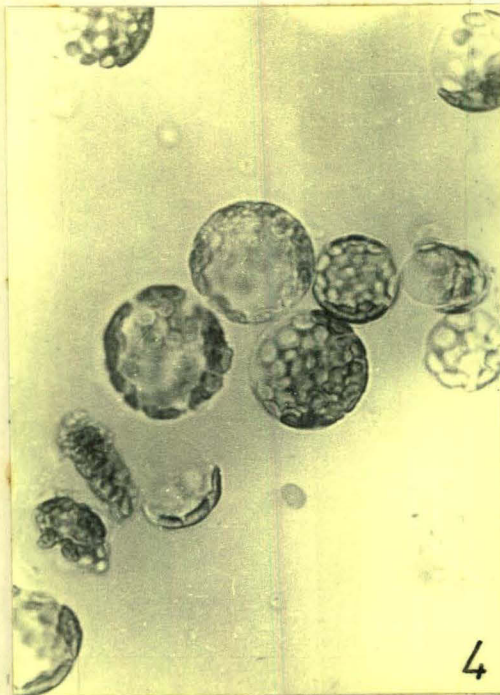
0.5M mannitol was added. The pH was adjusted to 5.6 and
the incubation carried out without vacuum infiltration
at 30C. The number of protoplasts released and the
conditions of contents inside them were recorded. From
Fig. 8, it is seen that there was no significant difference
in the yield of protoplasts in the three concentrations
of mannitol used. However, under the microscope, it was
found that protoplasts were most stable in 0.5M mannitol
solution. In 0.3M and 0.4M mannitol, the stability of the
protoplasts was adversely affected and many protoplasts
were seen to burst and throw out their contents after
swelling. Other osmotica like glucose, sucrose and
sorbitol were not found suitable enough for the isolation
of protoplasts. Salt solutions, like nitrates of K^+, Na^+,
Mg^{++}, Ca^{++} and calcium chloride in the concentration of
0.4M were also used in the isolation mixture for proto-
plasts and were found suitable for maintaining the stability
of the protoplasts for a short interval (ca. 5-6 hr). The
protoplasts were found to lose their shape after this
interval in all except Ca^{++} containing mixtures. Potassium
dextran sulphate, which is added to the isolation mixture
for the stability of protoplasts was also tested along with
mannitol as the osmoticum. In the incubation mixture, 1.25%
dextran sulphate was added, whereas the control was without
it. Figure 9 shows some reduction in the yield of proto-
plasts caused by inclusion of potassium dextran sulphate

Fig. 8. Kinetics of release of protoplasts from
the leaves of A. thaliana with different
concentrations of mannitol. Leaves were
incubated in the incubation mixture contain-
ing 0.3M, 0.4M or 0.5M mannitol as the
osmoticum and the number of protoplasts
siolated at various intervals of time was
recorded.

FIG.8
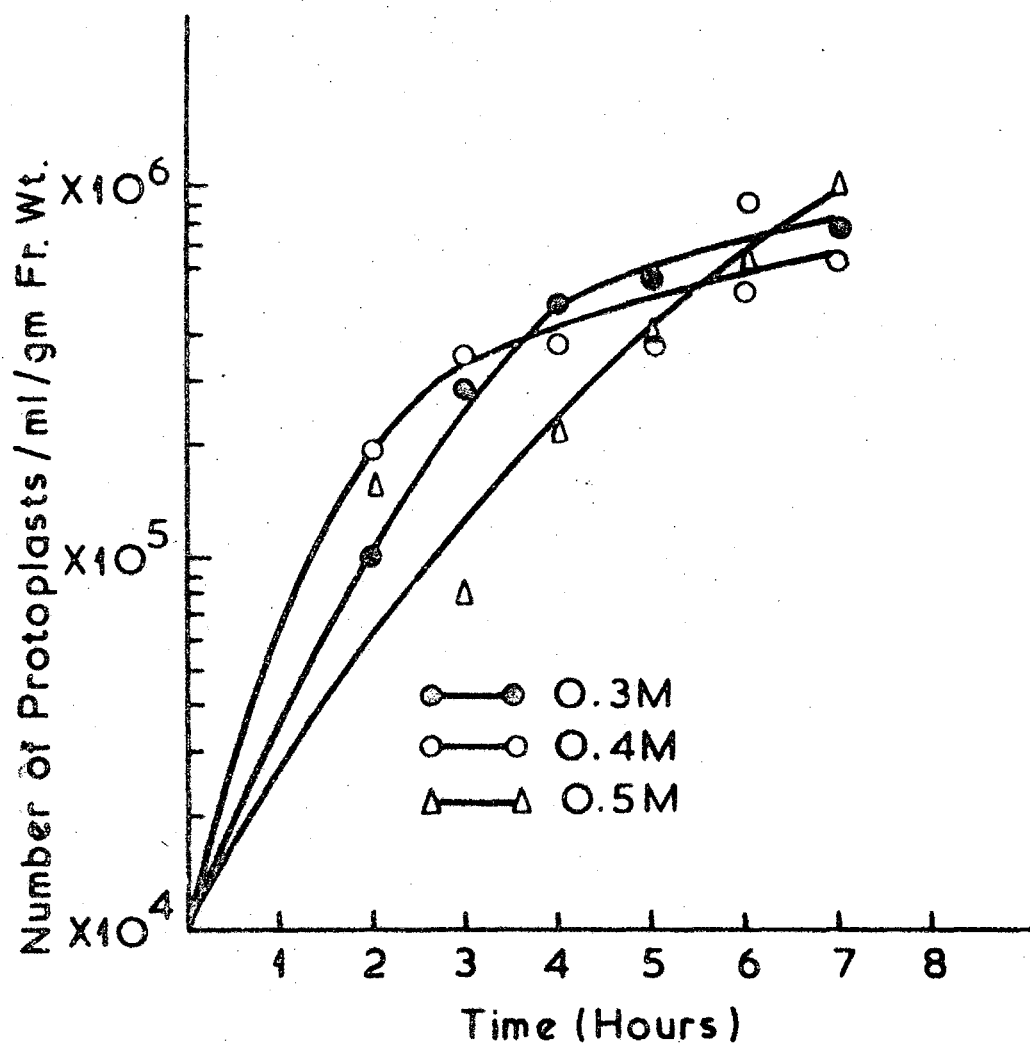
FIG. 9
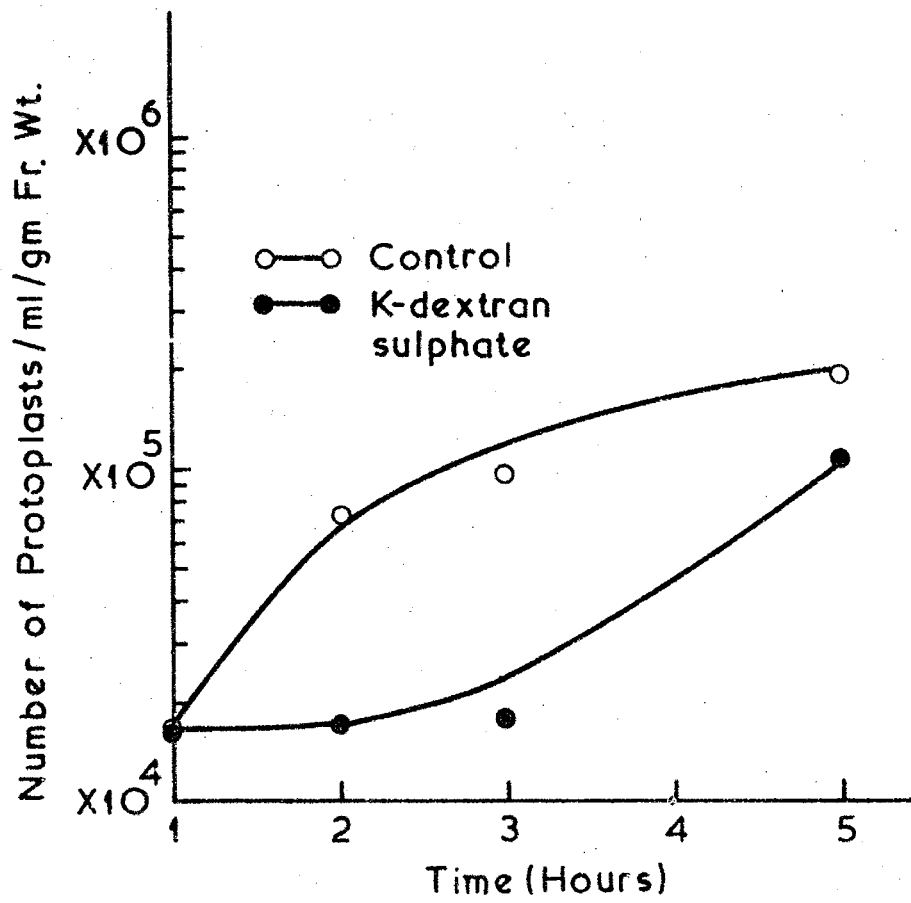
in the isolation mixture. This, thus shows that it is not useful in maintaining the stability of protoplasts of Arabidopsis.

2. Macerozyme - This is required to dissolve the middle lamella and separate the cells from each other. About 0.2 gm leaf material was incubated at 30C in 10 ml of 0.5M mannitol with the concentration of macerozyme varying from 0.1%-0.5%. The pH of the mixture was adjusted to 5.6. Figure 10 shows that 0.5% is the best concentration of macerozyme for releasing maximum number of cells, though a lesser number of cells is also released by lower concentrations. Figures 11 and 12 represent the single cells isolated from mesophyll and palisade layers, respectively. The presence of the enzyme, macerozyme, alone in the incubation mixture did not result in the release of any protoplast.

3. Cellulase - This enzyme is used to break down the cellulose of the cell walls. After one to two hours of incubation, the cell walls were partly digested and appeared slightly thin. As the enzyme penetrated further, the separation of the cell wall could be clearly seen (Fig. 13). Eventually the cell wall was totally digested resulting in the release of spherical protoplasts (Fig. 14). Inspite of the diversity in the shape of cells, the isolated protoplasts were all spherical. In order to

Fig. 10. Kinetics of release of cells from the leaves
of A. thaliana with different concentrations
of macerozyme. Leaves were incubated in the
incubation mixture containing 0.1, 0.2, 0.3,
0.4 or 0.5% macerozyme and the number of cells
isolated at various intervals of time was
recorded.

FIG.10
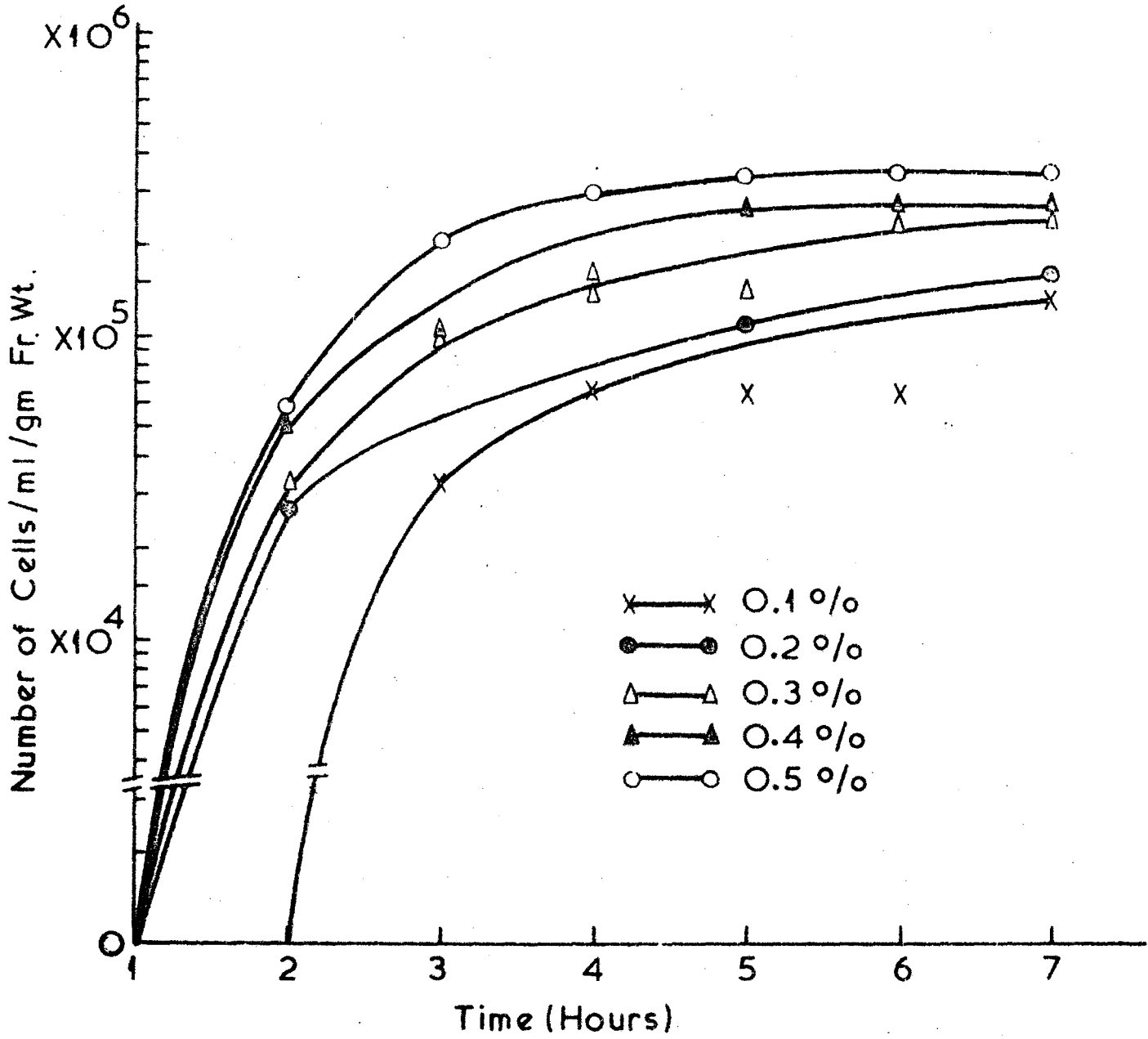
Fig. 11. A cell isolated from the mesophyll of
A. thaliana after macerozyme treatment.
X 800.

Fig. 12. A cell isolated from the palisade of
A. thaliana after macerozyme treatment.
X 800.
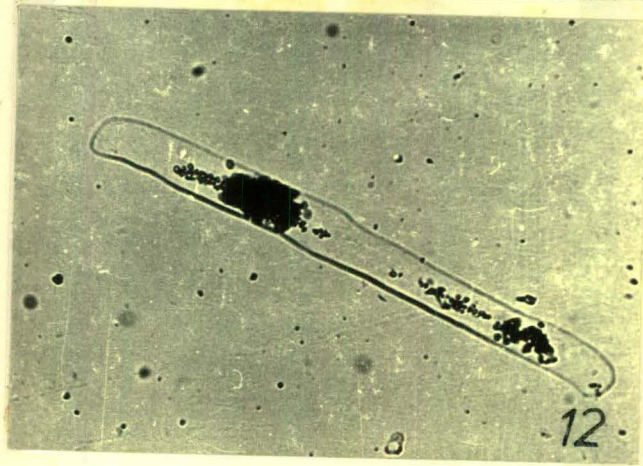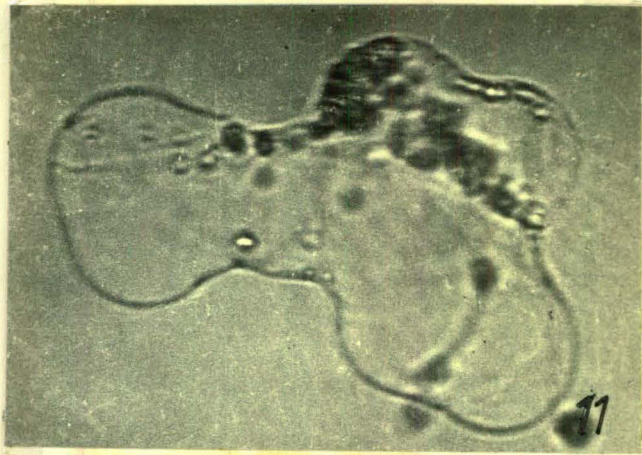
Fig. 13. Intermediate stage of cell wall digestion
following cellulase treatment. X 800.

Fig. 14. Protoplasts released after cellulase
treatment. Note the cytoplasmic strands in
one of them. X 800.
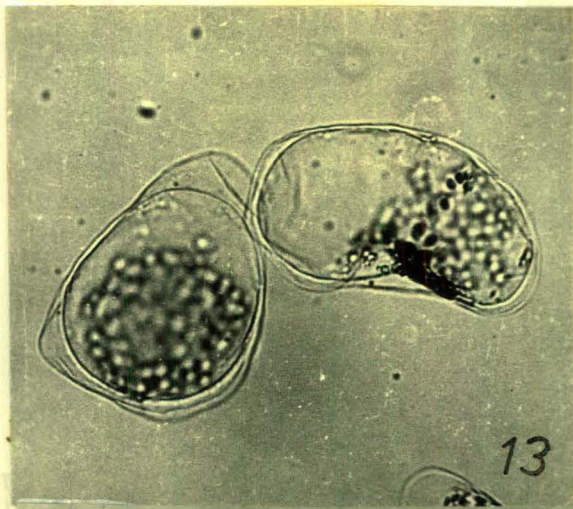
13

14

determine the best concentration of cellulase for isolating a large number of protoplasts in a short time, about 0.2 gm leaf material was incubated in 10 ml of a solution containing 0.5M mannitol, 0.5% macerozyme and 1%, 2%, 3%, 4% or 5% cellulase. The pH was adjusted to 5.6. From Fig. 15, it is seen that in 5% cellulase maximum number of protoplasts was isolated, though lower concentrations were also found effective in releasing quite a large number of protoplasts, and after seven hours of incubation, there was no significant difference in the yields in different concentrations.

4. pH - Since the enzymes used in the incubation mixture are active at particular pH values it was considered necessary to find out the optimum pH for the release of protoplasts. Approximately 0.1 gm leaf material was incubated in 5 ml of the enzyme mixture containing 0.5M mannitol, 0.5% macerozyme and 3% cellulase. The pH of the mixture was adjusted to 4.6, 5, 5.6, 6 and 6.6. The incubation was carried out at 25C and the kinetics of release of protoplasts recorded. As seen in Fig. 16, pH range from 5.0-6.0 was found to be most suitable for the isolation of protoplasts. Comparatively low yield of protoplasts was obtained below and above this range.

