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THE USE OF CELL SURFACE PROPERTIES FOR HYBRID PROTOPLAST SELECTION

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STATEMENT

The work on the use fluorescein diacetate as a viability stain for protoplasts was a development from an observation initially made by myself during my Honours study at the University of New South Wales. With this possible exception this thesis contains no material that has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief it contains no material previously published or written by another person, except when due reference is made in the text.

signed

P.J. LARKIN.

Acknowledgements

I am indebted to Professor C.J. Driscoll whose foresight beyond his own field initiated my interest in protoplasts. The value of his advice and interest and his sharing of my excitement over things learned have never diminished. Thanks are also due to Dr. W.R. Scowcroft who has given valuable advice despite geographical isolation. I must also acknowledge the extraordinary generosity of Dr. M.A. Jermyn in synthesising many chemicals to suit my needs.

My research project was dislocated by a transfer from Sydney to Adelaide. The potential trauma of this move was very much cushioned by the helpfulness of the people of the Department of Agronomy. Those who shared the dislocation with me also contributed the insanity needed to make the cytogenetics laboratory a suitable dwelling place for me. Thanks to Joan Sharpe for her typing.

I would like to thank my parents, John and Mancel, for their encouragement and support throughout my 25 years —— the first period of my education. The last 5 years and 20 months respectively have been made very much more fun by my two good friends : my wife, Elwyn, and son, Owen.

Abbreviations used

MES	=	2-(N-morpholino) ethanesulfonic acid
2,4-D	=	2,4 - dichlorophenoxyacetic acid
NAA	=	1-naphthaleneacetic acid
IAA	=	3-indoleacetic acid
ZEA	=	zeatin
KIN	=	kinetin = 6-furfurylaminopurine
BA	=	6 - benzylaminopurine
pCPA	=	p-chlorophenoxyacetic acid
PEG(x)	=	polyethylene glycol (average molecular weight x)
PVP	=	polyvinylpyrrolidone
BSA	=	bovine serum albumin
MW	=	molecular weight
PD	=	transmembrane potential difference
FDA	=	fluorescein diacetate
h	=	hour(s)
min	=	minute(s)
x		signifies a sexual cross
+		signifies a somatic (fusion) cross

TABLE OF CONTENTS

	Statement	ii
	Acknowledgements	iii
	Abreviations used	iv
	Table of contents	v
	Summary	vii
SECTION 1	GENERAL INTRODUCTION	1
SECTION 2	GENERAL LITERATURE REVIEW	6
2-1	DEVELOPMENT OF PROTOPLAST TECHNOLOGY	6
2-1-1	Early History and the Fundamentals of Isolation	6
2-1-2	Recent Advances in Protoplast Isolation	12
a)	Plant growth conditions	12
b)	Tissue pretreatment	15
c)	Osmoticum modifications	18
2-1-3	Protoplast Culture	25
2-1-4	Protoplast Fusion	46
2-2	GENETIC LOSS IN WIDE CROSSES	60
2-3	SOMATIC HYBRIDIZATION	64
2-4	PROGRESS AND PROBLEMS WITH MUTANT CELL LINES	76
SECTION 3	PURIFICATION AND VIABILITY DETERMINATIONS OF PLANT PROTOPLASTS	81
	Summary, Introduction , Materials and Methods, Results, Discussion.	
SECTION 4		92
4-1	MODEL 1 FOR HYBRID SELECTION USING ANTISERA	92
4-2	PLANT PROTOPLAST AGGLUTINATION AND MEMBRANE-BOUND β-LECTINS	94
	Summary, Introduction, Matterials and Methods, Results, Discussion.	
4-3	CONCLUSIONS FOR MODEL 1	122

SECTION 5			123
	5-1	MODEL 2 FOR HYBRID SELECTION USING β-LECTIN DIFFERENCES	123
	5-2	PLANT PROTOPLAST AGGLUTINATION BY ARTIFICIAL CARBOHYDRATE ANTIGENS Summary, Introduction, Materials and Methods, Results and Discussion, Conclusions	125
	5-3	CONCLUSIONS FOR MO DEL 2	143
SECTION 6			144
	6-1	MODEL 3 FOR HYBRID SELECTION USING LECTINS	144
	6-2	PLANT PROTOPLAST AGGLUTINATION BY CLASSICAL LECTINS Abstract, Introduction, Materials and Methods, Results, Discussion	146
	6-3	CONCLUSIONS FOR MODEL 3	163
SECTION 7			164
	7-1	MODEL 4 FOR HYBRID SELECTION	164
	7-2	EVALUATION AND CONCLUSIONS FOR MODEL 4	166
SECTION 8			171
	8-1	PLANT PHENOLIC AND TERPENOID GLYCOSIDES	171
	8-1-1	Introduction	171
	8-1-2	Materials and Methods	174
	a)	Plant tissue extractions	174
	b)	Preliminary purification of <u>Ceratonia</u> extract	174
	c)	Chemicals	176
	8-1-3	The Blocking of β-Lectin Effects on Protoplasts	178
	8-2	MODEL 5 AND 5a FOR HYBRID SELECTION USING β-LECTIN BLOCKERS	186
	8-2-1	Differential Abilities to Use Different Carbon Sources	189
	8-2-2	Materials and Methods	191
	8-2-3	Testing of Model 5a	192
	8-3	CONCLUSIONS FOR MODELS 5 AND 5a	203
SECTION 9		GENERAL CONCLUSIONS	205
SECTION 10		LITERATURE CITED	210

SUMMARY

The development of and the problems associated with somatic hybridization were reviewed. The most widespread approaches to selecting protoplast hybrids from fusion mixtures of 2 types of protoplast are based on (i) the additivity of antimetabolite resistances, or (ii) auxotrophic complementation. These approaches were criticized mainly on their non-generality and their inability to select for many partial hybrids. It was argued that many, if not all, of these criticisms would be avoided if hybrid protoplasts were selected by cell sorting procedures.

An initial model attempted to produce specific antisera which would differentially agglutinate one or other of the 2 species of protoplast. However massive agglutination was equally caused by control serum and by test serum. This phenomenon was investigated further. All animal sera tested were able to induce protoplast agglutination of all plant species with which they were tried. It was postulated that antibodies were not responsible for this agglutination and that β-lectins in the protoplast plasmalemma were binding to suitable multi-liganded glycosidic substrates in the sera. This postulate was supported by the observation that all protoplasts agglutinate in the presence of artificial carbohydrate antigens, called Yariv antigens, provided the glycosides are also suitable for binding by purified plant β-lectins. Also both serum- and Yariv antigen-induced agglutination could be partially inhibited by certain phenolic-β-D-glycosides.

Experiments were also conducted to assess whether extracted plant lectins could be used to sort hybrid protoplasts from parental protoplasts. Some lectins, namely soybean lectin, concanavalin A, peanut lectin and castor bean lectin II, were able to agglutinate protoplasts. However none displayed any capacity to differentiate between different protoplast species.