Fig. 15. Kinetics of release of protoplasts from the leaves of A. thaliana with various concentrations of cellulase. Leaves were incubated in the incubation mixture containing, 1,2,3,4 or 5% cellulase and the number of protoplasts released at various intervals of time was recorded.

FIG. 15
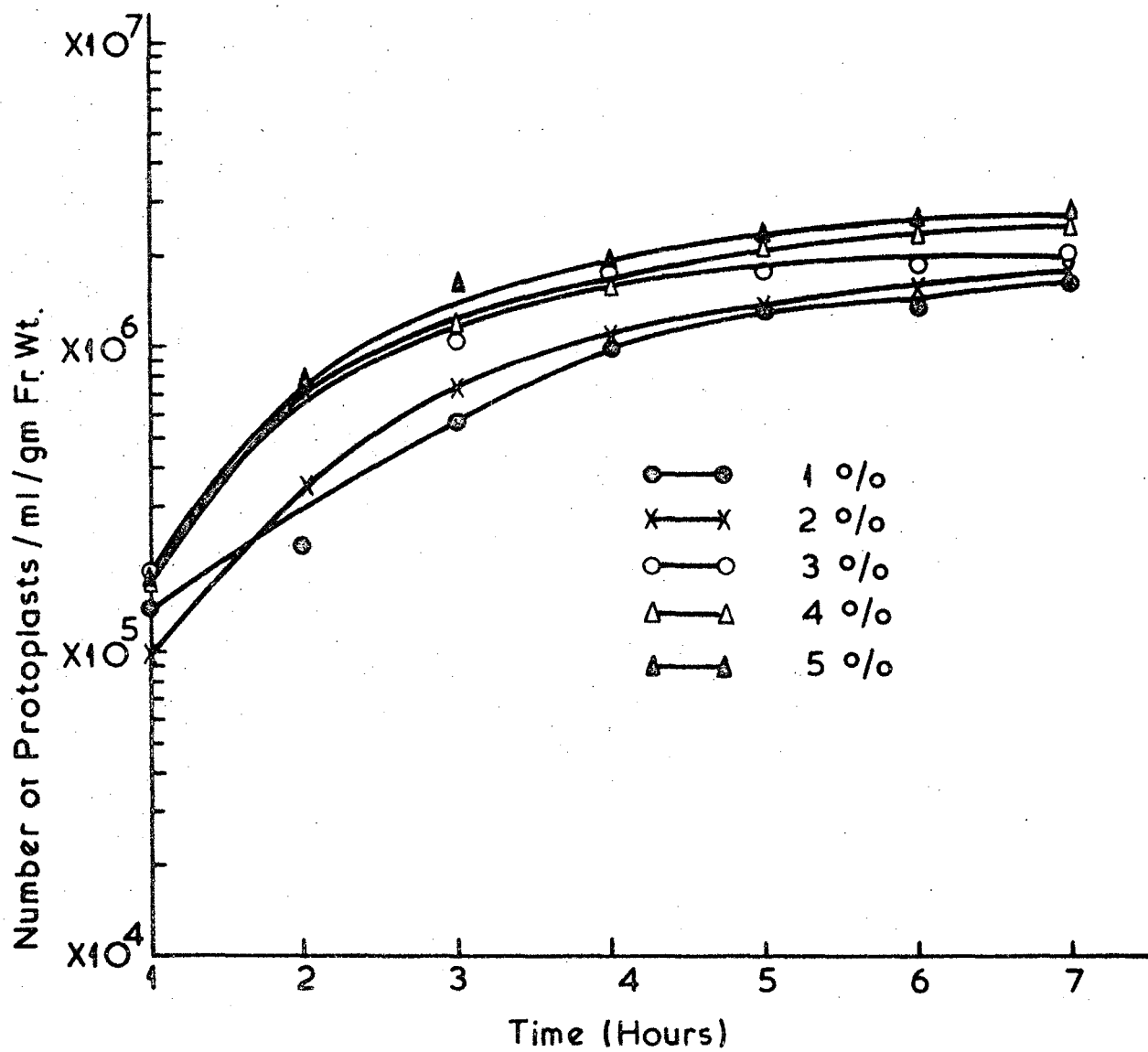
Fig. 16. Kinetics of release of protoplasts from the leaves of A. thaliana at different pH.

FIG. 16
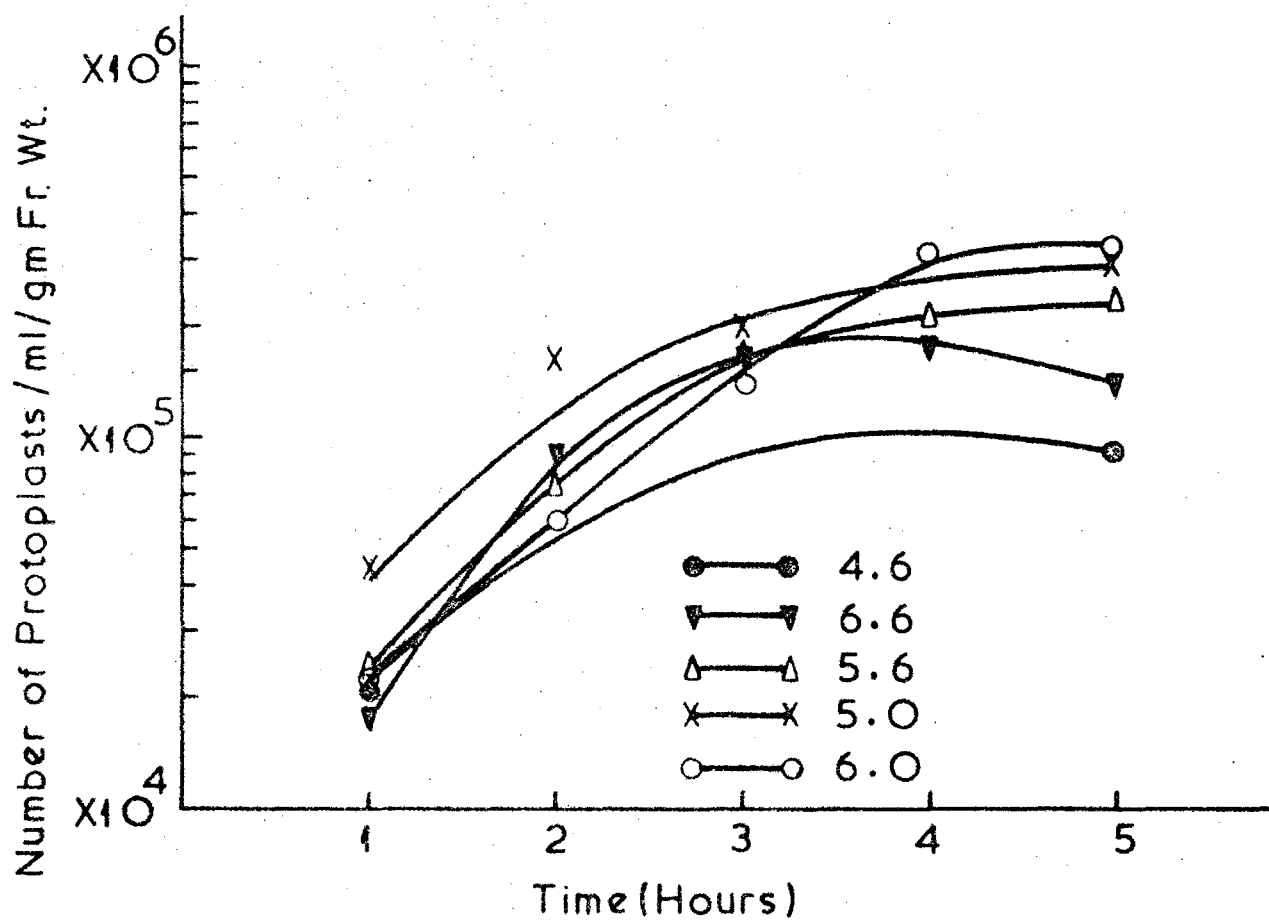
Influence of Physical Factors

1. **Light** - Two sets of experiments were carried out
to find the yield of protoplasts. In one set, 0.5 gm leaf
material was incubated in the incubation mixture containing
the enzymes and the osmoticum. In the other set, all
conditions were kept identical except that the incubation
was carried out in dark. One set was kept in a water-bath
shaker maintained at 25C in alight (ca. 2000 lux) and the
other set was maintained at 25C in a water-bath shaker
kept in dark and shaking at a low speed (45 strokes/min).
The kinetics of release of protoplasts was recorded. As
shown in Fig. 17, it was found that more protoplasts
were released in dark than in light and microscopic
studies revealed that protoplasts were comparatively
more stable in the former than in the latter.

2. **Temperature** - To study the most effective tempera-
ture for the release and stability of protoplasts, these
were incubated at different temperatures. It was found
that 30C was best for releasing protoplasts in short
duration (2-3 hr). At 22-25C, the release of a large
number of protoplasts required longer period varying from
12-16 hr. Protoplasts were killed at temperature
above 30C.

3. **Shaking** - A water-bath shaker (S.E.W.) shaking
at low speed (ca. 45 strokes/min.), was found to hasten

Fig. 12. Kinetics of release of protoplasts from the leaves of A. thaliana in light (ca. 2,000 lux) and dark.

FIG. 17
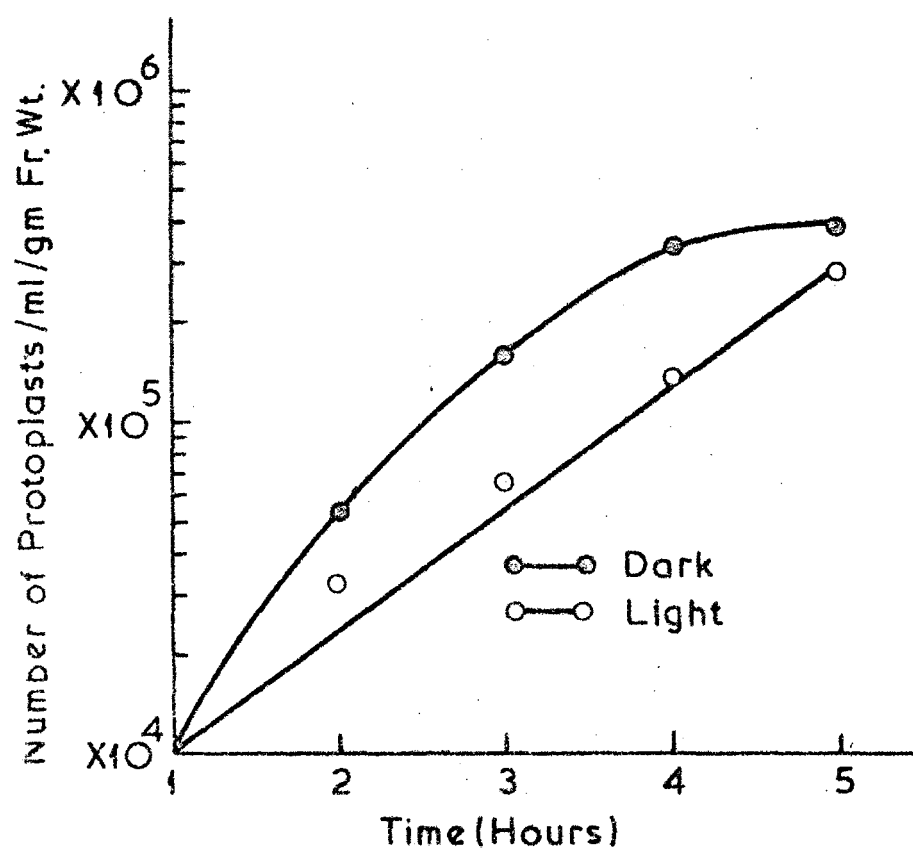
the release of protoplasts, though this caused slight
breakage too. At higher speeds, breakage of protoplasts
was more pronounced. When the incubation was carried
out for a long time (12-16 hr), shaking was not found
to be necessary.

4. **Kinetics of release** - The minimum time required
for release of protoplasts was found to be one hour. However,
the number of cells released after this period exceeded
the number of protoplasts. After 5-7 hr, a good yield
of protoplasts (Fig. 15) was obtained, but entire material
was digested only after 12-16 hr. Therefore, the maximum
yield of protoplasts was obtained only after this period.

5. **Age** - To find out whether there was any
correlation between the age of the plant and the yield
of protoplasts, ca. 0.5gm leaf material was incubated in
10 ml of the incubation mixture containing 0.5% macerozyme,
3% cellulase and 0.5M mannitol. The pH of the mixture
was adjusted to 5.6. Plants, varying in age from 25 days
to 56 days were used. The results are shown in Fig. 18.
It was seen that a high yield of protoplasts was obtained
from 56 as well as 36-day-old plants, though a good number
of protoplasts were also released from 25-day-old plants.
It was further seen that the protoplasts were released
within the first hour of incubation in the case of 25-day-
and 36-day-old plants. Protoplasts from 56-day-old plants

Fig. 18. Kinetics of release of protoplasts from the leaves of A. thaliana plants of different ages.

FIG.18
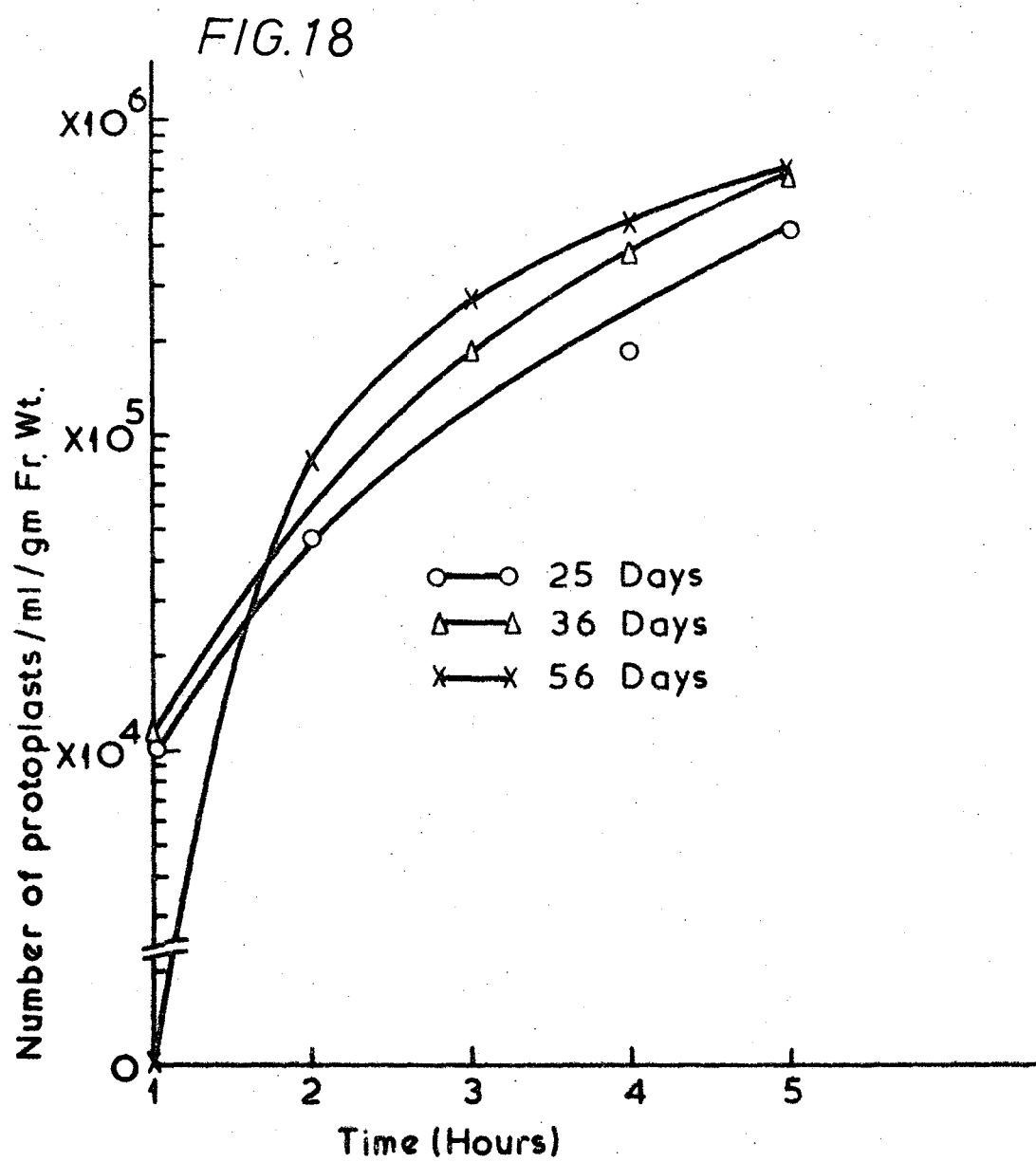
were not released in the first hour but in the second
hour of incubation. It was also observed that the
young plants yielded small, non-vacuolate or sparsely
vacuolate protoplasts which were full of the protoplasmic
contents; whereas protoplasts from older plants yielded
large, highly vacuolate protoplasts. It was difficult
to isolate protoplasts from plants below 22 days of age
and above 60 days.

6. Comparison of yield of protoplasts from different
organs - Protoplasts were isolated from roots, shoots,
leaves and flowers (flower buds were included along with
flowers) to find which of these yielded more protoplasts
with greater ease and how they were morphologicalèy
different. The incubation mixture contained 0.5M mannitol,
0.5% macerozyme and 3% cellulase at pH 5.6. As seen
from Fig. 19, it was observed that a comparatively high
yield of protoplasts was obtained from shoots, followed
by flowers, leaves and finally the roots. Roots proved
to be a tough material since the protoplasts were released
only after 7 hr of incubation. As regards the morphology
of protoplasts from different parts, it was found that
the protoplasts isolated from leaves were highly chloro-
phyllous and contained a large number of chloroplasts
(Fig. 20). Protoplasts isolated from shoots were the
largest in diameter (40-60 μm) and seemed highly

Fig. 19. Kinetics of release of protoplasts from roots, shoots, leaves and flowers (flower-buds included) of A. thaliana.

FIG.19
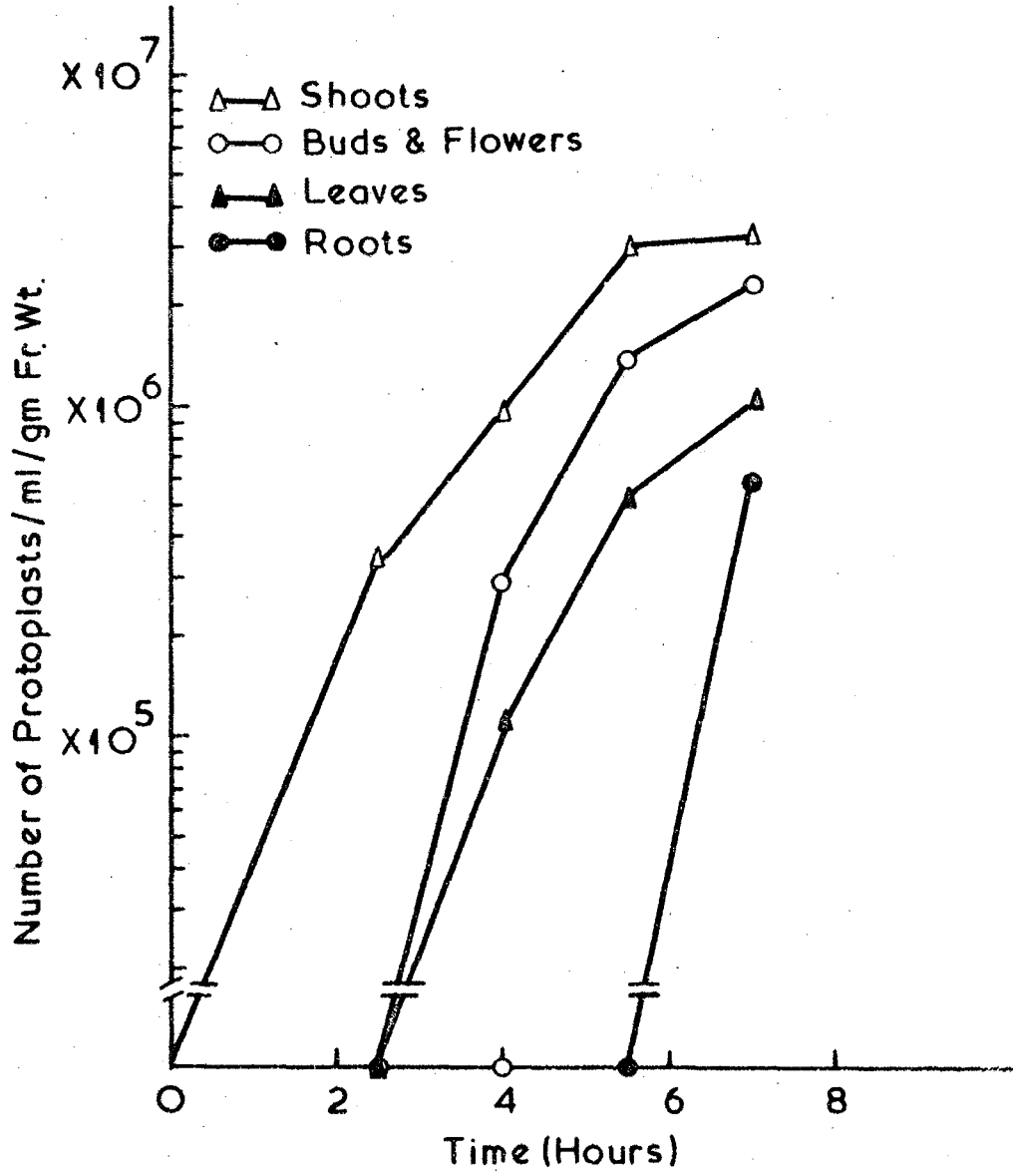
vacuolated (Fig. 21). Protoplasts from flower buds contained a few chloroplasts whereas those from roots were completely devoid of them. Protoplasts isolated from flowers and flower-buds appeared smaller than those isolated from leaves. Protoplasts isolated from the roots were the smallest in size (ca. 20 μm in diameter).

Isolation of Protoplast from Callus

Protoplasts were isolated from the haploid callus of Datura, Nicotiana and Petunia. Diploid callus of Arabidopsis and its Py mutant was also employed for the isolation. Isolation mixture was modified slightly in these (see Materials and Methods). Only fresh (one-week-old) subcultures of callus released a large number of protoplasts with ease. One-month-old callus, which was slightly hard, did not yield any protoplast even after various modifications of the isolation mixture. Isolation of protoplasts from diploid callus of Py mutants of Arabidopsis was better when sucrose was used as osmotic stabilizer rather than mannitol. The protoplasts isolated from callus were invariably spherical irrespective of the plant source utilized. They, however, differed in number and the distribution of vacuoles in the cytoplasm. Figures 22 and 23 represent the cells and intermediate stages of isolation of protoplasts from

Fig. 20. Protoplasts isolated from leaves of <u>A</u>. <u>thaliana</u>.
X 320.

Fig. 21. Protoplasts isolated from shoots of
<u>A</u>. <u>thaliana</u>. The larger protoplasts seem
to have formed as a result of spontaneous
fusion. X 320.

20

21
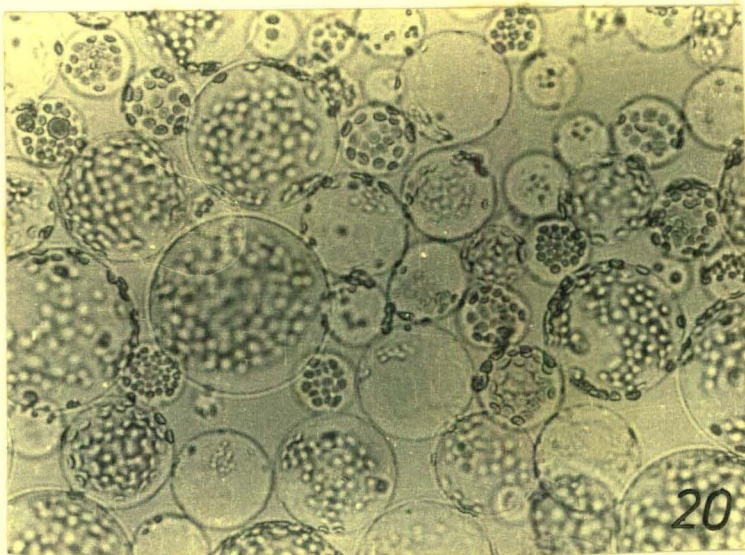
Py mutant callus. Figures 24 and 25 represent the protoplasts of Py callus of A. thaliana and haploid callus of Petunia, respectively. It was noted that whereas Py callus protoplast had 2-3 spherical vacuoles occupying different positions inside the protoplast, that of Petunia had a large central vacuole with cytoplasmic contents sparsely distributed on the periphery. Size differences were not prominent. Some of the protoplasts isolated from callus were found to contain chloroplasts as well. The kinetics of release of protoplasts from Py callus is shown in Fig. 26, from which it could be seen that the yield of protoplasts was 1.7×10^5 protoplasts/ml/gm after 5 hr of incubation.

STUDIES ON VIABILITY OF PROTOPLASTS

The protoplasts after isolation and washing were found to maintain their spherical shape. Protoplasts were deemed non-viable if they expanded too much in volume and burst or got shrunk. Protoplasts were also considered non-viable if they threw out their cytoplasmic contents. The protoplasts sometimes showed cyclosis (which is a particular kind of slow movement of the cytoplasmic contents manifested by a living cell) when observed under the microscope. Viability of protoplasts was also tested by the FDA fluorescence test (Larkin, 1976). To the protoplast suspension, FDA (flourescein diacetate),

Fig. 22.   Cells isolated from the callus of Py
          mutant of A. thaliana. X 320.

Fig. 23.   Intermediate stage of isolation of
          protoplasts from the callus of Py mutant
          of A. thaliana. X 320.

Fig. 24.   Protoplast isolated from the callus of
          Py mutant of Arabidopsis. X 800.

Fig. 25.   Protoplasts isolated from the haploid
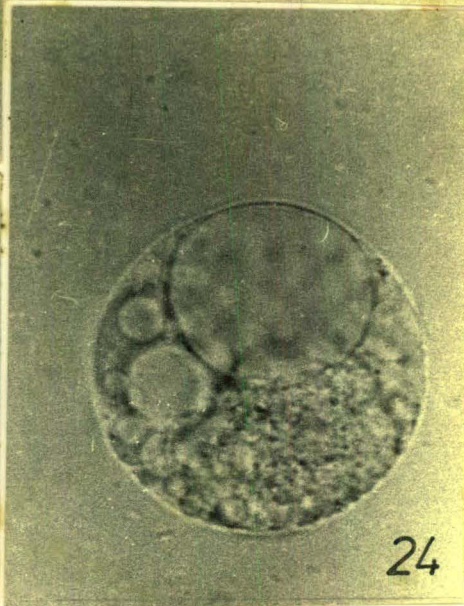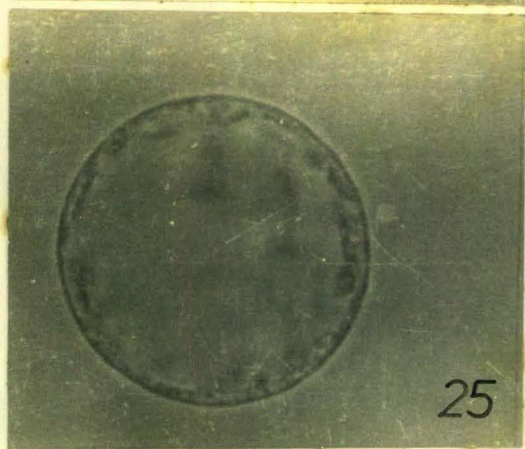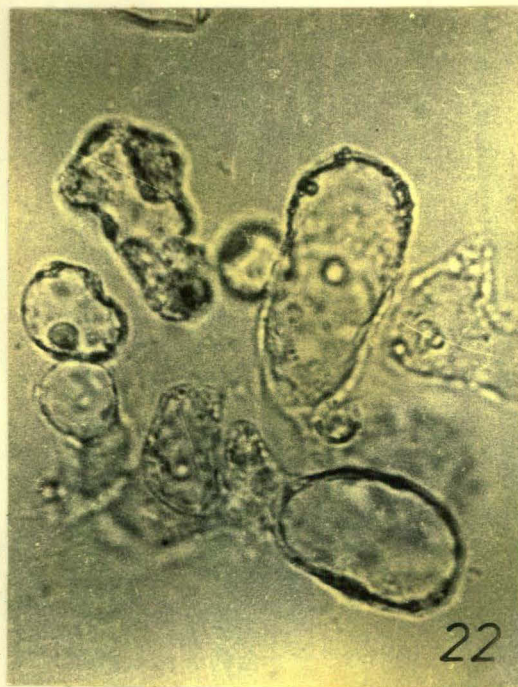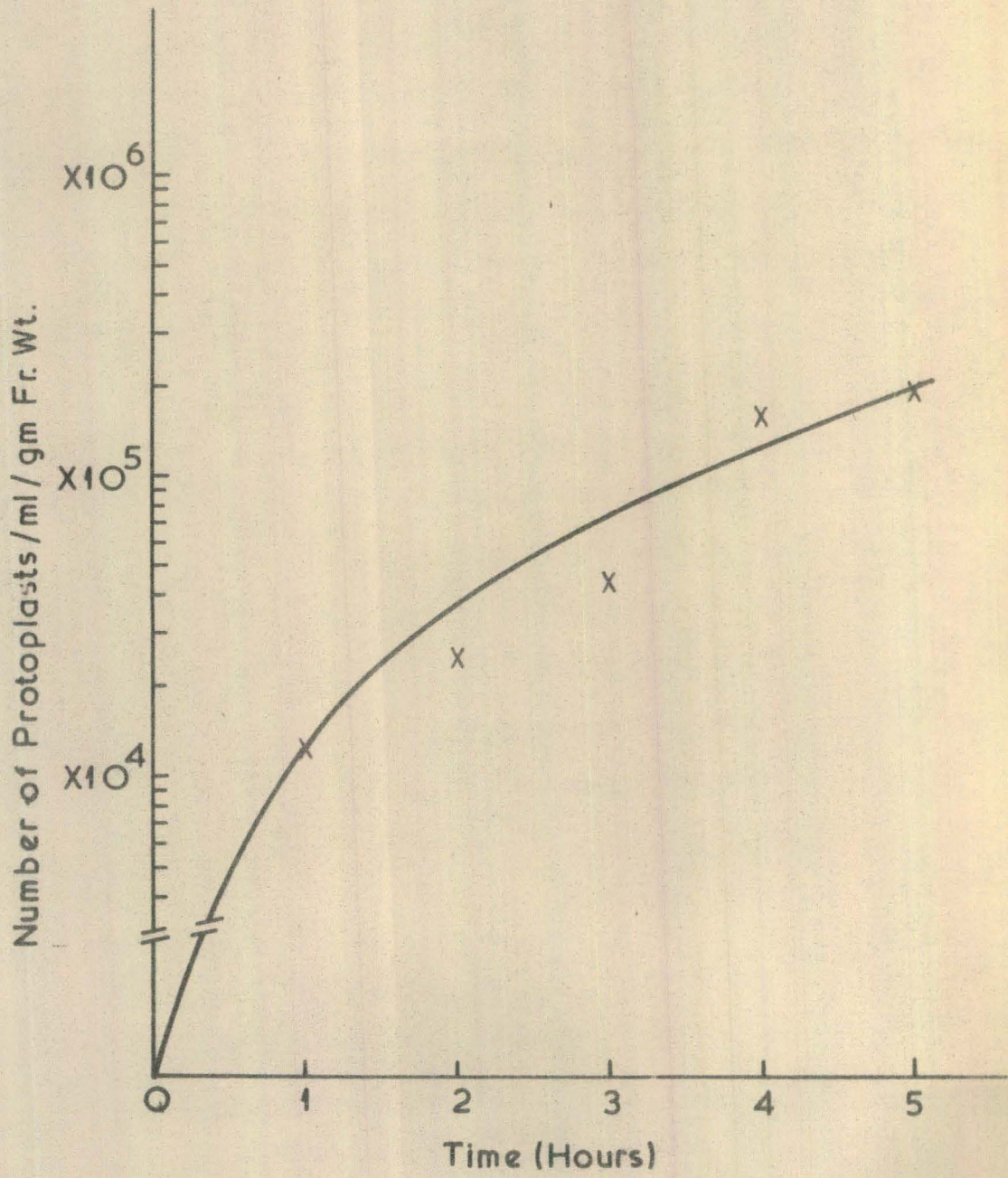   callus of Petunia. X 800.

22

23

25

24

Fig. 26. Kinetics of release of protoplasts from
the callus of Py mutants of A. thaliana.

FIG. 26

a viability stain, was added in the concentration of
0.01%. After 5 min, the protoplasts were examined for
flourescence using a Reichert Zetopan-Binolux fluorescent
microscope. The exciter filter "22" (transmission range,
300-400 nm) and UV absorpiton filter "2" (Transmission,
450 nm) were suitable. Protoplasts were considered via-
ble if they fluoresced and a yellow/green fluorescence
was obtained. Hundred percent protoplasts were found
to be viable according to this test. In addition, since
the incubation was carried out for the longest period
(16 hr) in Arabidopsis, radioactive incorporation studies
were done to see if these protoplasts were healthy enough
to synthesize RNA and proteins. From Fig. 27, it can be
seen that the protoplasts released after 16 hr incubation
in the enzyme mixture were able to incorporate a significant
amount of labels showing thereby that the protoplasts
isolated after a long period of incubation were still
alive.

CULTURE OF PROTOPLASTS

After the success in isolating a large number of
viable protoplasts, the next attempt was to grow them
in axenic culture. Various standardized media were
tried per se, as well as after modifications of the
hormone composition (see Materials and Methods). Small
aliquots were taken and examined on a slide under the

<u>Fig. 27.</u> Kinetics of RNA and protein synthesis by
protoplasts isolated from the leaves of
<u>A. thaliana</u>. The protoplasts were incubated
with ^{3}H uridine or ^{3}H leucine. The incubation
was stopped at different intervals by chilling
the protoplasts. The protoplasts were washed
and their TCA precipitate was collected on
filters and counted in a scintillation counter.