Certain crude plant phenolic extracts were able to inhibit protoplast agglutination (as induced by either serum or Yariv antigens). These extracts also blocked the β-lectin sites so that the protoplasts, even after repeated rinsing, were not agglutinated. Preliminary purification suggested flavonoid glycosides may be the blocking molecules. A model for hybrid selection was presented employing 2 antigenically distinct blocking molecules and 2 antisera specific for these blockers. Some of the essential presuppositions of this model were validated using a modified model. This model is general in the complete sense of the word because the surface differences between parent protoplasts are artifically created in the form of pretreatments with the blocking agents. Hybrid selection based on applied surface differences is independent of the genes present in the hybrid cells. Therefore this selection should also be able to recognise hybrids which have lost some or all of the genetic material of one of the parents.

SECTION 1 GENERAL INTRODUCTION

Hybridization is an essential feature of almost all crop breeding programmes enabling the introduction of new genetic material. Extensive application of this process is limited by reproductive barriers. One such barrier is the breakdown of the developing endosperm of a hybrid embryo. This may be overcome by culturing the embryo on synthetic medium. The failure of the parental chromosomes to pair in meiosis would lead to sterility in the hybrid plant. This can be avoided by chromosome doubling techniques. Sexual incompatibility is a more extreme form of reproductive barrier which limits attempts to tap new sources of genetic diversity. Alien pollen can be rejected at a number of stages including germination, pollen tube penetration of the stigma, pollen tube nutrition for growth down the style, and penetration of the egg cell. Somatic hybridization is a scheme for bypassing all sexual mechanisms and hence all sexual incompatibility. The scheme is illustrated in Fig 1-1.

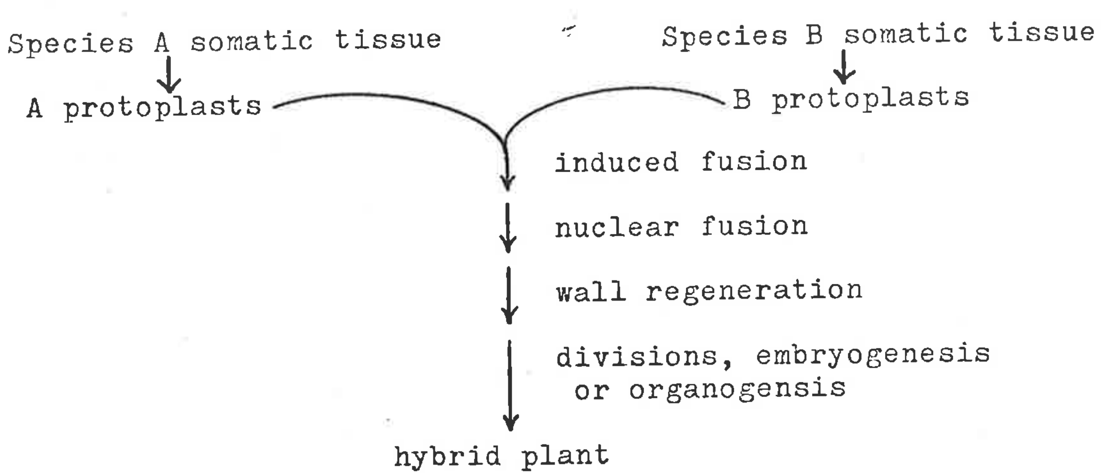

Fig 1-1. Somatic hybridization scheme.

Plant cells are surrounded by a rigid wall and cannot be fused. Protoplasts are formed by the removal of the wall.

Fusion of somatic cells from different plants is made possible with protoplasts.

Although sexual incompatibilities are bypassed it is likely that in very wide somatic crosses biochemical and chromosome organisation incompatibilities will occur. Experience with wide sexual crosses (see 2-2) suggests that extensive genetic loss can be expected from one of the parents. Somatic hybridization will allow the bringing together of two (or more) diverse genotypes at least for the duration of a few divisions. The worker seeking to use somatic hybridization will need to be able to screen not only for full hybrids but for chromosome additions or substitutions, single gene exchanges, or even cells with the nuclear genotype of one parent but a mixed cytoplasm.

Despite a number of successful applications of somatic hybridization (see 2-3) there are a number of troublesome points delaying general application. One of these is the failure to find appropriate culture conditions for protoplasts of many species. However the bottleneck causing the greatest difficulty is the need for a means of selecting hybrid cells from parental cells in the fusion mixture. Even with the more recent fusion technique (see 2-1-4) hybrid protoplasts will be in a minority relative to parental and self-fused protoplasts. If the fusion mixture is grown non-selectively the hybrids will be lost amongst the parental genotypes. The most widespread approach to this problem, following the animal cell culture pattern, is the use of selective media. This approach takes two forms a) Auxotroph complementation. Two plant cell lines are needed each requiring an exogenous supply of a metabolite (e.g. amino acid, nucleotide or vitamin) which the wild

type cell does not require. It is assumed that the fusion hybrid will be self-sufficient for both metabolites because one of the two genomes has non-mutant genes for the appropriate functions i.e. the two genomes complement. The fusion hybrid will, unlike the parental protoplast types, grow on minimal medium (in which the two metabolites are not supplied). b) Additive antimetabolite-resistances. Two plant cell lines are needed resistant to one each of two antimetabolites e.g. amino acid analogues, toxic levels of NaCl. It is hoped the fusion hybrid will inherit the resistances of both parent lines and thus have a selective advantage over parental-type protoplasts in media containing both antimetabolites.

Such approaches to hybrid selection have been and may continue to be useful (see 2-3) but suffer from certain disadvantages:

(i) In both cases mutant lines are needed. Mutant isolation is time-consuming and difficult. Many mutants prove to be unstable and/or "leaky" (see 2-4). These approaches are not general because new mutants would be required in each prospective parental genotype.

(ii) Most of the mutant lines are maintained in culture. Cultured cell protoplasts have proved more difficult to regenerate than protoplasts derived directly from plant tissues. Of the 22 plant species whose protoplasts have been regenerated to plants only 3 examples involved protoplasts of cultured cells (see 2-1-3).

(iii) There is the danger with auxotrophic mutants that in mixed culture of the two mutants one will "cross-feed" the missing metabolite to the other and vice versa thus allowing both mutants

to continue growing on minimal medium. Such cross-feeding may not be possible with certain types of metabolites which should be given preference in this approach.

(iv) In the case of additive anti-metabolite resistances it may be susceptibilities rather than resistances which are inherited by the fusion hybrids.

(v) In both these approaches selection depends on the presence in the fusion hybrids of a few particular genes from both parents throughout the selection period. However it has already been pointed out that in the wide crosses to which somatic hybridization will be applied extensive loss of genetic material can be expected. Thus a selection system based on a few particular genes may well overlook most of the potentially useful partial hybrids.