FIG. 27
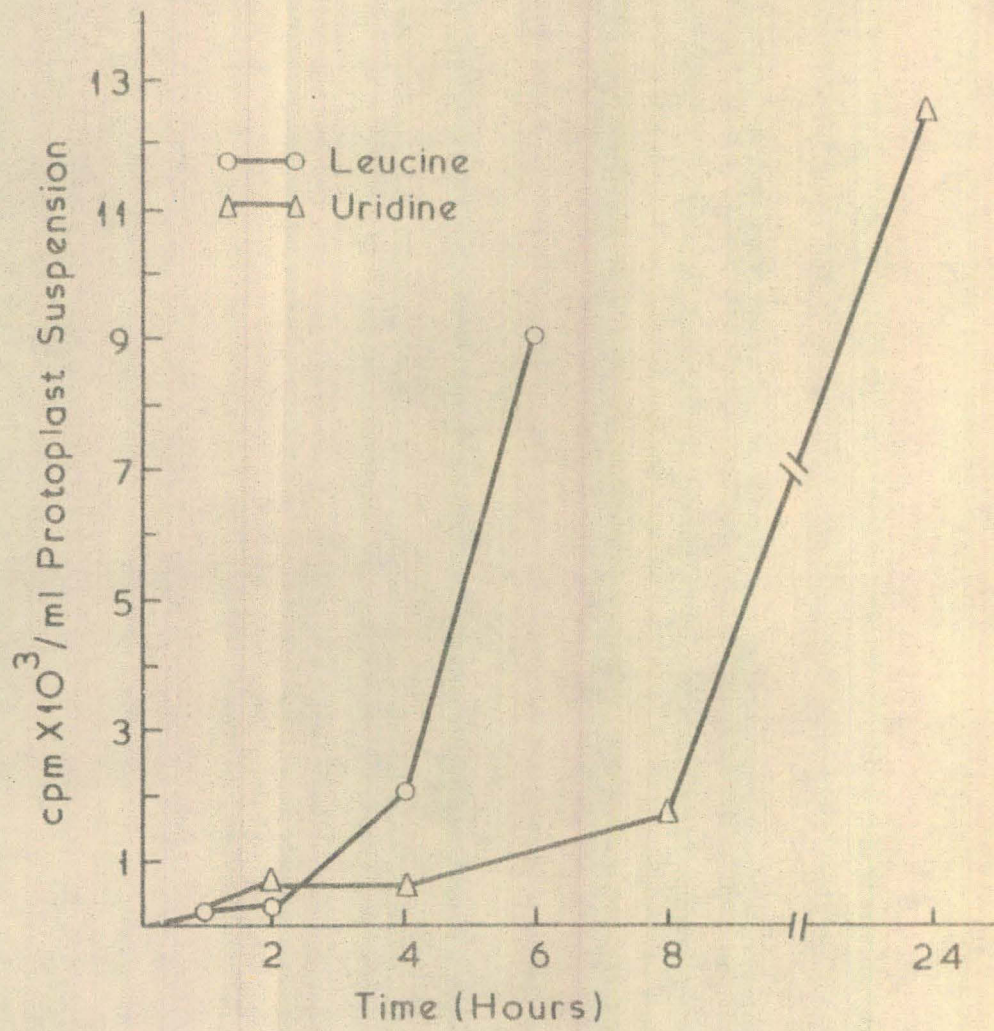
microscope from the day of culture onwards. The
protoplasts showed an increase in size after 24 hr and
sometimes did not appear absolutely spherical. Chloro-
plasts were found rearranged in a pattern differing from
the one observed on the first day of culture. They
seemed to be arranged on the periphery of the proto-
plasts or concentrated on one side or the other, rather
than distributed evenly inside the protoplasts. Accord-
ing to Nagata and Takebe (1970) this phenomenon suggested
an extensive development of cytoplasmic strands across
the central vacuole. After 48 hr of culture, some
of the protoplasts became oblong and others were seen
to have a thick boundary presumably the cell wall (Fig. 28).
These regenerated cells had spherical shape, in contrast
to the various shapes possessed by the cells, isolated
after the macerozyme treatment of the tissue. The
presence of the cell wall (seen after culturing the
protoplasts in Gamborg's B_5 medium with the addition of
10% coconut milk) was confirmed by subjecting the cells
to plasmolysis, when the protoplasm shrank away from
the wall and the cell wall was clearly visible (Fig. 29).
The percentage of protoplasts that regenerated a wall
was rather low, ca. 20-30%. These cells were sometimes
found to divide once on the fourth day of the culture,
in Nagata and Takebe (1971) liquid medium. This
phenomenon was, however, very rarely observed. In all

Fig. 28. Protoplast showing wall regeneration around
it. X 800.

Fig. 29. Shrinkage of protoplasm away from the
regenerated wall under the influence of
hypertonic solution. X 320.
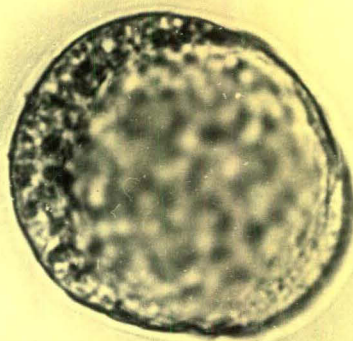
28
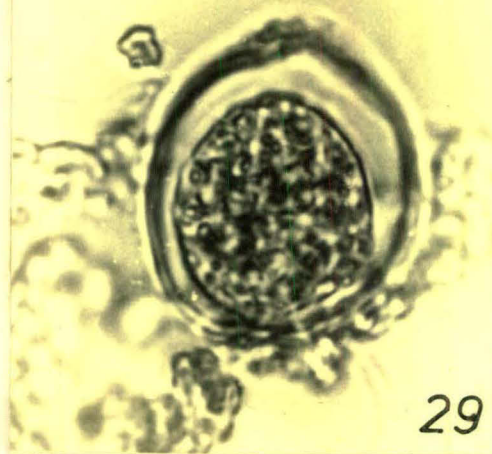
29

other cultures, the protoplasts either burst or somehow perished. Occasionally, colony formation was observed on Nagata and Takebe (1971) médium after one month of culture. Cells of these colonies proliferated and developed into a mass of callus.

INVESTIGATIONS ON FINDING CONDITIONS FOR FUSION

Fusion of protoplasts from the same species may occur spontaneously during isolation in the enzyme mixture. This phenomenon was observed very frequently in protoplasts isolated from Nicotiana (Fig. 3), and occasionally in Arabidopsis. Various substances, such as nitrates of Na^+; K^+, Ca^{++} and Mg^{++}; calcium chloride, gelatin, lectins and polyethylene glycol (PEG) were used to find out their suitability in bringing about fusions amongst the protoplasts of same tissue, same species (intraspecific) different species (interspecific) or different genera (intergeneric). The relative effects of the fusing agents are discussed in the following few pages.

Influence of Various Chemicals

1. Effect of sodium nitrate - Successful fusions were reported by Power et al. (1970) for the first time and then by Carlson et al. (1972) by sodium nitrate

treatment (see Previous Work). In the present investigation, it was seen that the leaf protoplasts of Datura, when washed on a millipore filter (see Flow-sheet No. 2), with 0.7M $NaNO_3$ and then suspended in 0.7M $NaNO_3$, aggregated and fused. The percentage of fusion varied from 2-3. In Arabidopsis, the leaf protoplasts were induced to fuse in a similar manner in 0.7M $NaNO_3$. The percentage of fusion varied between 4-5. But in this concentration of $NaNO_3$, the protoplasts looked highly shrunken. Therefore, the protoplasts were suspended in 0.4M $NaNO_3$ solution after washing. Aggregations took place amongst the protoplasts of Arabidopsis resulting in fusion between protoplasts from leaves (Figs.30-31); roots (Fig. 32) and also from callus (Fig. 33). Intraspecific fusions were also achieved in protoplasts isolated from cabbage and radish with the help of 0.5M $NaNO_3$. The percentage of fusion never exceeded 5 in all these tissues.

2. Effect of potassium nitrate - The protoplasts were isolated in the enzyme mixture in which 0.4M KNO_3 was used as the osmoticum. After 5 hr, these were washed and suspended in 0.4M KNO_3 solution. About 2-3% aggregation and 1-2% fusion was obtained in A. thaliana protoplasts.

3. Effect of magnesium nitrate - The protoplasts of A. thaliana were isolated in the enzyme mixture containing
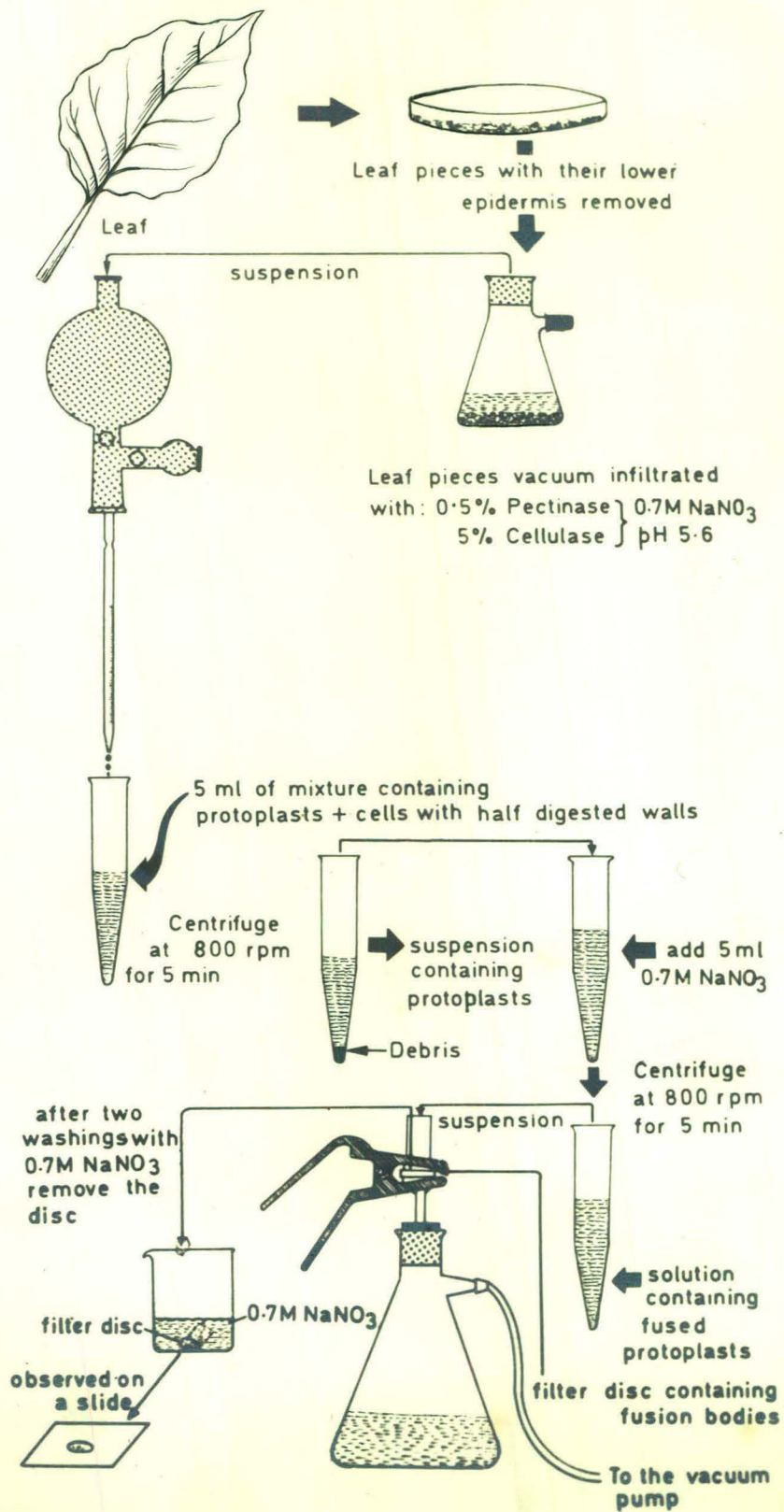
Flow Sheet NO. 2

The Fusion Of Protoplasts

Leaf

Leaf pieces with their lower epidermis removed

suspension

Leaf pieces vacuum infiltrated with: 0·5% Pectinase ⎱ 0·7M NaNO$_3$
5% Cellulase ⎰ pH 5·6

5 ml of mixture containing protoplasts + cells with half digested walls

Centrifuge at 800 rpm for 5 min

suspension containing protoplasts

add 5ml 0·7M NaNO$_3$

Debris

after two washings with 0.7M NaNO$_3$ remove the disc

suspension

Centrifuge at 800 rpm for 5 min

filter disc

0·7M NaNO$_3$

solution containing fused protoplasts

observed on a slide

filter disc containing fusion bodies

To the vacuum pump

Fig. 30. Aggregation of protoplasts isolated from leaves of A. thaliana in the presence of 0.4M NaNO$_3$. X 800.

Fig. 31. Intermediate stage in the fusion of protoplasts isolated from leaves of A. thaliana in the presence of 0.4M NaNO$_3$. X 800.

Fig. 32. Fusion of protoplasts isolated from roots of A. thaliana in the presence of 0.4M NaNO$_3$. X 320.

Fig. 33. Fusion of protoplasts isolated from the callus of Py mutant of A. thaliana. X 800.

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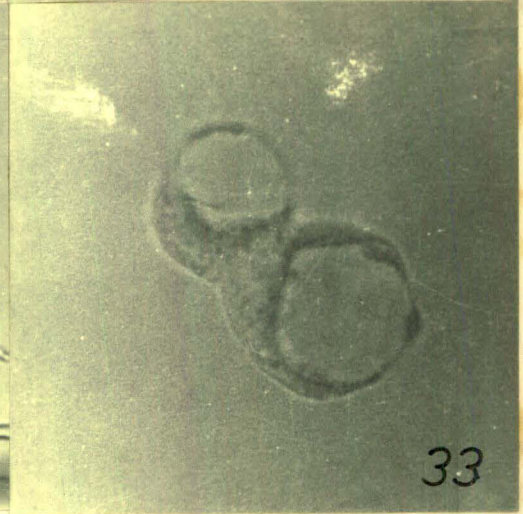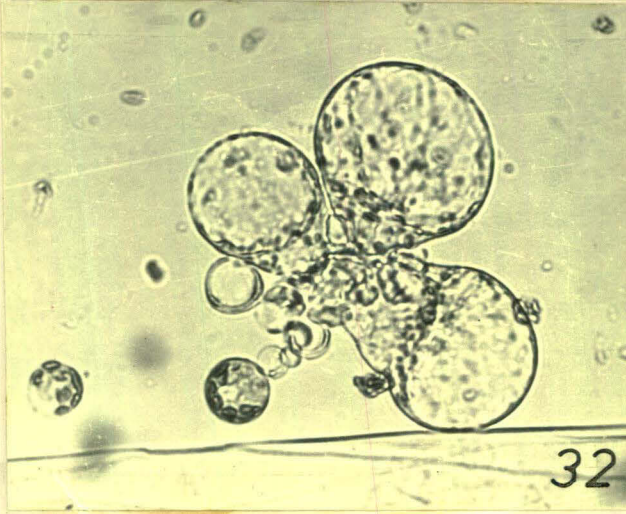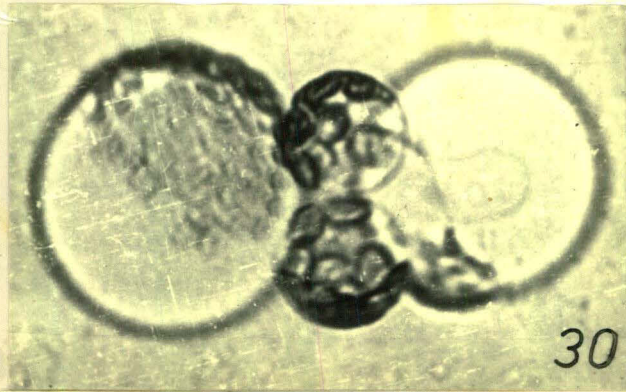
$0.4M$ $Mg(NO_3)_2$ as osmoticum. They were washed after 5 hr on a millipore filter with $0.4M$ $Mg(NO_3)_2$ and then suspended in the same solution. The protoplasts were found to aggregate after 15 min and the percentage of aggregation was 6-7. The percentage of fusion was not more than 5 in any case.

4. <u>Effect of calcium nitrate</u> - The enzyme mixture utilized for the isolation of protoplasts of <u>A</u>. <u>thaliana</u> contained $0.4M$ $Ca(NO_3)_2$ as osmoticum. The protoplasts were washed after 5 hr incubation and suspended in a solution containing $0.4M$ $Ca(NO_3)_2$. About 6% aggregation and 5% fusion was observed after fixation and staining.

It was also observed that when the protoplasts were washed on a millipore filter by low suction pressure, some of the protoplasts, being fragile, collapsed. Therefore, while investigating the effect of other fusing agents, this practice was discontinued and the washing was done by centrifugation alone or by centrifugation and floatation method (<u>see</u> Materials and Methods).

5. <u>Effect of calcium chloride</u> - The protoplasts after washing were suspended in the fusion inducing solution which contained $0.05M$ $CaCl_2.2H_2O$, $0.4M$ mannitol in $0.05M$ glycine, NaOH buffer, the pH of which was set as high as 10.5. The protoplasts were pelleted and the

centrifuge tubes were placed in a water-bath shaker
set at 30C. Aliquots were drawn out and observed
under the microscope. The protoplasts were seen to
aggregate after half an hour of incubation in the
fusion inducing solution (Figs. 34-35). Fusion was
observed after the protoplasts had aggregated together.
It was found that 10-15% fusion took place amongst
the 30-40% protoplasts of A. thaliana which had
aggregated. At a temperature higher than 30C, the
protoplasts were adversely affected and died. At low
pH (5.6) and low temperature (25C), $CaCl_2$ was found to
be ineffective in inducing fusion.

6. Effect of gelatin - Protoplasts after isolation
in the enzyme mixture were washed with 0.5M mannitol
and suspended in 1% or 2% gelatin (E. Merck), respectively.
Loose aggregates of 2-10 protoplasts were formed in
1% gelatin solution but none of these were found to fuse.
In 2% gelatin solution, the protoplasts were found to
adhere loosely within 15 minutes. After 2 hr, the
aggregates agglutinated tightly. Fusion was observed in
some of these tightly agglutinated protoplasts by the
dissolution of the membranes between the protoplasts
(Fig. 36). The percentage of fusion varied from 2-5.

7. Effect of lectins - These are proteins which
were earlier utilized to agglutinate different types of

Figs. 34-35. Aggregation of protoplasts isolated from
leaves of $\underline{A}$. $\underline{thaliana}$ in the presence of
$CaCl_2$ at pH 10.5. X 800; X 320.