The concern of this thesis is the investigation of alternative hybrid selection systems based on cell surface properties and the physical sorting of cells. A fusion-hybrid protoplast will have a hybrid plasma membrane bearing the combination of surface properties of the parent protoplasts. A cell sorting procedure utilizing the hybrid surface properties of these protoplasts would have the advantage of effecting hybrid selection prior to genetic loss and thus not overlooking potentially useful partial hybrids. In addition to this advantage, no mutants are required and protoplasts can be isolated from whichever tissues are most likely to allow regeneration. Furthermore, if the membrane differences being exploited are artificially applied to the parental protoplasts prior to mixing and fusion, then the selection technique is universal and can be applied equally to any two parents.

However selection based entirely on cell sorting has the disadvantage that none of the alien genome is being required to function and thus there is no pressure to retain any of the alien genome. The ideal selection regime may begin by enriching for fusion hybrids by cell sorting, followed by gentle nutritional pressure for certain desirable functions of both genomes.

A number of potential cell sorting approaches are evaluated in this thesis. Firstly antisera are examined to see if they can be produced with the specificity required to differentiate between the protoplasts of 2 species and their fusion hybrid. Exogenous lectins are examined for the capacity to differentiate between protoplasts on the basis of surface antigens. Endogenous membrane-bound lectins are similarly considered to see if they differ sufficiently between protoplasts of different species so that they respond differently to exogenous antigens. Attempts are also made to utilize the characteristics of the membrane-bound lectins to artificially coat the protoplasts of 2 species with different moieties prior to fusion. When these moieties are certain phenolic glycosides they appear to be sufficiently stable to allow protoplast sorting on the basis of differential agglutination.

SECTION 2 GENERAL LITERATURE REVIEW

2-1 THE DEVELOPMENT OF PROTOPLAST TECHNOLOGY

2-1-1 Early History and the Fundamentals of Isolation

The word 'protoplast' will be used here to denote the isolated plant cell denuded of its cell wall. This term has also been used for a cell which has plasmolysed away from the wall but is still contained within the wall. Despite this minor point of ambiguity and despite the suggested alternative of 'gymnoplast' (Frey-Wyssling, 1967), common usage overwhelmingly supports the term protoplast being used for a naked isolated cell.

Prior to 1960 the only means available for releasing protoplasts was by mechanical manipulation. Klercker (1892 in Cocking, 1972) was the first to isolate intact protoplasts. The method involved plasmolysis of the tissues of the water warrior (*Stratiotes aloides* L.) and then sectioning of the tissue at thicknesses designed to cut the cells only once. Yields were low and many cells were damaged. Such techniques are only feasible with tissues whose cells are highly vacuolated and able to shrink away from the cell wall. Epidermal protoplasts of onion and radish have been isolated mechanically (Chambers and Hofler, 1931, and Tornava, 1939, both in Evans and Cocking, 1975). Whatley (1956) similarly obtained beet storage parenchyma protoplasts.

Since the advent of enzymatic methods, mechanical production is rarely used. Pilet *et al* (1972) isolated onion root protoplasts both mechanically and enzymatically and compared the RNase and transaminase activities. The activities were higher with the mechanical method. However they could not

discount the possibility that the two preparations represented different subpopulations of the root cells. It was also possible that the 12h. required for enzymatic isolation caused the relative drop in activities. Giles (1974 b) produced protoplasts from the coenocytic, non-septate alga, Bryopsis plumosa, by plasmolysis and fracture. Mature algae could be regenerated from such protoplasts. Similarly, protoplasts were prepared from the moss, Polytrichum juniperinum and leafy gametophytes regenerated (Gay, 1976). In this latter case the available enzymes were totally ineffective. Occasionally mechanical procedures are used to produce single cell suspensions which are then digested enzymatically to protoplasts (Miksch and Beiderbeck, 1976; Harada, 1973; Bui-Dang-Ha and Mackenzie, 1973).

The first protoplasts produced with enzymes were of tomato roots and fruit locule and oat coleoptiles using culture filtrates of the fungus, Myrothecium verrucaria (Cocking, 1960; Gregory and Cocking, 1965; Ruesink and Thimann, 1965). The chief advantages were immediately apparent in that the yields were relatively much larger and tissues were used which are unsuitable for mechanical methods. Cellulytic enzymes are now available commercially. The most common sources are Trichoderma viride (Onazuka,Cellulysin and Meicelase P), a Basidicomycete (Driselase) and snail (Helix pomatia) gut juice (Glusulase and Helicase). These are described as cellulases but their activities are more complex than this designation conveys (Taiz and Jones, 1971). Rhozyme HP150 is described as a Rhizopus hemicellulase though its activity is complex. Similarly the Rhizopus 'pectinases' (Macerozyme and Macerase) and Aspergillus 'pectinases' (Pectinol R10, Sigma

pectinase and Serva pectinase) are mixtures of various activities (Evans and Cocking, 1975; Thomas and Davey, 1975). Cassals and Barlass (1976) demonstrated that Serva pectinase had pectinase activity but also as much cellulase activity as Driselase.

In addition to these enzymes there have been some less-widely used preparations. These include Zymolase (ex-*Arthrobacter luteus*) used for pollen tetrad protoplasts (Wakasa, 1973), Xylanase (ex-*Aspergillus niger*)(Landova and Landa, 1975; Li-Su-Nam *et al*, 1976; Mezentsev *et al*,1976) and Colonase (Wakasa, 1973). Also an enzyme from *Penicillium citreo-viride* has recently been used (with Onazuka, Macerozyme, and Driselase) to isolate barley callus protoplasts (Koblitz and Hirte, 1976; Koblitz, 1976). Cereal callus cultures have been particularly refractory to digestion prior to these reports.

Cell walls of different species and tissues can differ greatly in composition (Keller *et al*, 1970; Gamborg and Eveleigh, 1968; Burke *et al*, 1974). The hemicelluloses such as xylans, galactomannans and glucomannans adsorb to the cellulose microfibrils and can obstruct cellulase action (Northcote, 1972). The uronic acid- and arabinose - containing side chains of the xylans in particular vary between species. It is therefore significant that Keller *et al* (1970) found a correlation between arabinose, galactose and xylose composition and the inability to form protoplasts with purified cellulase, pectate lyase, and endopolygalacturonase. Lignins are high molecular weight products of dehydrogenation and polymerisation of coumeryl, coniferyl and sinapyl alcohols. During lignification these compounds penetrate the wall replacing the water of hydration in

the matrix (Northcote, 1972). Such a wall is refractory to enzymatic digestion. Leaf parenchyma and cell cultures have low levels of lignification and uniformity of cell type and are the most common sources of protoplasts.