Fig. 36. Apparent fusion of protoplasts isolated
from leaves of $\underline{A}$. $\underline{thaliana}$ in the presence
of gelatin. X 800.
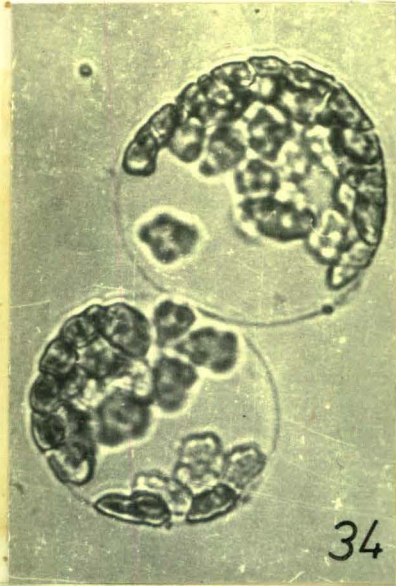
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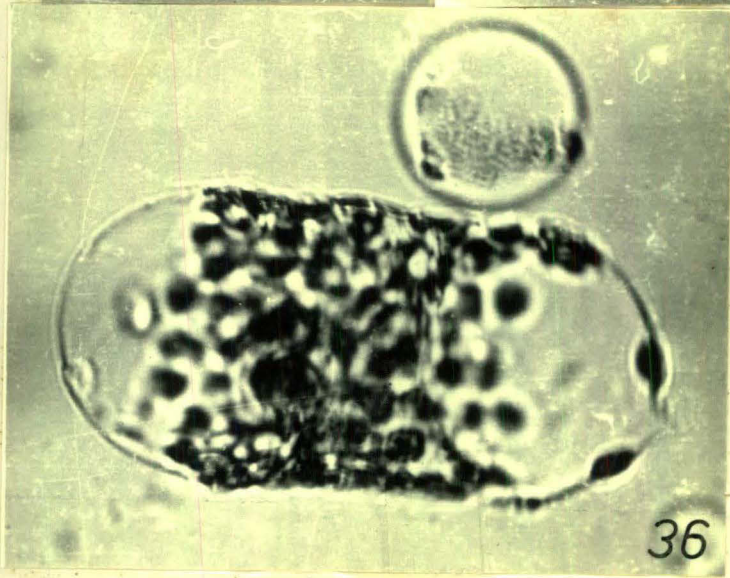
36

animal cells, but are now being used to agglutinate
plant cells too. Agglutinins derived from plant source
are referred to as phytohemaglutinins. In the present
investigation, for fusion experiments, phytohemaglutinin
(of kidney bean origin) was obtained from Patel Chest
Institute, Delhi and concanavalin A commonly referred
to as Con A (of jack bean origin) from Sigma. Whereas
phytohemaglutinin did not induce aggregation or fusion,
Con A proved quite effective. A solution containing
0.25M KCl, 0.01M $CaCl_2$ and 0.05M Tris, adjusted at
pH 7.2, was used for washing and suspending the protoplasts.
After this, the protoplasts were induced to fuse by
adding equal volume of protoplast suspension in buffer
solution, and Con A solution (prepared in the same
buffer in which protoplasts were suspended), such that
the final concentration of Con A was 20 µg/ml or 40 µg/ml.
Whereas 20 µg/ml Con A did not induce much agglutination
(only small percentage of aggregates was occasionally
observed), 40 µg/ml Con A was quite effective. About 16%
of the protoplasts were found to be agglutinating under
the influence of 40 µg/ml Con A (Figs. 37-39). In the
same concentration, however, with the passage of time,
protoplasts were seen to burst. Apart from observing
under the microscope, a new technique was employed to
establish aggregation of protoplasts. Small samples,
ca. 2.5 ml of protoplasts, with and without Con A

Figs. 37-39. Aggregation and partial fusion of
protoplasts isolated from leaves and
shoots of A. thaliana in the presence
of 40 ug/ml Con A.

Figs. 37-38 X 320.
Figs. 39   X 800.
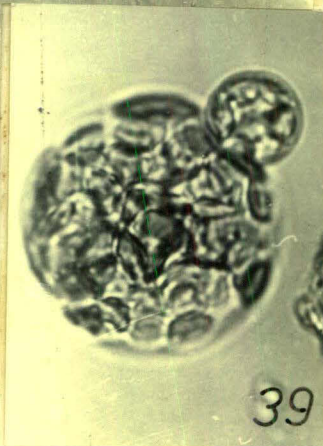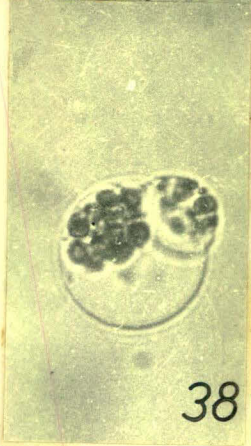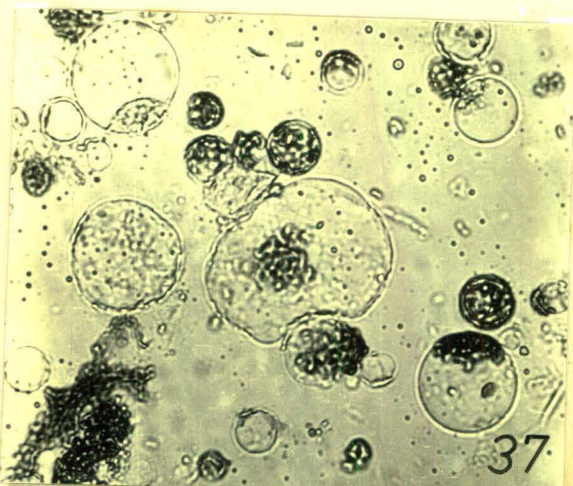
(serving as control) were poured in two cuvettes respectively, and their optical density (O.D.) was recorded at 720 nm at various intervals (Fig. 40) in a Carl Zeiss spectrophotometer. The decrease in optical density was taken as a measure for aggregation of protoplasts. The results from Fig. 40, indicate that as compared to the control, only the initial rate of aggregation is enhanced in the treated sample.

8. Effect of polyethylene glycol - Protoplasts were isolated in an incubation mixture containing 0.175M mannitol, 0.175M sorbitol, 3mM $CaCl_2$, 0.5mM CaH_2PO_4, 0.5% macerozyme and 3% cellulase. The pH of the mixture was maintained at 5.6. The protoplasts were then washed and suspended in solution A containing, 0.4M glucose, 3.5mM $CaCl_2$ and 0.7mM KH_2PO_4. The fusion mixture was made by adding 0.5g polyethylene glycol (PEG 6000, obtained from Polysciences, Warrington, U.S.A.) to 1 ml of solution A, enriched in some cases by 5mM sodium citrate buffer at pH 5.6. Few drops of protoplast suspension were put on the haemocytometer and the observations recorded. Interestingly, within 10-15 min of introduction of the PEG solution, protoplasts started coming near each other and establishing contact by throwing out a protuberance (Figs. 41-43). Very tight agglutinations of 2-20 protoplasts were observed after half an hour. About

Fig. 40. Effect of Con A on aggregation of protoplasts
isolated from leaves of A. thaliana. Small
samples (ca. 2.5 ml) of protoplasts with and
without Con A (serving as control) were poured
in two cuvettes, respectively and their optical
density (O.D.) was recorded at 720 nm on a
spectrophotometer at different time intervals.

FIG. 40
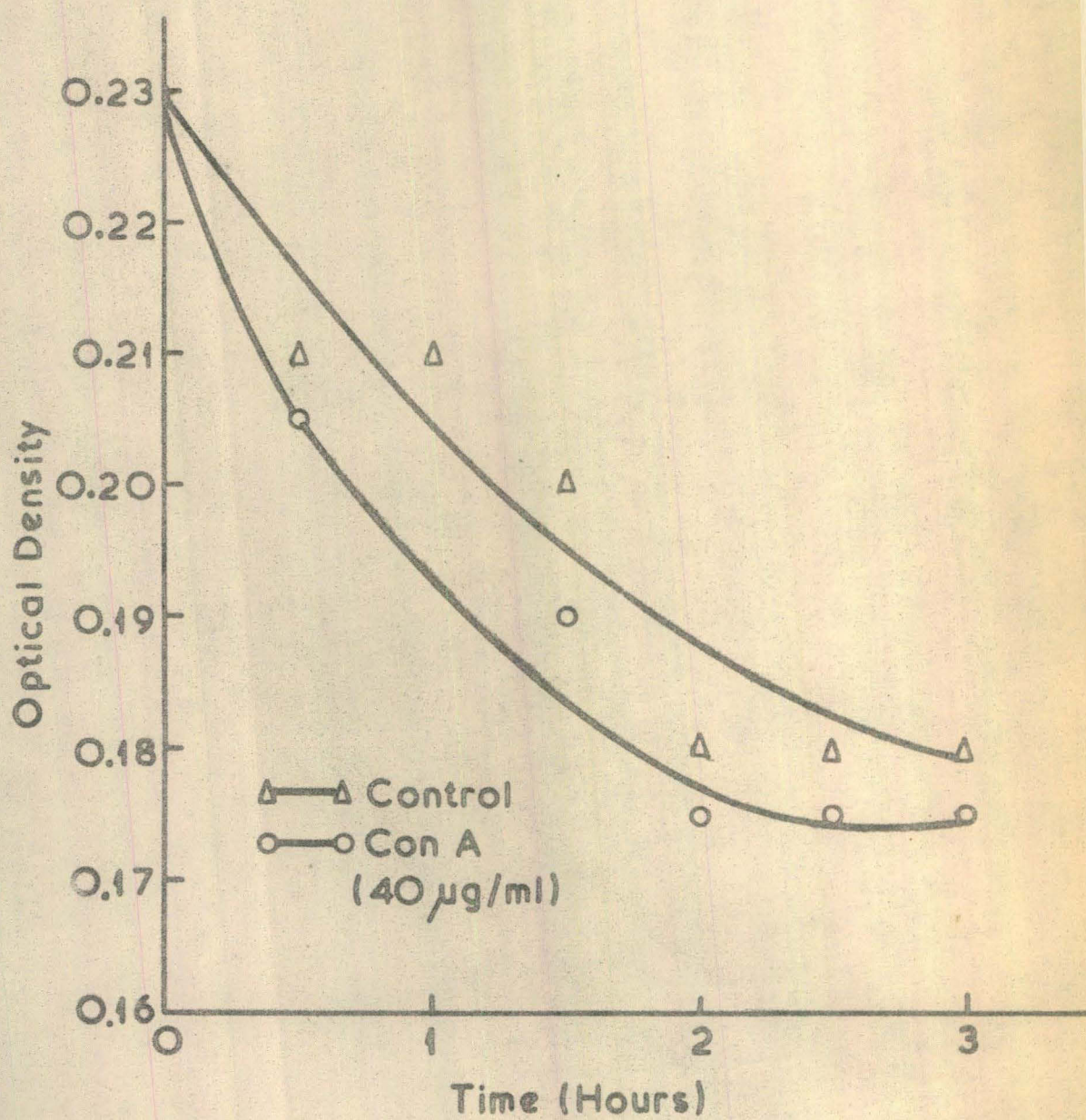
Figs. 41-43. Initial stages in the aggregation and fusion
of protoplasts isolated from shoots of
A. thaliana with PEG. (Note the protuber-
ance thrown out by one of the protoplasts
to make contact with the other protoplast).
X 126.

41

42

43
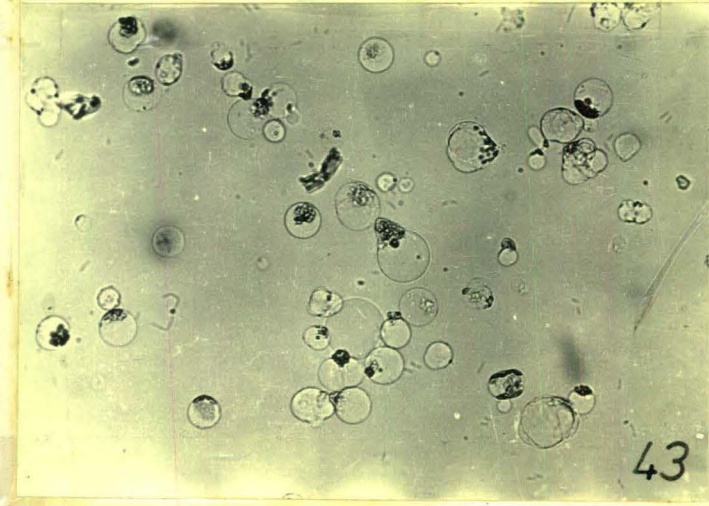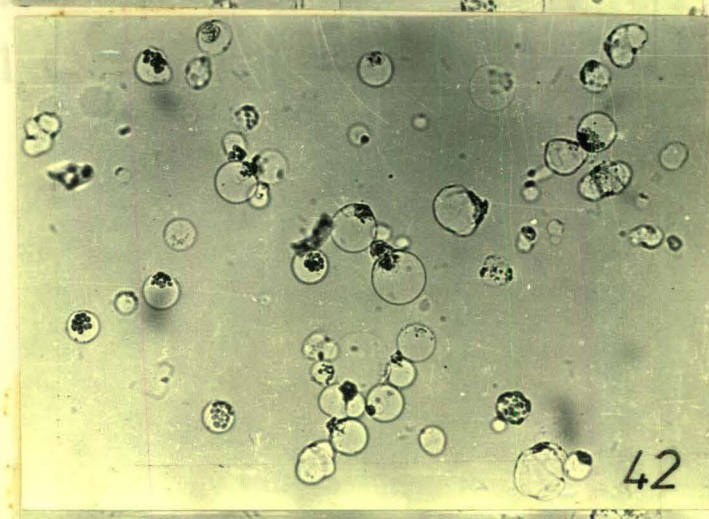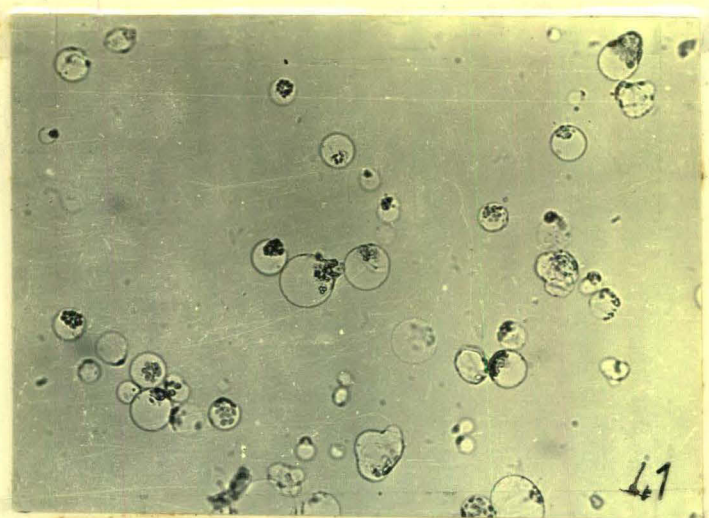
50-60% protoplasts were involved in the process of homoplasmic fusion. Enrichment by sodium citrate buffer, did not help in fusion, which was achieved without it. Fused product was recognized because of its considerably large size (Figs. 44-46). Intra-specific fusion was achieved in A. thaliana leaf proto-plasts (Fig. 47, d-g) with high molecular weight PEG. Low molecular weight PEG (1,540) was not found to be effective in fusing the protoplasts. High molecular weight PEG was also found to fuse protoplasts from, callus of Petunia, callus of Arabidopsis, Py mutant, leaf of cabbage with leaf of radish and leaves and radicles of pea (Figs. 48-52). Moreover, aggregations took place between protoplasts of leaves of A. thaliana and callus of Py mutant (Figs. 53-54); callus of Petunia and callus of Arabidopsis, Py mutant; apical buds of pea and leaves of pea (Figs. 55-56); leaves of pea and radicles of pea (Fig. 57) and between leaves of pea and etiolated shoots of pea (Fig. 58). Though PEG was found to induce fusion non-specifically, it was also noted that, in a suspension containing protoplasts from two different species, aggregation and fusion was favoured amongst the identical protoplasts or protoplasts origina-ting from the same species (homoplasmic fusion). This was identified from the characteristic morphological features of protoplasts of one species which differed

Figs. 44-45. Intermediate stages of fusion of
protoplasts isolated from leaves of
A. thaliana in the presence of PEG.

Fig. 44 - X 320.
Fig. 45 - X 800.

Fig. 46. A single protoplast formed as a result
of fusion of two or more protoplasts.
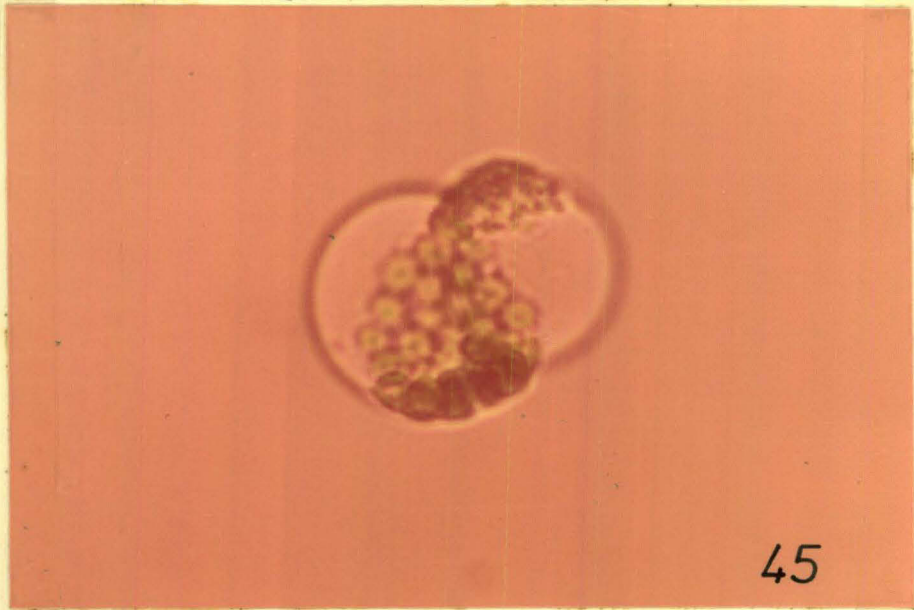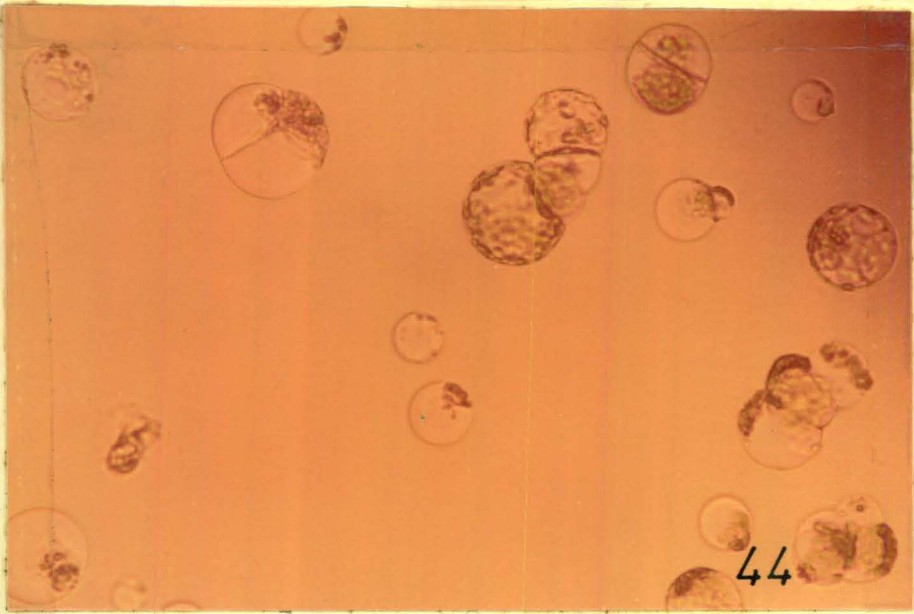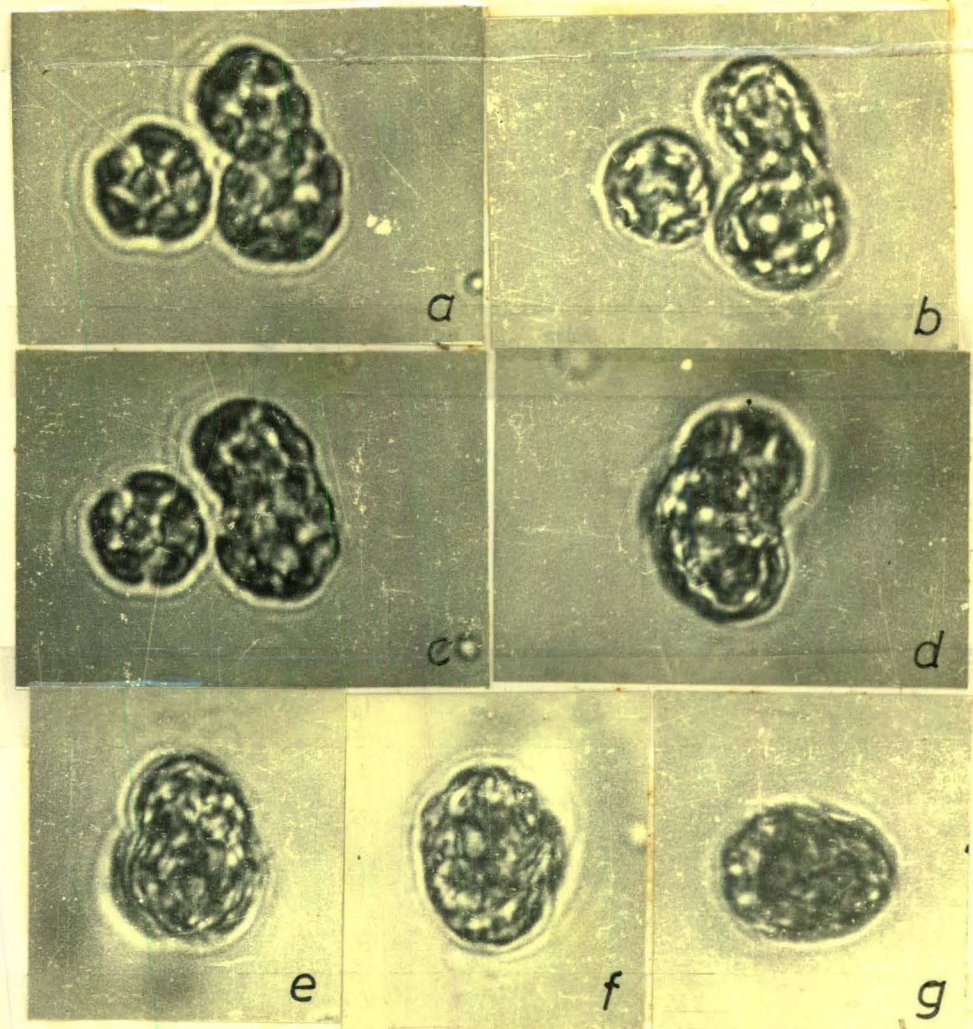X 2000.