There has been some concern over the impurity of commercial cellulytic enzymes. Schenk and Hildebrandt (1969) claimed that pretreatment of the enzymes at pH 0.8 (or 1NHCl in acetone at -20°C) reduced the toxic effects. They suggested this pretreatment was inactivating peroxidases by removal of heme groups. However Ruesink (1971) treated oat coleoptile protoplasts with horseradish peroxidase for 1h with no detrimental effect. He also found no correlation between the low levels of peroxidase in a number of cellulases and the yield or viability of the protoplasts they produced. A number of impurity enzyme activities can be present including DNase, RNase, protease, lipase, phospholipase, β-1, 3-glucanase and chitinase (Cocking, 1972). Ruesink (1971, 1973) concluded that any strongly basic protein, irrespective of enzyme activity, could bind to the negatively-charged protoplast surface causing a destabilization. It may be partly for this reason that Ca^{++} ions are so widely useful for protoplast stability since they neutralize the protoplast surface. Maretzki and Nickell (1973) noted a detrimental effect of Glusulase on protoplast respiratory rates which could not be prevented by prior dialysis or Sephadex G25 purification.

Attempts to purify the enzymes often results in increased activities in the specific assay being used, but simultaneously a decline in ability to release protoplasts. Various column chromatographic fractionations of cellulytic enzymes have resulted

in a number of specific activities but cell wall degradation was only possible when some or all of the fractions were remixed (Schenk and Hildebrandt, 1969; Karr and Albersheim, 1969, 1970; Selby, 1973). Von Arnold and Eriksson (1976) found that all attempts to purify the enzymes resulted in fewer surviving pea leaf protoplasts.

Eriksson et al (1974) claimed that a brief treatment of Driselase with 10mg/ml activated charcoal reduced its toxicity although there was a concomitant reduction in activity. It is commonly presumed that the desalting of the enzymes on Sephadex G25 or Biogel P6 will decrease toxicity. However Cassals and Barlass (1976) found that Biogel P6 desalting of Macerozyme, Onazuka SS and R10, Serva pectinase and Driselase did not increase protoplast yields nor viability and certainly decreased wall-degrading activities relative to crude solutions of equivalent protein concentrations. It is quite clear that despite advances in our understanding of the composition of the plant cell wall (Burke et al,1974; Albersheim, 1974) it is not yet feasible to define in terms of pure enzymes what is required to degrade various species.

The most frequent isolation method involves incubation of the tissues in cellulase/pectinase mixtures. However the first enzymatic isolations with leaf protoplasts used a 2 step sequential method. Free walled-cells were first released with Macerozyme and then digested with Onazuka cellulase to release protoplasts (Otsuki and Takebe, 1969; Nagata and Takebe, 1971). Similar sequential isolations have been used with tobacco leaf (Burgess and Linstead, 1977; Pisetskaya et al, 1975), tomato leaf (Mühlbach et

al, 1977) and mulberry leaf (Ohyama and Oka, 1975). A number of workers have employed multiple enzyme treatments but without a distinct non-protoplast, free-cell stage. Examples include Citrus sinensis L. callus (Vardi et al, 1975), Brassica napus L. leaf (Kartha et al, 1974 a), Pisum sativum L. shoot apex (Gamborg et al, 1975 a) Hordeum vulgare L. callus (Koblitz, 1976) and Pharbitis nil L. cotyledons (Messerschmidt, 1974).

Upachya (1975) appears to be the only author to have compared single-and multi-step isolations. He concluded for Solanum tuberosum L. leaf that the single step procedure gave more consistent yields. Also less manipulations may lessen the risk of contamination.

The application of relatively standard techniques to more and more species does not require analysis. There are however a number of instances of the use of tissues which are not normally used and reference will be made to these. Petal epidermis protoplasts contain coloured vacuoles and thus may be very useful for observing fusion events. Similar enzyme treatments are required for these as for the leaves (Potrykus, 1973; Landova and Landa, 1975; Wakasa, 1973). Protoplasts have also been obtained from pollen and pollen mother cells of Lilium L. and Trillium L. (Ito, 1973 a,b; Ito and Maeda, 1973, 1974; Takegami and Ito, 1975), tobacco (Bhojwani and Cocking, 1972; Rajasekhar, 1973; Bajaj, 1974), petunia, wheat, rye, Datura L., tomato, Atropa L., Solanum L., Antirrhinum L., maize, Luffa Mill., Cajanus Adans. mut. DC. and Dianthus L. (Bhojwani and Cocking, 1972; Bajaj, 1974; Rajasekhar, 1973; Wakasa, 1973; Deka et al, 1977).

2-1-2 Recent Advances in Protoplast Isolation

2-1-2 a) Plant growth conditions. There has been concern over the seasonal variations of protoplast yields. Plants grown in particular seasons give much better yields than those in other seasons depending on the location of the laboratory (Power and Cocking, 1970; Schilde-Rentschler, 1972; Morel et al, 1973; Potrykus, 1973). Watts et al, (1974) attempted to more carefully define appropriate growth conditions for tobacco (cv. White Burley) leaf protoplast isolation. They concluded that growth needed to be rapid and uninterrupted. Plants were supplied regularly with a high nitrogen fertilizer and sodium vapour lamps used to maintain 10,000-20,000 lux (15h day, 22°C) throughout the year. During summer the plants were shaded to avoid illumination exceeding 30,000 lux. By using leaves that were fully expanded but not senescing from 40-60 day old plants, protoplasts were stable and could be produced throughout the year.

Morel et al (1973) claimed that controlling the temperature did not negate the seasonal variation. However Kartha et al (1974a) found that rape leaves grown at 19-21°C with 40-45% relative humidity produced good protoplast yields while those grown at 26°C with 70% humidity gave low yields of unstable protoplasts. They did not state whether the light conditions were the same for this comparison.

Recently Cassals and Barlass (1976) gave what appears to be an important insight into the mechanism for plant growth condition effects on protoplast yields. They grew tomato plants in the glasshouse during summer either with full sunlight ("hard plants") or under muslin shade ("soft plants"). The yields from

hard plants were low and inconsistent. The protoplast yields from soft plants were consistently higher (even in other seasons). The hard leaves had a 10 fold increase in calcium pectate relative to soft leaves. Sodium citrate (1.9mM), though not other chelators, increased yields from all leaves when included in the enzyme mixture. They supposed that the citrate loosened the calcium pectate which otherwise obstructed wall degradation. El Hinnawy (1974) has also noted the ability of chelators such as cyclohexane-1,2-diamine tetraacetic acid (CDTA) to weaken calcium pectate. EDTA (0.01M) has been reported to accelerate the enzymatic maceration of certain leaf species with high calcium contents (Otsuki and Takebe, 1969). Hanke and Northcote (1974) found sodium citrate to be essential for the isolation of soybean callus protoplasts, although they were apparently unaware of any possible connection with the pectate intercellular cement. Low irradiance is an important factor in reducing the leaf 'hardening' caused by calcium pectate. It is interesting that Morel _et al_ (1973) also implicated the calcium content of walls as being involved in the seasonal effects.

There is now a much greater awareness of the need to state the plant growth conditions even when no effort has been made to optimize them. Controlled environment chambers are being more extensively used. The lighting used in such chambers is such that the irradiance is lower than the levels expected in a glasshouse without shading. In the Waite Institute's glasshouses on a sunny day even in July the irradiance can exceed 1000 μE/sec. m^2 (equivalent to 80,000 lux of cool white fluorescent tube illumination according to McCree,1972). Low illumination of

4500-16000 lux was used in the growth of the following plants which gave consistent high protoplast yields : rape (Kartha *et al*, 1974a); barley, ryegrass, meadow fescue, lucerne and red clover (Hughes *et al*, 1976; Mezentsev *et al*, 1976); oats (Brenneman and Galston, 1975; Kaur-Sawhney *et al*, 1976a; Fuchs and Galston, 1976); *Ranunculus* L. (Dorion *et al*, 1975); *Petunia* Juss. (Power *et al*, 1976a); and tobacco (Shepard and Toten, 1975).

Other authors have used conditions which produce etiolated leaves. Taylor and Hall (1976) obtained stable protoplasts from 4-7 day old maize leaves and roots grown with only 312 lux. Wheat and rye epicotyls grown in total darkness were good sources of protoplasts (De La Roche *et al*, 1977).

It is probably very significant that for plants grown in the high and variable illumination of glasshouses it has often been found essential to transfer tissues to total darkness for a few days before protoplasts can be isolated. Examples are *Saccharum spp*.L. (Krishnamurthi, 1976), *Pisum sativum* (Constabel *et al*, 1973, 1975a), *Medicago sativa* L. and *Caragana arborescens* Fabr. (Constabel *et al*, 1975b), and *Melilotus officinalis* Mill. (Fowke *et al*, 1976). Similarly Pelcher *et al* (1974) moved *Phaseolus vulgaris* L. from the glasshouse to very low light conditions (200-400 lux) for at least 24h before stable protoplasts could be obtained in high yield. It may be that a period of darkness is able to partially reverse the high-light induced 'hardness' of tissues.

At least in the case of pea (*Pisum sativum*) the need for a darkness pretreatment is negated by growing the plants in a

controlled low irradiance environment. Von Arnold and Eriksson (1976) obtained consistent high yields from leaves of pea plants grown at 22°C, 60% relative humidity with a 16h day of 500 $\mu W/cm^2$ cool white fluorescent light (equivalent to 13,650 lux).

An alternative approach to the need to produce plant material which will routinely produce good protoplast preparations has been the use of axenic shoot cultures. Tobacco and petunia shoots (Binding, 1974a, 1974b, 1975, 1976) and Datura innoxia L. shoots (Schieder, 1975) were cultured and subcultured in sterile synthetic media under entirely reproducible environments. Leaves could be regularly harvested and used for protoplast isolation. Using petunia, Binding (1974a) very carefully optimized illumination, sucrose concentration and temperature for the growing cultures to produce maximum protoplast yields. The optimum illúmination was 6000 to 8000 lux even when other parameters were varied.

2-1-2 b) Tissue pretreatment. The standard pretreatment for leaves is to peel the lower epidermis off before floating the leaf pieces, peeled surface down, on the enzyme solution (Nagata and Takebe, 1970). Epidermis peeling very greatly improves enzyme penetration and protoplast yields. Some have gone to the trouble of peeling both the upper and lower epidermis (Adiri and Lavee, 1975, with olive leaves). Others, however, have devised means of avoiding this tedious procedure altogether. Schilde-Rentschler (1972) found that β-glucuronidase (ex-E.coli) or pectinglycosidase (ex-Aspergillus) digested the cutin on Nicotiana tabacum L. and Crepis capillaris L. leaves obviating the need for peeling. Rohament P, another pectinglycosidase, could replace

peeling for tobacco (Sarkar *et al*, 1974) and potato (Upadhya, 1975). Dusting with carborundum dust or brushing with a nylon brush have been recommended as less tedious means of allowing enzymes access to the leaves (Coutts and Wood, 1977; Shepard, 1975).

An alternative approach to allow enzyme access has been to section the tissue transversely or longitudinally with razors (Huber *et al*, 1975; Evans *et al*, 1972; Dorion *et al*,1975). Meyer and Abel (1975a) both peeled tobacco leaves and then preincubated in pectin acid transeliminase (PATE)(ex-*Bacillus polymyxa*). This preincubation allowed more rapid cellulase penetration. Von Arnold and Eriksson (1976) observed an increase in pea leaf protoplast viability if the leaves were peeled when submerged in osmoticum rather than in air or distilled water. Such simple procedural modifications may have profound physiological effects.

Recently Kaur-Sawhney *et al* (1976a,b) have suggested that leaf protoplast yields from cereals have been limited by the release of senescence enzymes during plasmolysis and wounding. They were able to significantly increase yields, integrity and synthetic activity of oat leaf protoplasts by pretreating the peeled leaves for 18h with one of the following senescence retardants: 0.5-1.0mg/l cycloheximide, 1-5mg/l kinetin, 10mg/l methyl-2-benzimidazole-carbamate, or 50mM arginine. Gatenby and Cocking (1977) successfully isolated and grew *Brassica oleraceae* L. leaf protoplasts but only after preincubation of the peeled leaves on callus-inducing medium for 7 days. It may be that this preincubation either inhibited the senescence enzymes or allowed them to diffuse away while the wounded tissue healed.

Cultured cells can be excellent sources for protoplasts, not least because they can be maintained indefinitely by subculture in the medium and growth conditions found favourable (Miller et al, 1971). However many cultures have proved completely unyielding to the available enzymes. There has been very little effort to optimize culture growth conditions for protoplast production. Generally the cultures should be in the mid-long phase of growth. This has been illustrated in soybean (Kao et al, 1970a; Hanke and Northcote, 1974), onion, *Convolvulus* (Bala Bawa and Torrey, 1971), *Gossypium* L. (Bhojwani et al, 1977), carrot (Grambow et al, 1972), *Vicia hajastana* L. (Kao and Michayluk, 1975), and tobacco (Uchimiya and Murashige, 1974). Reid and Galston (1975) demonstrated that soybean callus protoplast yields were higher if the subculture prior to isolation was on auxin-less medium.

Another aspect of tissue pretreatment is preplasmolysis. Plasmolysis of the tissue prior to the enzyme treatment greatly reduces the spontaneous fusion of the resulting protoplasts (Frearson et al, 1973). Cocking (1972) has also suggested that preplasmolysis reduces the uptake of the enzymes into the protoplasts. Plasmolysis causes an infolding of the plasmalemma and formation of vesicles. If plasmolysis occurs in the presence of the exogenous enzymes then they will be included in these vesicles and possibly result in some toxicity. Many researchers routinely use preplasmolysis treatments from 30min (Banks and Evans, 1976) to 4h (Coutts and Wood, 1977). Binding (1974a) investigated preplasmolysis at 9 different mannitol concentrations from 0.2 to 1.0M. The enzyme incubation was conducted at 0.6M mannitol. Preplasmolysis at concentrations below 0.5 resulted in higher percentages of multinucleate protoplasts resulting from spontaneous

fusion. The highest yield occurred when preplasmolysis was with 0.6 M mannitol.