44

45

46

Fig. 47(a-g). Events in fusion of protoplasts isolated from leaves of A. thaliana after PEG treatment. X. 800.

FIG. 47 a-g

Figs. 48-52. Stages in fusion of protoplasts isolated from pea leaves,(Figs. 48-50) and radicles (Figs. 51-52) in the presence of PEG. x 800.

48   49   50   51   52
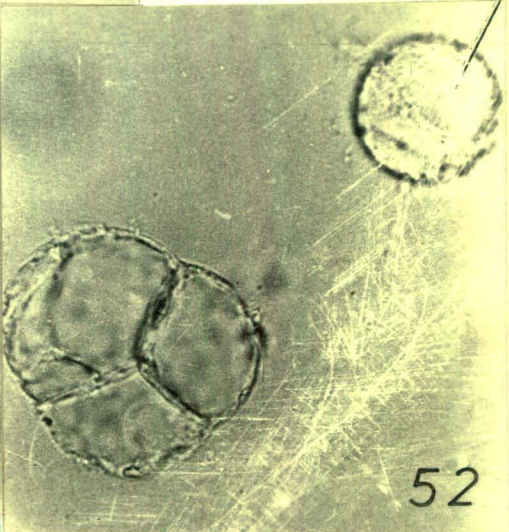
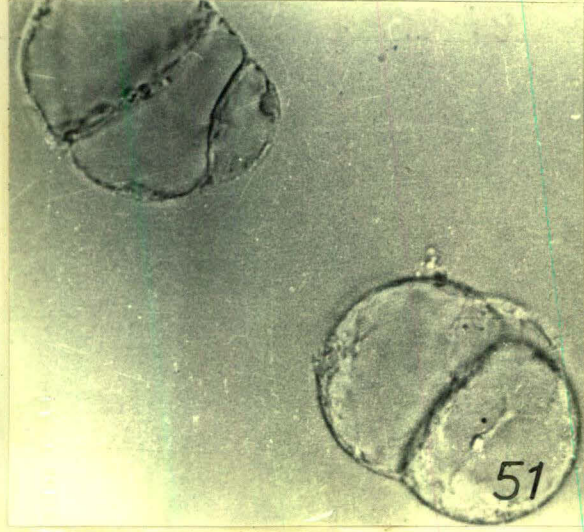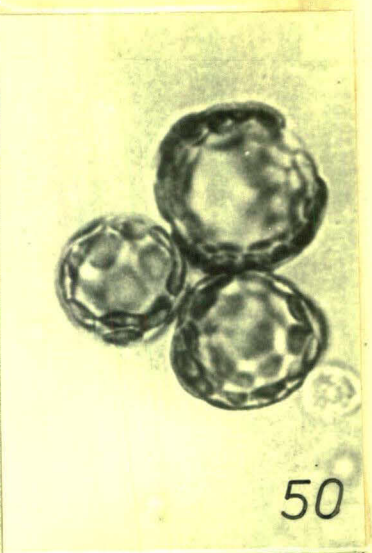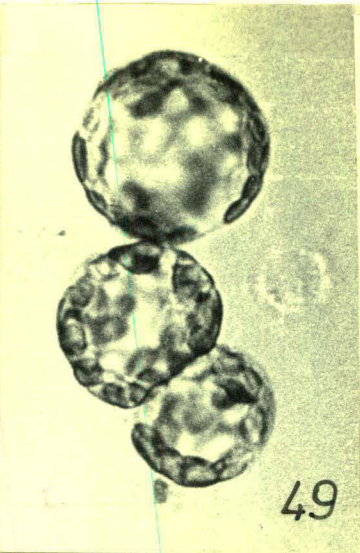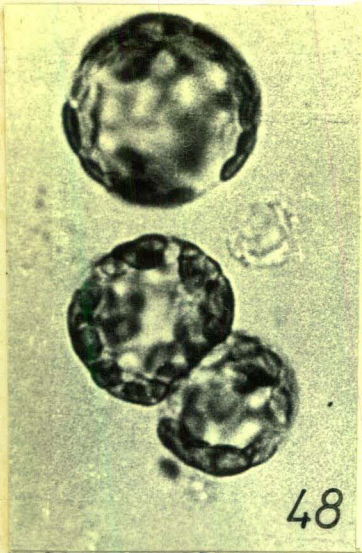Fig. 53. Aggregation of protoplasts isolated from leaves of A. thaliana and callus of Py mutant of the same. The arrow shows the protoplast from leaf. X 2000.

Fig. 54. Partial fusion of protoplasts isolated from leaves of A. thaliana and callus of Py mutant of the same. X 2000.

53

54
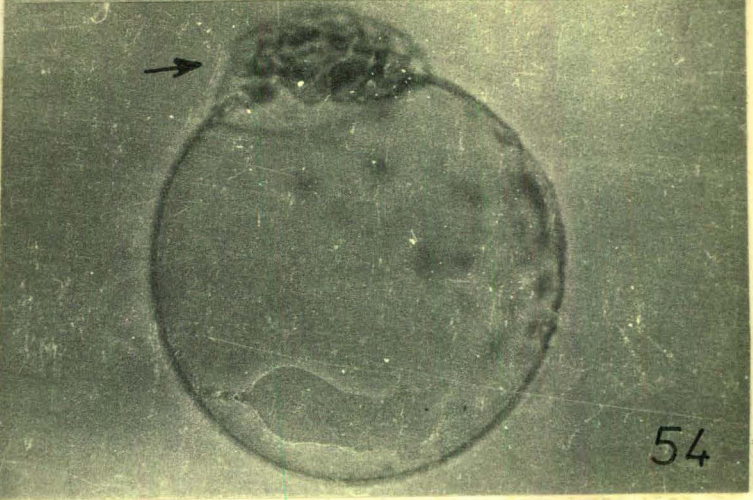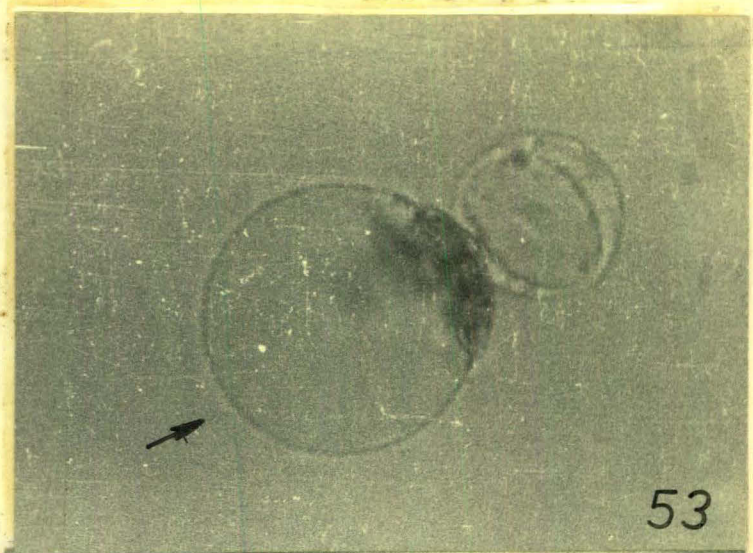
Fig. 55. Aggregation of protoplasts from pea-leaf
and apical bud of pea (shown with an arrow).
X 800.

Fig. 56. A single protoplast isolated from apical bud
of pea, enlarged to show its morphology. X 800.

Fig. 57. Fusion of protoplasts isolated from leaves
and radicles of pea (the latter shown with
an arrow). X 2000.

Fig. 58. Fusion of protoplasts isolated from leaves
and etiolated shoots of pea (the latter shown
with an arrow). X 2000.

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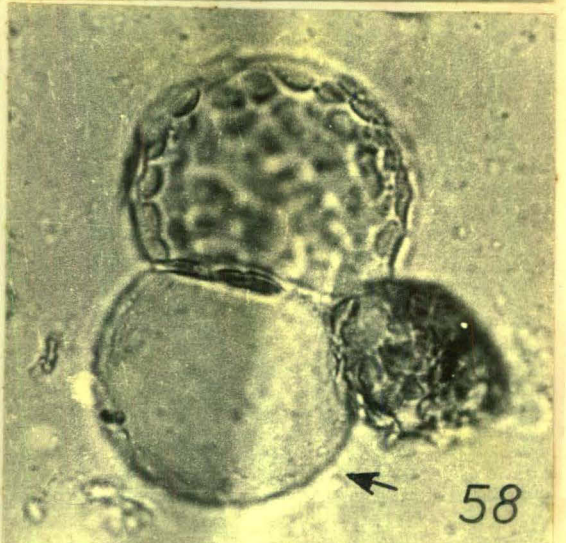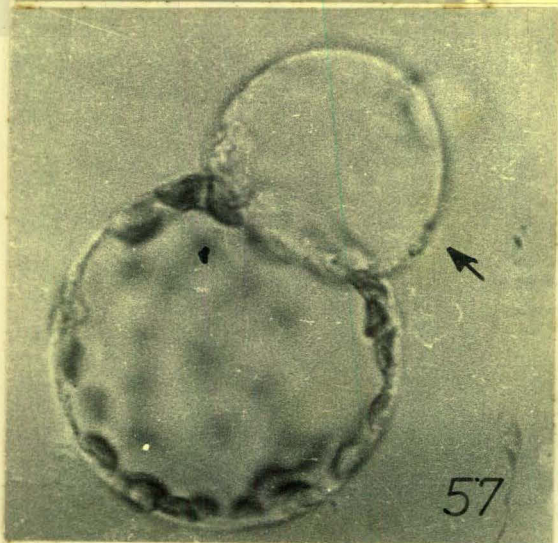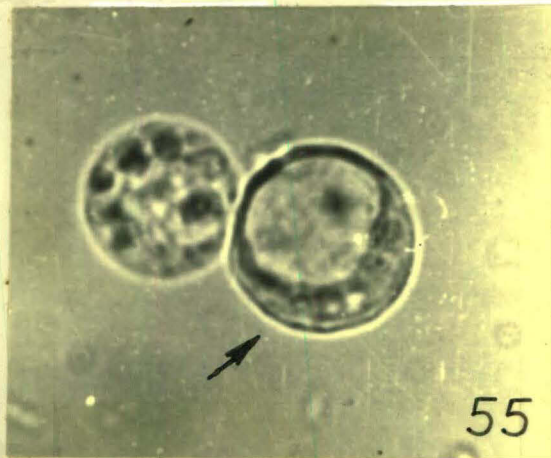
from those of the other, in a mixed population.

Effect of Slow Deplasmolysis

The molarity of mannitol solution in which protoplasts were suspended was brought down gradually to 0.3M by washing the protoplasts with a solution whose concentration was decreased in each step by 0.05M. Percentage aggregation of protoplasts isolated from leaves of A. thaliana plants of various ages was determined as shown in Table I. It was seen that, the protoplasts from leaves of younger plants aggregated more readily than those from older ones. The percentage aggregation induced by slow deplasmolysis was quite high (21-23%) in the leaves from younger plants.

TABLE I

Effect of slow deplasmolysis on percentage aggregation of protoplasts isolated from leaves of A. thaliana plants of various ages.

S.No.	Age of plant (days)	% aggregation in sample subjected to slow deplasmolysis	% aggregation in control
1.	25	21	1.2
2	35	23	2.0
3	56	14.2	0
4	69	5.5	0

Fusion of Protoplast Membranes

While isolating the protoplasts of <u>Arabidopsis</u>, it was observed that, if the concentration of osmoticum (i.e. mannitol) was maintained at 0.3M in the isolation mixture, instead of protoplasts, contour of the protoplast membrane, devoid of any contents was isolated (Fig. 59). These membrane ghosts were found to undergo division forming two hemispherical ghosts which did not separate (Figs. 60-61). The membrane ghosts were also found to undergo spontaneous fusions in the enzyme mixture (Figs. 62-63).

Fixation and Staining

The protoplasts were fixed after the fusion treatment (<u>see</u> Materials and Methods). Untreated protoplasts were also fixed to serve as the control. They were then stained with modified carbol fuchsin (<u>see</u> Materials and Methods) and observed under the microscope. The nuclei had taken up a dark stain as compared to the cytoplasm. The protoplasts showing more than one nucleus were photographed and are presented in Figs. 64-67.

ISOLATION AND FUSION OF MUTANTS

Isolation

The plants, that had emerged after sowing ethylmethane sulfonate (EMS)-treated seeds (<u>see</u> Materials

Fig. 59.   Membrane ghosts released from the protoplasts
                isolated from leaves of <u>A</u>. <u>thaliana</u> in 0.3M
                mannitol. X 320.

Figs. 60-63.   Stages in the fusion of membrane ghosts. Note
                that one of them has divided (shown with an
                arrow) and is seen fusing with a bigger
                protoplast. X 320.

Fig. 63.   The bigger protoplast has disintegrated. X 320.

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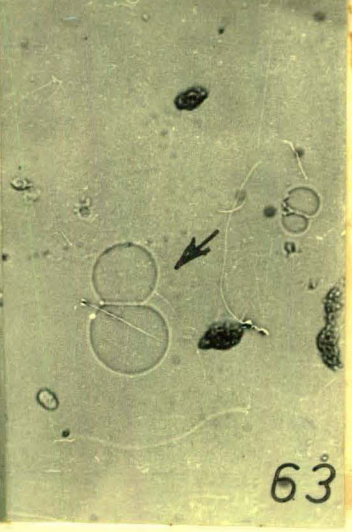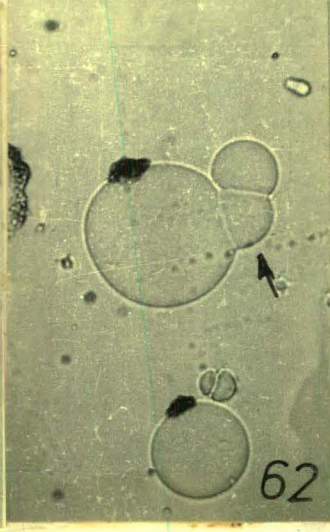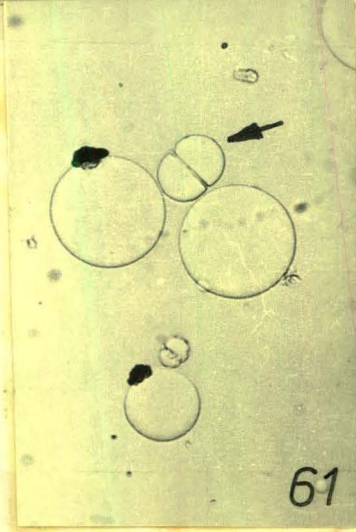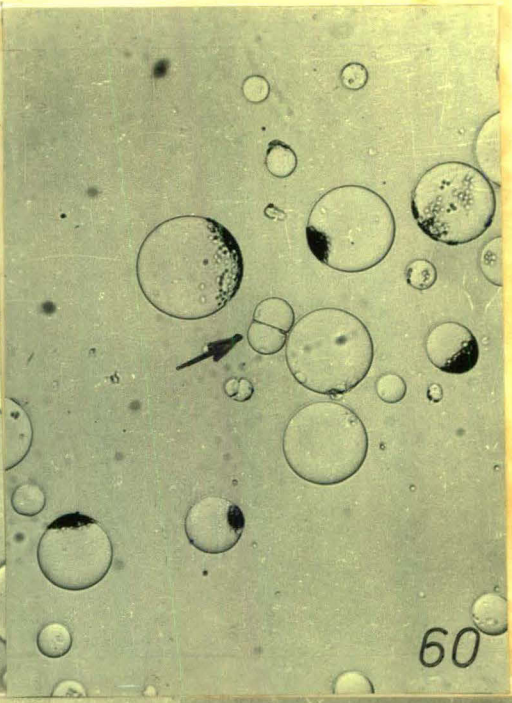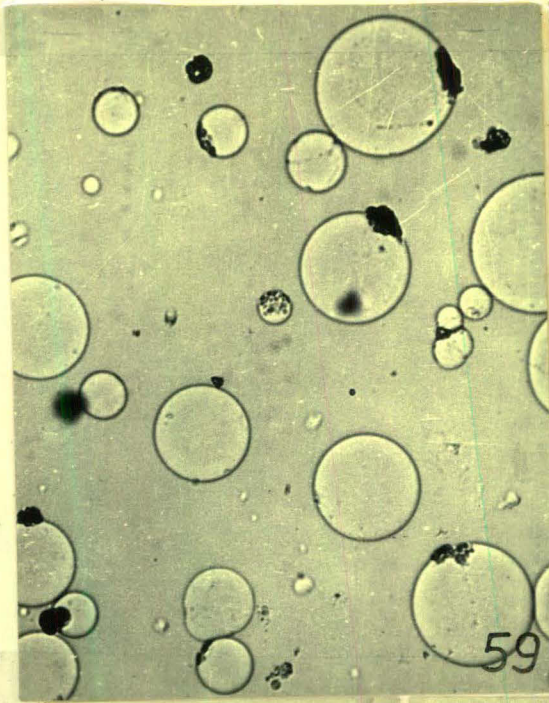
Figs. 64-67.   Protoplasts fixed and stained with

carbol-fuchsin to show the nuclei in

A. thaliana. X 2000.

Fig. 64.   Dinucleate formed after $CaCl_2$ treatment

of protoplasts.