Coutts and Wood (1975) observed that the omission of a 2 or 3 h preplasmolysis of cucumber leaves resulted in poor digestion and non-viable protoplasts. It may be of some advantage to plasmolyse the tissues gradually. Frearson et al (1973) used a sequential treatment with 1 h in each of 5%, 9% and 13% mannitol before enzyme incubation in 13% mannitol.

2-1-2 c) Osmoticum modifications. Plant cell protoplasts, having lost their rigid wall, are highly susceptible to lysis. They are protected from rupture by suspending in solutions called osmotica which provide a slightly plasmolysing environment. Optimum osmotic levels vary with the source of protoplast. Using mannitol as the osmotic agent the following optimal concentrations were observed for protoplast yields: 0.475 M with haploid Petunia shoots, 0.6 M with diploid Petunia shoots (Binding, 1974a), 0.4-0.7 M for tobacco cultures (Uchimiya and Murashige, 1974), 0.78 M for tomato leaves (Mühlbach et al, 1977), 0.45-0.6 osmolality (Schenk and Hildebrandt, 1971), 0.7 M for orange ovular callus (Vardi et al, 1975). Gamborg et al (1975a) found 0.4 M optimal for pea protoplasts when either mannitol or sorbitol was the osmotic agent but 0.3 M was optimal with glucose.

By far the most widely used osmotic agent is mannitol (Frearson et al,1973; Bui-Dang-Ha and Mackenzie, 1973) although this can often be replaced by sorbitol (Wallin et al,1974; Fowke et al, 1975; Grout, 1975) or sucrose (Harn, 1973; Giles, 1972; Pearce and Cocking, 1973) or glucose (Dorion et al,1975). Uchimiya and Murashige (1974) directly compared the yields of cultured tobacco

cell protoplasts using various osmotic agents at 0.3 and 0.7 M concentrations. Glucose, fructose, galactose, sorbitol and mannitol were effectively interchangeable. Sucrose, however, gave considerably lower yields. Sucrose also appeared to be detrimental to pea protoplasts (Gamborg et al,1975a). Michayluk and Kao (1975) demonstrated that xylose could replace sorbitol or glucose for soybean, bromegrass and *Vicia hajastana* protoplasts. Sucrose was not as good for soybean and *V.hajastana* but was the best osmotic agent for bromegrass. It appears therefore that species will differ in preference for osmotica. Mixtures of sugars and hexitols have also been used (Kartha et al,1974a; Kao and Michayluk, 1975; Vardi et al,1975). Indeed Eriksson and Jonasson (1969) found sorbitol/sucrose more suitable than sucrose, mannitol or $CaCl_2$ osmotica for *Haplopappus gracilis* Cass. corr. Endl. culture protoplasts.

A number of authors have included nutrient media salts in the isolation osmotica (Eriksson and Jonasson, 1969; Constabel et al, 1973; Hanke and Northcote, 1974; Dorion et al, 1975; Power et al, 1976a). However Uchimiya and Murashige (1974) reported that media salts gave no advantage. Even more frequently are particular salts included to increase protoplast stability. The calcium salts, calcium tetrahydrogen phosphate ($CaH_4(PO_4)_2.2H_2O$) and calcium chloride ($CaCl_2.2H_2O$) alone or in combination in concentrations of 1 to 6 mM appear to stabilize the protoplasts of many species (Kao et al, 1970a; Kao et al, 1971; Bright and Northcote, 1974; Kartha et al, 1974a; Gamborg et al, 1975a; Von Arnold and Eriksson, 1976; Gamborg and Shyluk, 1976).

A logical extension is to use salts to replace hexitols

and sugars as the major osmotic agents. Ruesink and Thimann (1966) used 0.10 molal $CaCl_2$+ 0.14 molal KCl (=3 x 0.1+2x0.14 = 0.58 osmol) for oat coleoptile protoplasts and claimed it improved yield and viability relative to 0.5 molal mannitol. Skene (1974, 1975) also used this salt osmoticum for grape callus protoplast isolation. Mezentsev et al (1976) showed 0.25 M $CaCl_2$ to be a better osmoticum than 0.34-0.50 M mannitol for protoplasts from the leaves of ryegrass, red clover, meadow fescue, lucerne and barley. Carrot root protoplasts were successfully prepared in 3.5% KCl + 0.5% $CaCl_2.6H_2O$ (total calculated osmolality of 1.01)(Kameya and Uchimiya, 1972). Similarly KCl/$MgSO_4$ and KCl/$MgCl_2$ mixtures have been used as osmotica for tobacco and potato mesophyll protoplasts (Meyer, 1974; Meyer and Abel, 1975a,b; Sarkar et al, 1974; Upadhya, 1975).

Even NaCl has been used as a suitable alternative to mannitol in protoplast isolation (Pinto da Silva, 1969). Binding (1974a) found that the three salt osmotica (i) 0.25 M NaCl (+ media salts) (ii) 88% seawater (iii) 0.12 M KCl + 0.18 M NaCl + 0.003 M $CaCl_2$ all gave higher recoveries of petunia protoplasts than the two mannitol osmotica tested. It was also claimed that cell debris remained in the supernatant of the ionic osmotica when the suspensions were centrifuged thus allowing more thorough cleaning.

The most frequently used macromolecular additive to osmotica has been potassium dextran sulphate (MW2850), a strongly anionic molecule bearing up to 3 sulphate groups per glucose moiety. It was first used by Takebe, Otsuki and Aoki (1968) at 0.3%(W/V) to increase the yields of tobacco mesophyll cells in

macerating enzyme treatments. They speculated that the polyanionic molecule was inactivating some toxic component of the enzyme mixture since dextran plus inorganic salts could not mimic the effect. Otsuki and Takebe (1969) found dextran sulphate to be beneficial to cell yields in 14 out of 19 species but positively detrimental to Zinnia L. and Coleus Lour. yields. This species difference argues against the hypothesised mechanism. Some authors have continued to use dextran sulphate (0.25-5.0%) in the first step of 2-step sequential protoplast isolation (Ohyama and Nitsch, 1972; Watts et al, 1974; Ohyama and Oka, 1975; Upadhya, 1975).

The use of dextran sulphate has extended to single-step protoplast isolation of a number of species (Motoyoshi, 1971; Evans et al, 1972; Coutts and Wood, 1975; Raveh and Galun, 1975; Vardi et al, 1975). Only two critical evaluations of its use in this way exist and they differ in their conclusions. Uchimiya and Murashige (1974) found 0-0.3% to be without effect and 3% to be detrimental to protoplast yields in single-step isolation from cultured tobacco cells. Binding (1974a) claimed 0.5% dextran sulphate (range 0-2%) was optimal for the yield of petunia mesophyll protoplasts. However even with 0.5% the difference in yield from the control was not great. Recently Mühlbach et al (1977) examined the effect of dextran sulphate when present only in the first step of a 2-step sequential production of tomato leaf protoplasts. They did not record whether the yield of freed cells was greater but they did note that intact protoplasts could only be obtained from cells previously isolated in the presence of the dextran sulphate. The effect was optimal at 0.5%(W/V).

It would be interesting to determine whether the effect, if real, relates to the ability of dextran sulphate at a higher M.W. (500,000) and higher concentration (20%) to agglutinate and fuse protoplasts (Kameya, 1975a)(see 2-1-4).

Another polymer which has attracted some interest as a constituent of isolation osmotica is polyvinylpyrrolidone (PVP). Shepard and Toten (1975) reported that 2% PVP (MW 10,000) consistently increased protoplast yields of tobacco leaf protoplasts. However this work was using a particularly low osmolality (0.2) for isolation and the beneficial effect of PVP may have simply been due to its contribution to the osmotic pressure which by the Donnan effect would have been out of proportion to its molarity. Indeed Binding (1974a) has shown that even 0.1% PVP (MW 10,000) had a consistently detrimental effect on viable petunia protoplast yields when the basal osmoticum was 0.6 M mannitol. Bovine serum albumin (BSA)(0.05%) and sodium ascorbate (50 mM) improved the yields from leaves of a number of species including wheat and barley (Rathnam and Edwards, 1976). Beier and Bruening (1976) claimed that 0.1% BSA improved protoplast yields from cowpea leaf.

Another aspect of osmoticum modification is the pH and use of buffering agents. Schenk and Hildebrandt (1971) found the optimal pH for protoplast survival was 5.7-6.0 (range 5.0-7.7 tested). Uchimiya and Murashige (1974) claimed that pH 4.7-5.7 (range 3.7-7.2 tested) was optimal for conversion of tobacco cultures to protoplasts. However in poorly-buffered solutions the pH can change during the digestion. Pelcher _et al_ (1974) observed a drop in pH of one unit and found an initial pH of 7.0 to

be optimal in their system for bean mesophyll protoplast isolation. Conversely Shepard (1975) reported a rising pH during tobacco leaf digestion.

A number of buffering systems have been used including phosphate buffers (Pinto da Silva, 1969; Pisatskaya et al, 1975), and citrate buffers (Wallin and Eriksson, 1973; Reid and Galston, 1975), and 2-(N-morpholino) ethanesulphonic acid (MES) (Giles, 1972; Kao et al, 1974; Kanai and Edwards, 1973; Kao and Michayluk, 1975; Dudits et al, 1976b). Coutts and Wood (1977) noted that 3 mM MES was beneficial to cucumber mesophyll protoplast survival. By contrast Mühlbach et al (1977) claimed that 10 mM MES reduced the yield of isolated tomato leaf cells in the first step of a 2-step sequential protoplast isolation. The only author to compare two buffer systems was Shepard (1975) working with tobacco. Potassium citrate buffer (10 mM, pH 5.6) significantly damaged and reduced yields whereas MES(10 mM, pH 5.6) not only stabilized the pH but reduced the digestion time needed and did not reduce the yield or plating efficiency of the protoplasts.

Antibiotics have sometimes been added to the enzyme solutions to eliminate or reduce the risk of contamination. Some antibiotics are too toxic to plant protoplasts e.g.aureomycin and kanamycin (Watts and King, 1973; Sarkar et al, 1974; Schilde-Rentschler, 1973). Chloramphenicol was recommended by Hess and Potrykus (1972) and Coutts and Wood (1977) although it was not effective against the contaminants encountered by Watts and King (1973). Other recommended antibiotics are carbenicillin, benzylpenicillin, ampicillin, tetracycline and gentamycin (Watts and King, 1973; Cocking et al, 1974; Davey et al, 1974; Power et al 1976b). Watts and King (1973) also found nystatin and

amphotericin B to be suitable anti-fungal agents. Good asceptic technique is preferable to the addition of anti-microbial substances.

2-1-3 Protoplast Culture

There has been a dramatic proliferation of literature in the last 7 years with attempts to culture plant protoplasts. It has become apparent that removal of the cell wall does not irreparably damage the cell's capacity to divide. However the isolation procedures do cause an osmotic shock from which the protoplasts need to recover. Premecz et al (1977) observed high proline levels in tobacco protoplasts which is a traditional indicator of osmotic stress associated with water loss. Gigot et al (1973) reported a temporary change in the condensation of chromatin and the granulation of nucleoli and a temporary hypertrophy or elongation of mitochondria after 3h in protoplast osmoticum. These changes reversed after 6h. Respiratory rates of isolated protoplasts also indicate a shock condition (Hoffmann et al,1975). Eriksson et al (1974) have also pointed out that the transfer of leaf cells from a relatively aerobic environment to a solution of high osmotic pressure could result in "waterlogging" symptoms such as changes in abscisic acid levels and production of ethylene which could inhibit division. It is perhaps for these reasons, at least in part, that suitable culture media for protoplasts often can not be predicted from the media used for corresponding cell cultures (Scowcroft et al, 1973).

The variations on culturing media and procedures are as numerous as the reports dealing with this subject. However it is useful to investigate some of the important parameters especially where alternatives have been critically compared. Much of what was discussed in 2-1-2 regarding isolation of protoplasts also applies to their culture. For example, plant growth conditions

are also critical for the eventual culture of protoplasts derived from those plants and it can generally be assumed that conditions optimal for protoplast yield and stability are also optimal for protoplast culture. The importance of leaf age for protoplast culturability has been amply demonstrated (Gamborg _et al_, 1975a; Nandi and Eriksson, 1977). An interesting development from this has been the suggestion that protoplasts culture better when the isolation involves leaves of the one age. Von Arnold and Eriksson (1976) observed a dramatic decrease in viability when pea leaves of different ages, from the same or different plants, were included in the one batch. Similar results were obtained with maize, wheat and rape. It was further shown that a similar inhibition of growth resulted when protoplasts were cultered in media 'conditioned' for 24h by protoplasts of a leaf of a different age. These diffusible inhibitory factors were not inactivated by boiling. Only when protoplasts were cultured in media conditioned by other protoplasts of similar leaf age would they proceed to divide and form cell clusters.

There is a division of opinion on the relative merits of culturing protoplasts embedded in agar-solidified medium or suspended in liquid medium. In most cases where a direct comparison was made it was found preferable to culture protoplasts in liquid medium for 1 or more weeks rather than immediate plating in solidified medium (tobacco: Takebe _et al_, 1971 and Ohyama and Nitsch, 1972 ; _Petunia_ spp.: Power _et al_, 1976a ; _Atropa belladonna_ L: Gosch _et al_, 1975a). After the 6th day of culture 30% of _Brassica oleraceae_ L. leaf protoplasts had divided in liquid medium but less than 1% in agar-solidified medium (Gatenby and Cocking, 1977). Shene (1975) achieved sustained divisions of grape callus

protoplasts in liquid but not in solid medium. Despite these contraindications, immediate plating in solid media has been successfully used for tobacco (Watts *et al*, 1974; Gleba *et al*, 1974; Raveh and Galun, 1975; Shepard and Toton, 1975), *Petunia* spp. (Banks and Evans, 1976; Haywood and Power, 1975; Hess and Potrykus, 1972), cowpea, potato, rice and *Lycopersicon* spp. Mill. (Davey *et al*, 1974; Upadhya, 1975; Deka and Sen, 1976; Zapata *et al*, 1977).