Fig. 65-67.   Multinucleate protoplasts formed after PEG-

treatment.

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66
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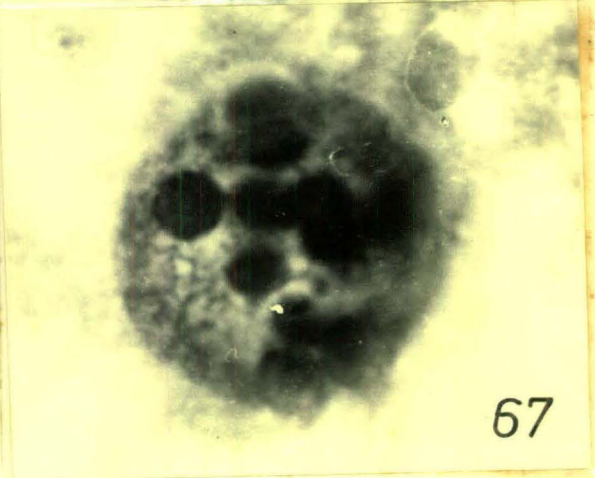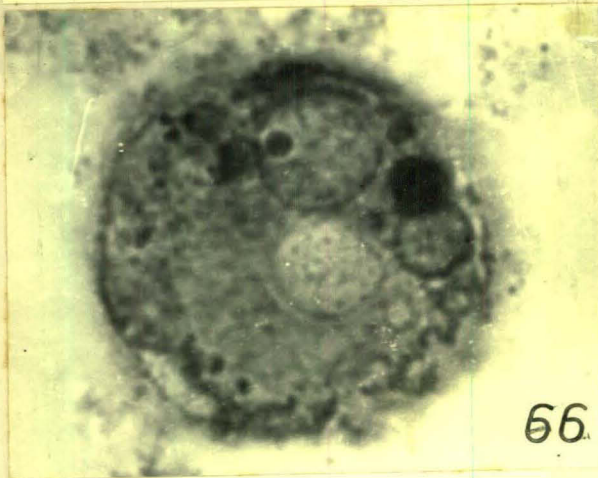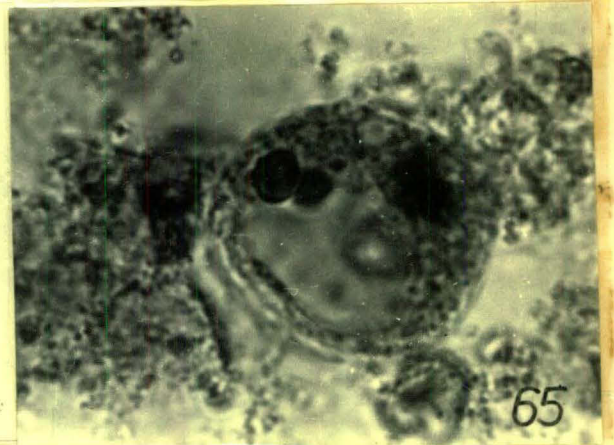
and Methods), were closely observed. Plants growing poorly as compared to the control, were suspected to be mutants (Figs. 68-69). These were picked up and cultured on the original medium enriched with 500ppm casein hydrolysate (containing all the essential amino acids). Poorly growing plants showed remarkable healthy growth in this amino acid rich medium (Fig. 70). Attempts to grow the callus of these mutants on various combinations of media eliminating one amino acid each time to establish the identity of mutants resulted in some degree of success.

Fusion

Protoplasts isolated from the callus of Py, and leaf mesophyll of Tz mutants (see Materials and Methods) were induced to fuse by PEG treatment. The percentage of fusion was very low (ca. 2%). The fused products could not, however, be individually picked up and regenerated on a selective medium without thiamine (on which the hybrids should normally grow), to confirm the fusion.

Fig. 68.   Normal plant of A. thaliana flanked by
           suspected mutants on either side. X 1.

Fig. 69.   Suspected mutant of A. thaliana. X 4.

Fig. 70.   Suspected mutant growing normally following
           transfer to amino acid-rich medium. X 2.

68

69

70
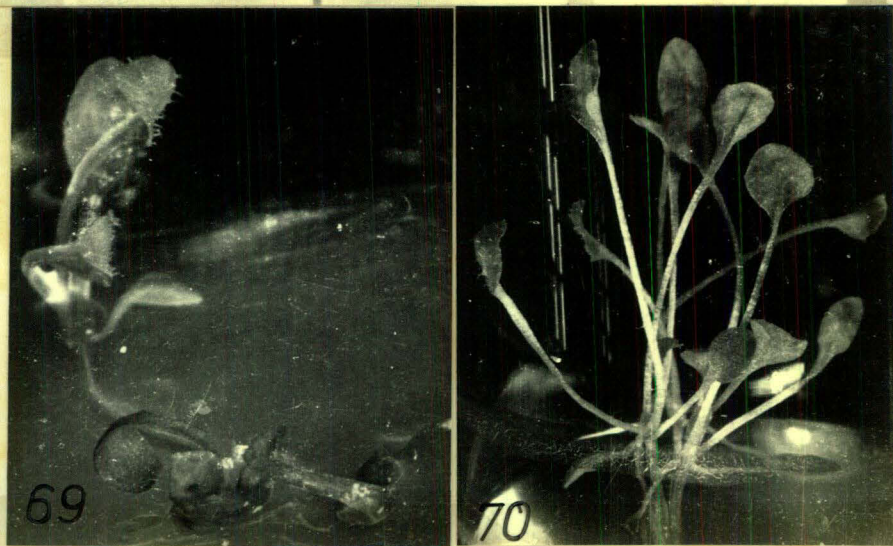
DISCUSSION

ISOLATION OF PROTOPLASTS

For the isolation of protoplasts, it has been found essential to add a suitable osmoticum and enzymes, such as pectinase and cellulase, besides other stabilizing agents, (Gamborg, 1976). Protoplasts are very sensitive to changes in the physical conditions too. In the next few pages, results of the present investigation have been discussed in relation to other work regarding standardization of the techniques.

Influence of Osmoticum

Various osmotic agents have been used for the isolation of protoplasts from different plants. Most commonly used are sugars, such as sucrose and glucose, sugar alcohols, such as sorbitol and mannitol and inorganic salts.

Effect of mannitol and sugars - In the present investigation, a slightly plasmolysing concentration of mannitol proved to be a better osmoticum than sucrose, glucose, sorbitol or inorganic salts. Plasmolysing concentration of the osmoticum was used to minimize any deleterious effects on the protoplasts due to the impurities present in the enzyme used. Moreover, it has been found by Raj and Herr (1970) and Bawa and Torrey (1971), that the isolated protoplasts are more stable in slightly

hypertonic than in isotonic solution, and therefore, the former concentration was used. In _Datura_ and _Nicotiana_, sucrose was equally effective as mannitol as an osmoticum, but protoplasts were more stable in the latter. This could be attributed to the fact that sucrose is a physiologically active sugar and might get metabolized, thus bringing a change in the original concentration of the osmoticum. This difficulty is not encountered when mannitol is used as an osmoticum, since it is physiologically inert.

Effect of inorganic salts - Various inorganic salts, such as nitrates of Na^+, K^+, Ca^{++} and Mg^{++}, when used as the osmoticum, were found to be beneficial for the isolation of protoplasts in the present investigation. This is in consistence with the results obtained by Meyer (1974) who reported high yield of protoplasts, irrespective of the physiological condition of the plants used, by using salt solutions to maintain suitable osmotic conditions. Protoplasts obtained in salt solutions, however, were not found to be stable for a long time which could be due to the rapid exchange of ions across the protoplast membrane, finally resulting in the bursting of protoplasts. Potassium dextran sulphate which is known to enhance the separation of cells and stabilize the isolated protoplasts (Otsuki & Takebe, 1969), possibly by

binding to some of the proteins present as contaminants
in the crude enzyme solutions, was not found to be effect-
ive for the isolation and stability of protoplasts in the
present investigation; possibly because the enzymes used
were comparatively pure. In fact, the addition of
potassium dextran sulphate changed the concentration of
the original osmoticum so that the latter was not as
effective in maintaining the stability of protoplasts
as when used without the addition of potassium dextran
sulphate.

Influence of Enzymes

Effect of macerozyme or pectinase - It was found that
macerozyme or pectinase is effective in the release of
cells from the leaf mesophyll tissue of Datura, Nicotiana,
pea, radish, wheat, potato, Petunia and Arabidopsis.
Generally, 0.5% was the concentration found best for the
isolation of cells. Many workers (Takebe et al., 1968;
Usui & Takebe, 1969 and Nagata & Takebe, 1970) also
preferred to use 0.5% pectinase in their experiments.
There are some, who used 1% pectinase (Chupeau & Morel,
1970) and yet others (Raj & Herr, 1970) who used
concentrations as high as 12%. The results reported by
these workers and of the present investigation do not
necessarily represent the maximum achievement for
individual species because, both the rate of maceration
and the yield of intact cells vary considerably with

species, age and the physiological conditions, of the
tissues used. It is, however, desirable to use as low
a concentration of enzymes as possible. This is so,
because any impurity such as nucleases, lipases, proteases
etc. that might be present in the enzyme would be present
in reduced quantity in lower concentrations of enzyme
preventing killing of many cells. This is why some
workers (Schenk & Hildebrandt, 1969a; Kao et al., 1971;
Cocking & Evans, 1973; and Vasil et al., 1975) preferred
to purify the enzymes before use. Since in our prepara-
tion, the enzymes were comparatively pure, further
purification was not found necessary. Many other workers
have also used the commercially available enzymes without
any further purification to obtain good yields of
protoplasts which were capable of further growth and
development (Nagata & Takebe, 1970; Kao & Michayluk, 1975;
Raveh & Galun, 1975 and Power et al., 1976).

Some workers reported the release of protoplasts by
pectinase alone from placental tissues of Lycopersicon
esculentum (Gregory & Cocking, 1965) and Solanum nigrum
(Raj & Herr, 1970) berries. These were exceptional
cases because the cell walls in such tissues are known to
be partially or completely hydrolyzed during the ripening
process. In the present investigation, all cells and no
protoplasts were released by using pectinase/macerozyme

alone. Slightly higher concentration i.e. 1-2% pectinase/
macerozyme was used to isolate cells from the fresh callus
of Datura, Nicotiana, Petunia and Arabidopsis, but it
failed to release cells from hard and old callus. Generally
pectinase/macerozyme acts by dissolving middle lamella
(the cementing material between the cells of a tissue)
which largely consists of calcium pectate. In hard callus,
possibly further deposition occurs on the middle lamella,
besides the calcium pectate and therefore, even increasing
concentration of pectinase/macerozyme could not help in
releasing the cells from hard callus.

Effect of cellulase- The effect of cellulase is to
degrade the callulose cell wall, thus releasing protoplasts.
This was used either separately, after the cells were
isolated by pectinase treatment (Otsuki & Takebe, 1969)
or along with pectinase (Power & Cocking, 1969). Most of
the workers have preferred to use Cocking's method, with
slight modification in the cellulase concentration,
depending upon the tissue used for quick isolation. Being
fast, this method reduces the changes of infection of
protoplasts. Moreover, since the usefulness of protoplasts
lies in their further culture and growth, it was thought
to expose them to crude enzyme -- cellulase (which might
be contaminated with toxic substances such as ribonucleases,
lipases, proteolytic enzymes etc.) for as short a period
as possible. In present investigation, quite a high yield

of protoplasts of the order of 10^5 protoplasts/ml/gm
fresh wt. of the tissue was obtained after 5 hr
incubation by using Cocking's method. Nevertheless,
at this stage many cells were also seen. Therefore,
often the incubation period was prolonged to 16 hr and
the protoplasts were still found to be viable. This
could be because the enzymes used were comparatively
pure and even a longer period of incubation did not
affect their viability.

It was also found that adequate penetration of the
enzymes is essential for isolation of protoplasts. This
was facilitated by initial vacuum infiltration of the
leaf pieces of all tissues except of Arabidopsis (which
are very thin), by which the air in the tissues was
replaced by enzyme mixture.

Cellulase was found to release protoplasts from
fresh callus of Arabidopsis, Datura, Nicotiana and
Petunia but proved ineffective in releasing protoplasts
from hard callus of the same. This is probably due to
the fact that the cell wall of higher plant cells
consists of cellulose, hemicellulose, pectin and protein
in different proportion, which changes during the
development and differentiation of a tissue. Cellulose
is present in two forms, i.e., amorphous and crystalline.

The amorphous cellulose can be more readily degraded
because of the availability of glucosidic bonds in
cellulose for hydrolysis by cellulase (a hydrolytic enzyme).
But how exactly and readily the cellulase attacks the
crystalline portion of cellulose is not understood.
In hard callus, there could be deposition of other
substances in addition to more crystalline cellulose
which could not be hydrolysed by cellulase.

Influence of pH

It has been established that the optimum pH range
of macerozyme is 5.0-6.0 and that of cellulase is, 4.0-
5.0. Therefore, the pH range from 4.6-6.6 was tested in
the incubation mixture (where both the enzymes were used)
for its suitability for isolation of protoplasts. The
pH between 5.0-6.0 was found most suitable and at pH
below 5.0, protoplasts did not survive. This accounts
for the decrease in number of isolated protoplasts at
pH lower than 5.0. All other workers have also used this
particular pH range for the isolation of protoplasts. A
pH of 6.5 was found effective by Ruesink (1965), for the
isolation of protoplasts from Avena coleoptiles.

Influence of Physical Factors

Effect of light - In the present investigation, number
of protoplasts which were stable for a longer time was much
larger, when isolated in dark rather than in light. This

could possibly be attributed to the increased ion flux from the protoplasts to the incubation mixture in light resulting in their death. Some of the workers like Ruesink (1965), Motoyoshi (1971), Bhojwani and Cocking (1972) also preferred to keep the incubation mixture in dark rather than in light for the isolation of protoplasts.

Effect of temperature - Though the optimum temperature range for the activity of the enzymes -- macerozyme and cellulose lies between 40C-50C, the incubation temperature was maintained at 30C for A. thaliana, since this proved to be the most effective temperature for the rapid release of protoplasts without damaging them. For Datura and Nicotiana, however, 36C proved to be better. Most of the workers have preferred to use a temperature range between 25C-27C, whereas in some instances temperatures as high as 37C have been used (Motoyoshi, 1971).

Effect of shaking - This is not obligatory for the isolation of protoplasts, though it hastens their release. But sometimes the faster release is not desirable on account of the damage that can be caused by shaking. Generally these protoplasts are very fragile, and after some of them have been released, in the first hour, shaking needs to be stopped, to prevent the released protoplasts from breakage. Other workers have also opted either to

continue (Takebe et al., 1968) or discontinue (Power &
Cocking, 1969) shaking of the incubation mixture, depend-
ing on their material.

i.   Kinetics of release - Though the minimum time
required for the release of protoplasts was found to be
one hour, not many protoplasts were released until 5-7 hr
of incubation. This is in contrast to the results
obtained by most other workers who have used 30 min
(Ito, 1973) to 2 hr (Bhojwani & Cocking, 1972) for the
maximum release of protoplasts. There are, however,
workers (Evans et al., 1972) who carried out the incuba-
tion for 5-12 hr for isolating maximum number of proto-
plasts. In the present investigation, complete
digestion of the plant material was achieved only after
12-16 hr. This length of time is not desirable since it
is preferred to expose the protoplasts to the enzymes
for as short a period as possible.

Source and the Physiological State of the Material

As observed by other workers, it was confirmed in
the present investigation, that leaves are usually
ideal for the isolation of large quantities of uniform
populations of protoplasts (Cocking, 1972). Protoplasts
can also be isolated from callus but it is more advant-
ageous to isolate protoplasts directly from the plant.

Besides the organ, the growth conditions of the plants
also have an effect on the isolation of protoplasts e.g.
in tobacco, Watts et al. (1973) reported that, illuminat-
ion, temperature and insecticides used, exerted an
important influence on the isolation of protoplasts and
their further growth. The age of the callus, and of the
plants also play an important role in the isolation of
protoplasts. Lignification of the walls of callus cells
makes it difficult to isolate protoplasts from them.
This problem has, however, been remedied by frequent
subculturing and inducing rapid growth of the callus.

The most important factor responsible for the
variability in the yield of protoplasts, appears to be
the physiological conditions of the source tissue or
plant, which affects its susceptibility to hydrolysis
of the cell wall and the stability of protoplasts
(Uchimiya & Murashige, 1974; Watts et al., 1974; Zaitlin
& Beachy, 1974 and Shepared & Totten, 1975). The nutrition
and age of the plant also directly affect the isolation
of protoplasts and their suitability for further growth.
These parameters were found to vary from species to species,
In most cases, e.g in Nicotiana, Datura and Arabidopsis,
older leaves were not found to yield protoplasts. This
problem is apparently caused by differences in the chemical
and/or physical nature of the cell wall and the osmotic

conditions of cells and tissues, which are known to vary according to age. Variations in the amount and nature of the hemicellulose and lignin content of the cell wall -- which are age related and apparently quite common -- and the presence of enzyme inhibitors in the wall, interfere with the accessibility of the wall degrading enzyme -- cellulase to the cellulose microfibrils. These problems, however, could be overcome by growing the experimental plants under carefully controlled nutritional and environmental conditions, by using young leaves from plants differentiated *in vitro* or by using rapidly growing callus cultures, grown in well defined nutrient media.

Viability

Out of the various methods tested, it was found that cyclosis is difficult to observe especially in protoplasts which have a peripheral layer of chloroplasts (Larkin, 1976). As regards the phenomenon of size variation with osmotic changes, it is rather tedious and allows only a few protoplasts to be assessed. There are other workers (*see* Previous Work) who used dye exclusion and oxygen uptake and photosynthetic activity as the criterion for testing the viability of isolated protoplasts. The former of these can only be applied to individual protoplasts, and the latter to whole suspensions. In the present investigation, where above mentioned methods were not used, the staining by fluorescein diacetate proved quite

useful because of its suitability at the individual protoplast level as well as at the population level. Fluorescein diacetate (FDA) is a non-fluorescing, nonpolar substance which is freely permeable across the intact plasmalemma. Fluorescein, which is a fluorescent polar product of esterase activity of FDA can not be transported freely across the intact plasmalemma. Therefore, fluorescein accumulates only in viable cells and not in dead cells making it useful in the identification of viable protoplasts.

Macromolecular synthesis by isolated protoplasts as demonstrated by Fuchs & Galston (1976) is another technique which can be used to demonstrate the viability of protoplasts. This has been successfully exploited.

Further proof of viability of protoplasts can only be obtained if these can regenerate a wall, divide and grow in culture.

Culture of Protoplasts

There appeared to be no difficulty as regards the cell wall regeneration by protoplasts which was successfully achieved in the present investigation. This has been reported by almost all the workers attempting culture of protoplasts (see Previous Work). However, in contrast to the results obtained by other workers, colony formation

was a rare phenomenon. Most of the times, the protoplasts did not show any further activity in the culture medium. This could be due to the improper nutritional medium which needs to be modified largely by trial and error. In this time limited study, it was, therefore, not possible to devise a suitable medium for the further growth of protoplasts.

FUSION OF PROTOPLASTS

Effect of Ions

Since Na^+, K^+, Mg^{++} and Ca^{++} are the ions involved in the regulation of membrane permeability, it was thought that altered electrical and physiological properties of the membrane might affect the fusion of protoplasts. Moreover, $NaNO_3$ has been successfully utilized for bringing about fusions amongst protoplasts of various species (Power et al., 1970; Potrykus, 1971; Carlson et al., 1972). The mechanism by which sodium nitrate brings about fusion is not understood. Question arises whether it is the sodium or the nitrate ion which is important for bringing about fusion. Kameya and Takahashi (1972) after investigating the effects of different salts such as KCl, KNO_3, NaCl, $NaNO_3$, $CaCl_2$ and $Ca(NO_3)_2$ reported that it is the sodium ion which is effective in bringing about fusion. They also reported that removing of protoplasts from potassium salts to sodium salts increased fusion rate.

Of course, the mechanism of action of these salts is not understood. More detailed studies regarding the structure of membranes and the role of ions have yet to be done in order to understand and bring about fusion of protoplasts from widely divergent species. However, sodium nitrate did not gain much importance as a fusing agent because high fusion frequencies were not achieved amongst vacuolated protoplasts such as the leaf protoplasts (Power & Cocking, 1971; Cocking, 1972). In the present investigation, sodium nitrate was found to induce a small percentage of fusion (ca. 5%). Also calcium nitrate was found to induce fusion. This is in consistence with the results of Schieder (1975) and Keller and Melchers (1973). The latter have also reported the absence of either protoplast aggregation or fusion if magnesium was substituted for calcium. In the present investigation, $Mg(NO_3)_2$ induced aggregation but no fusion. In comparison with all other salts used, $CaCl_2$ was found to be the best. In fact, quite a high percentage of fusion (10%-15%) was achieved using $CaCl_2$ at high pH. Keller and Melchers (1973) have reported 20-50% protoplast fusion after $CaCl_2$ treatment at a high pH and high temperature (37C). The use of $CaCl_2$ at high pH and temperature for bringing about fusion is based on the following grounds. It was suggested by Toister and Loyter (1971) that high pH conditions induced the formation of intramembranous

lysophospholipids such as lysolecithin, and lysophosphati-
dylethanolamine. Lysolecithin has been shown to induce
animal cell fusion (Lucy, 1970). Keller and Melchers
(1973) thought that it was possible that intramembranous
lysolecithin might induce fusion between the plant proto-
plabts too. According to them, the high pH conditions
might be changing some of the characters of membranes
by disturbing the surface negative charge on the proto-
plasts towards conditions favoured by aggregation.
Calcium might be acting by stabilizing the protoplasts
against lysis and by linking the membranes between
different protoplasts. Higher temperature (37C) was
found to hasten the process (Keller & Melchers, 1973).

Effect of Gelatin

In the present investigation, A. thaliana, small
amount of fusion of protoplasts was observed by using
2% gelatin as a fusing agent. Kameya (1973) reported
aggregation and fusion of protoplasts by using 2-5%
gelatin. In fact, he found that similar results were
obtained by using any of the highly water soluble, high
molecular weight compounds like gelatin or dextran sulphate
(or a combination of these two). He postulated some changes
in the electrical properties of the plasma membrane by
these compounds which finally resulted in fusion of
protoplasts. However, the exact mechanism of action of

gelatin with respect to induction of protpplast aggregat-
ion and fusion is not clear.

Effect of Lectins

In the present investigation, agglutination of
protoplasts was obtained by using 40 μg/ml Con A. This
is in confirmation with the results of Glimelius et al.
(1974) who reported the agglutination of protoplasts with
various concentrations of Con A. The results seem more
significant from the microscopic observations because,
as compared to the control, in which 1% aggregation was
observed, the treated samples showed 16% aggregation on
an average. However, the spectrophotometric observations
showed quite a large decrease in O.D. (which was due to
agglutination of protoplasts) in the control also. This
was, however, lesser than the decrease in O.D. observed
in the treated sample.. It is possible that in the treated
sample, Con A did not agglutinate the protoplasts very
tightly and while handling the suspensions for spectro-
photometric analysis, the aggregates could have separated
out. Since some spontaneous aggegate formation occurred
in the control too (which was apparently quite high in
this case), it could account for the small difference
obtained in the control and in the treated sample. Therefore,
the microscopic method even though tedious, was preferred
to the spectrophotometric method for observing agglutina-
tion of protoplasts. It is speculated that lectins act by

binding on specific sites (probably carbohydrate groups)
on the membranes and induce cytoagglutination by cross-
linking the receptors on different cells (Davis et al.,
1976). The details of the mechanism of agglutination by
lectins in plants cells need to be worked out.

Effect of PEG

This has been successfully utilized to bring about
intraspecific fusion, as also the intergeneric aggregation.
Fusion in the latter case was not confirmed probably
because of the insignificant number of heteroplasmic
fusions that might have occurred as compared to the
homoplasmic ones. Kao et al. (1973) have also reported
that even though adhesion of protoplasts by PEG is non-
specific, yet hompplasmic fusions are favoured over
heteroplasmic ones. Amongst all the chemicals tested,
PEG was found to be the best for bringing about fusion
amongst a large number of protoplasts of different origin
in shortest time. The exact mechanism of PEG induced
fusion is not clear though some theories have been proposed
(Constabel & Kao, 1974; Grout & Coutts, 1974; Kao &
Michayluk, 1974 and Wallin et al. 1974). According to
Constabel and Kao (1974) agglutination of protoplasts in
the presence of PEG occurs due to the attraction of proto-
plasts by electrostatic forces. Polyethylene glycol (PEG),
(which is slightly polar and preferentially exhibits a
negative charge like the negative charge on the plasmalemma

of plant protoplasts) acts by forming a sort of bridge between the surfaces of adjacent protoplasts either directly or indirectly through bivalent ions like Ca^{++}. Fusion is presumed to result due to the disturbance and redistribution of electrical charges when PEG is diluted out. The membrane contact which might extend to large areas might be discontinuous, forming intervening spaces. When PEG solution is diluted, the protoplasts which are shrunken, expand, erupting the opposite plasma-lemma at various points such that there is an establishment of cytoplasmic continuity between adjacent protoplasts. Finally the membranes of the fusing protoplasts join at their outer edges and the intervening sections form vesicles which degrade gradually.

Effect of Slow Deplasmolysis

Unlike Keller et al. (1973) who achieved protoplast fusion by a deplasmolyzing osmotic shock, the fusion was achieved in the present investigation, only by bringing down the concentration of mannitol slowly from 0.5M to 0.3M. The numerous protoplasts, when they are in close proximity with each other and slightly shrunken, expand on deplasmolysis. In doing so, the membranes of the protoplasts lying close to one another are brought in contact over smaller or larger areas, finally resulting in fusion. This principle later gained importance while

treating the protoplasts with PEG. Actual fusion was achieved only when PEG was diluted out resulting in the expansion of protoplasts and finally their fusion.

Fusion of Mutants

Fusion of thiamine mutants, Py and Tz (see Materials and Methods) of A. thaliana was tried according to the scheme shown in Fig. 71. As seen from the results, only a limited success has been obtained in this scheme. The fusion does occur, but may be the fusion product requires some specific nutritional conditions which need to be standardized further in order to achieve somatic hybridization.

Fig. 21. The scheme followed for somatic hybridization of Py and Tz mutants of A. thaliana.

FIG. 71

Thiamine

$\underbrace{\qquad\qquad}$ Pyrimidine $\underbrace{\qquad\qquad}$ thiazole

Py mutant
(required thiamine
or Pyrimidine for
growth)

Tz mutant
(required thiamine or
Pyrimidine for growth)

Protoplasts
P_y

Protoplasts
T_z

PEG (Fusion)

Hybrid
$(P_y \, T_z)$
(Should not require Thiamine
for growth)
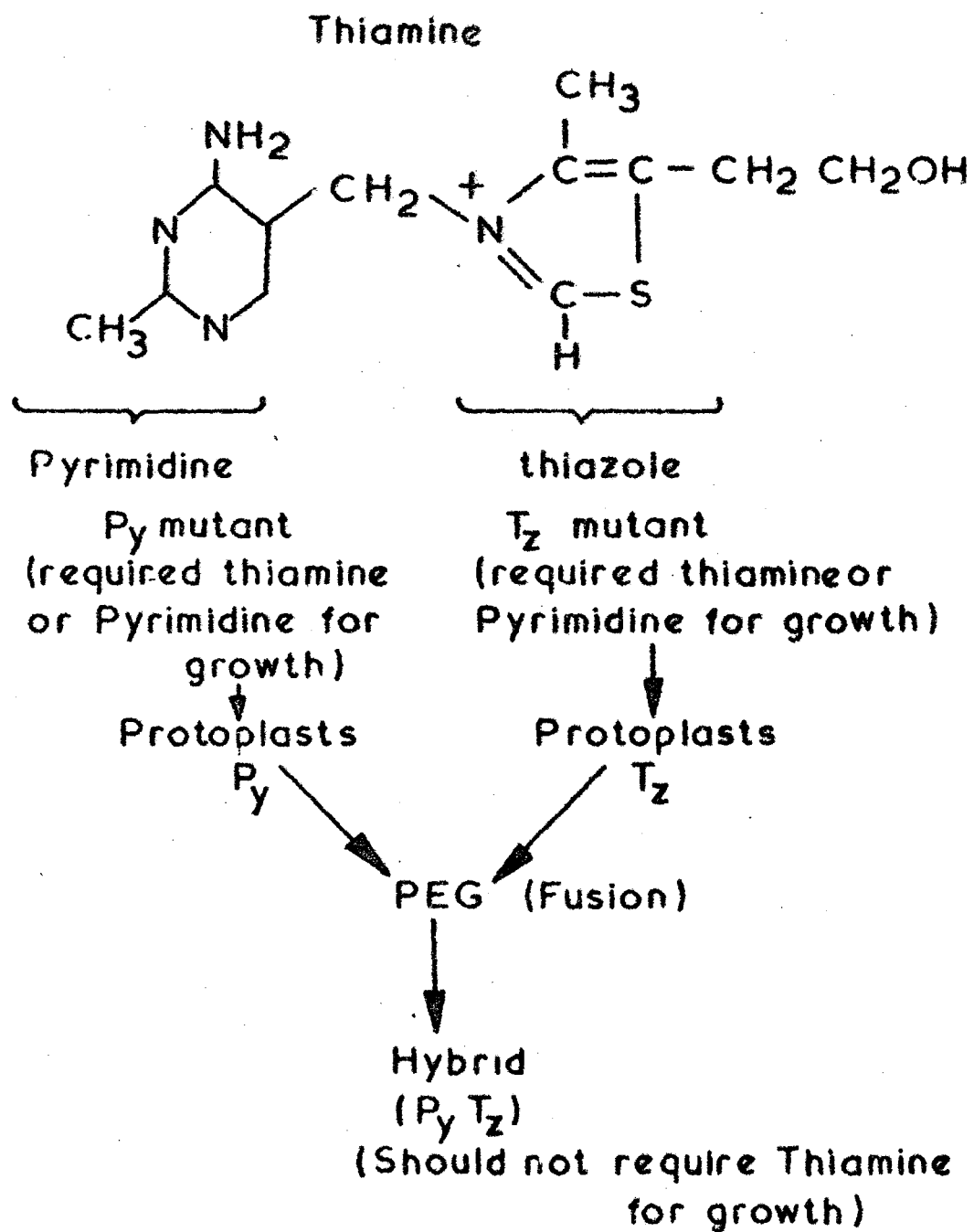
SUMMARY

The work in this laboratory was started to standardize the conditions for, i) the isolation of viable protoplasts from various materials and tissues and ii) fusion of these protoplasts. The effects of different chemical and physical factors for the isolation and fusion of protoplasts have been studied. In addition, an attempt has been made to standardize the conditions for the culture of protoplasts. The experimental plants were, Nicotiana, Datura, Arabidopsis, Petunia, pea, radish, cabbage, potato and wheat.

Chemical factors - For the isolation of protoplasts, tissue required to be treated with an osmoticum, macerozyme and cellulase. However, the conditions for the isolation of protoplasts from various tissues need to be standardized in each laboratory, as modifications from the established conditions are generally required, depending upon the genetic variations in tissues.

Mannitol, 0.6M was found to be a better osmotic stabilizer than sucrose for the isolation of Nicotiana and Datura leaf protoplasts. For all other materials used, e.g. leaves of Arabidopsis, pea, wheat, radish, cabbage, potato and Petunia, 0.4-05M mannitol was found to be suitable for the stability of protoplasts. Potassium dextran sulphate, when used along with mannitol, for the isolation of protoplasts of A. thaliana was found to affect the yield adversely.

Macerozyme, 0.5% was effective for isolation of single cells from leaves. This concentration had to be raised to 1% for the isolation of cells from callus.

Cellulase, 5% was found to release protoplasts most effectively. Cellulase, 2-3% was also found to release protoplasts, but a longer (>5 hr) incubation period was required.

pH, A range 5.0-6.0 was found suitable for all materials.

Physical factors - Amongst the physical factors the isolation was better when the incubation was carried out in dark.

For the isolation of Nicotiana and Datura protoplasts, 37C was found to be the optimum temperature, whereas for all other materials 25-30C was found to be suitable.

Shaking at 45 strokes/min was found to hasten the release of protoplasts, but it caused a little damage.

Generally, the leaves from younger plants were found to be more suitable for the isolation of protoplasts rather than from older ones. In Arabidopsis, the leaves of plants varying in age from 25-36 days were found to yield larger number of protoplasts than the leaves from plants either younger or older than this age.

Leaves were found to be the most suitable material for the isolation of protoplasts. Callus proved slightly tough. In the latter case, fresh subcultures yielded better results.

Distinct morphological differences were observed in protoplasts from one species to another. These were exhibited in terms of size, number and distribution of chloroplasts, position and number of vacuoles and distribution of cytoplasm.

Seasonal variations in the temperature and humidity conditions were found to affect the yield of protoplasts significantly in the case of naturally growing plants. That is why, controlled culture conditions of the plant materials utilized were found to be essential for reproducible results.

Some characteristic protuberances of protoplasts were observed in **Nicotiana**. These were found to arise from one of the protoplasts lying in between two other protoplasts. These are not reported by other workers. The significance of these is not known.

Viability - The isolated protoplasts were proved to be viable by the movement of contents inside them (cyclosis), maintenance of shape of regulation of osmotic conditions, incorporation studies and the fluorescent dye staining technique. In some instances, protoplasts regenerated walls and showed limited division

proving thereby that they were viable.

Wall regeneration - In A. thaliana wall regeneration was noticeable after 48 hr of culture in Gamborg's B_5 medium enriched with 10% coconut milk and also in Nagata and Takebe medium.

Cell division - This, and occasional colony formation was achieved on Nagata and Takebe -- liquid and solid medium respectively.

Fusion medium - Amongst all the chemicals tested for bringing about fusion of protoplasts, high molecular weight PEG(6,000) was found to be most effective. High pH and $CaCl_2$ treatment, as well as slow deplasmolysis were also found to help in fusion. Inorganic salts like, $NaNO_3$, $Mg(NO_3)_2$, $Ca(NO_3)_2$, and gelâtin could bring about 2-5% fusion. Potassium nitrate was found to be only slightly effective and brought about 1-2% fusion.

The specific lectin, Con A (of jackbean origin) induced aggregation but no fusion. The new technique of utilizing spectrophotometêr to determine aggregation by Con A was found to be less suitable than the microscopic method.

These studies on the isolation and fusion of protoplasts form the preliminary steps for achieving the goal on somatic hybridization.

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