Frearson *et al* (1973) have pointed out the advantage of solidified culture for following the progress of particular protoplasts. Binding (1974a) routinely used liquid culture for petunia protoplasts but investigated the effects of 0.2, 0.3, 0.4, 0.5 and 0.6% agar on their development. Quite dramatically the highest yield of dividing protoplasts was in 0.2% agar. It may be an advantage to use such low levels of agar to prevent cross-contamination of developing colonies which ideally should derive from single protoplasts.

The majority of workers have used sucrose as the chief nutrient sugar. Many, however, have successfully used glucose as the major carbon source (Constabel *et al*, 1973 ; Pelcher *et al*, 1974 ; Kartha *et al*,1974a; Gosch *et al*, 1975b). Other authors have employed mixtures of glucose and sucrose as nutrient sugars (Upadhya, 1975; Thomas *et al*, 1976; Zapata *et al*,1977; Gatenby and Cocking, 1977). Grambow (1972) found that carrot culture cell protoplasts grew better when he cultured them first with glucose or cellobiose and gradually replaced these with sucrose, rather than when cultured immediately in sucrose. By constrast Uchimiya and Murashige (1976) reported that sucrose was a slightly superior

carbon source to glucose and cellobiose for tobacco protoplasts. Galactose was totally ineffective. In addition it was shown that 1.5% was the optimum sucrose level and 3% was excessive and detrimental.

There are some reports where glucose is used as both the major carbon source and the major osmotic agent (Gamborg et al, 1975a ; Kao and Michayluk, 1975; Gamborg and Shyluk, 1976; De La Roche et al,1977). However mannitol continues to be the most widely used osmotic agent for protoplast culture. Most of the discussion in section 2-1-2c on protoplast isolating osmotica is also applicable for culturing. Some investigations are of particular relevance to protoplast culture osmotica and will be introduced here. Pea shoot protoplasts divided better when the osmoticum was glucose rather than sorbitol or mannitol (Gamborg et al, 1975a). Similarly Gamborg and Shyluk (1976) claimed glucose to be a more beneficial osmoticum during flax protoplast culture than sorbitol/mannitol mixtures. By contrast Von Arnold and Eriksson (1976, 1977) reported sorbitol (or mannitol) to be clearly superior to glucose or sucrose for pea leaf protoplasts. In Nicotiana leaf protoplasts Meyer (1974) and Ohyama and Nitsch (1972) found sucrose more satisfactory than mannitol and Nagy and Maliga (1976) found sucrose more satisfactory than glucose or mannitol as osmotic agents for the culture media. By contrast, concentrations of sucrose exceeding 0.3 M inhibited carrot protoplast growth (Wallin and Eriksson, 1973). In each case the available osmotic agents should be compared.

When developing a protoplast culture medium it will also be necessary to optimize the osmolarity. Appropriate culturing

osmolarities are genotype-dependent and do not necessarily correspond to optimum isolation levels. Binding (1974a) examined mannitol concentrations from 0.3 to 1.0 M and found 0.4 and 0.4 to 0.6 M to be the respective optima for division of haploid and diploid petunia leaf protoplasts. The isolation yields of these protoplasts had been optimal in 0.475 and 0.6 M mannitol respectively. Nicotiana tabacum cv. Xanthi nc leaf protoplasts divided best with 0.7 M mannitol (Nagata and Takebe, 1971) but N.tabacum cv. Samsun protoplasts regenerated and divided much better at 0.4 M mannitol (Gleba et al, 1974). Isolation of Citrus callus protoplasts was optimal with 0.7 M mannitol while 0.6 M was best for culture (Vardi et al,1975). The optimal osmolarity may vary depending on which osmotic agent is used. Gamborg et al (1975a) found 0.4 to be optimal with mannitol or sorbitol but 0.3 gave the best results with glucose in the culture of pea protoplasts.

Suitable salt compositions for protoplast culture vary with different species. Zapata et al (1977) found Lycopersicon protoplasts could only be cultured with B5 salts(Gamborg et al, 1968) not MS salts (Murashige and Skoog, 1962) nor FPC salts (Frearson et al,1973) which have been suitable for other species such as tobacco and petunia respectively. Dorion et al (1975) successfully cultured Ranunculus sceleratus L. leaf protoplasts with White's salts (White, 1943) but could produce no divisions with Heller's salts or MS salts at full or half strength. Shepard and Toten (1975) found NT salts (Nagata and Takebe, 1971) to be unsuitable for tobacco protoplasts at densities less than 10^4/ml. They demonstrated that NT was toxic since ½ and 1/3 NT gave considerably better results. This toxicity was also

observed by Uchimiya and Murashige (1976) who concluded that the MS salt formulation was clearly superior to NT, Heller's, White's and SH salts (Schenk and Hildebrandt, 1972). These studies do not go far to define the critical characteristics of the salt formulations which make them suitable or unsuitable for a given species, however they do demonstrate the importance of making a systematic evaluation of a number of alternative basal media.

One of the important parameters of the salt media which has achieved more detailed examination is the Ca^{++} concentration. In one experiment Binding (1974a) optimized the $CaCl_2.2H_2O$ concentration for both haploid and diploid petunia protoplasts at 5 mM (750 mg/l) as in V47 medium. In another experiment (less reliable since the overall plating efficiencies were much lower) the optimal concentration was 12mM (1760 mg/l) as in V60 medium. Kao et al (1973) observed a stimulation of soybean and bromegrass protoplast division when the Ca^{++} level was raised to 5.3 mM. Von Arnold and Eriksson (1977) cultured pea leaf protoplasts with varing $CaCl.2H_2O$ levels and found 1760 mg/l to be the optimum. It is significant that a great deal of the successful culturing of protoplasts has been with $CaCl_2.2H_2O$ at 600 to 1000 mg/l (Potrykus, 1973; Frearson et al,1973; Pelcher et al,1974; Kartha et al, 1974a; Nagy and Maliga, 1976) and up to 1500 mg/l (De La Roche et al, 1977). Bhojwani et al (1977) specifically found B5 salts to be far more suitable for cotton protoplast culture when the $CaCl_2.2H_2O$ concentration was raised to 780 mg/l. Meyer (1974) and Meyer and Abel (1975) used 12,000 and 10,800 mg/l $CaCl_2.2H_2O$ in their tobacco protoplast media in which salts were the major osmotic agents. Such concentrations were certainly

toxic to pea protoplasts (Von Arnold and Eriksson, 1977). A number of authors have combined $CaCl_2.2H_2O$ and $CaH_4(PO_4)_2.H_2O$ in order to supply Ca^{++} for protoplast media (Gamborg et al, 1975a; Gamborg and Shyluk, 1976; Dudits et al, 1976b).

However the factors involved in the relative worth of salt media are considerably more involved than simply the Ca^{++} concentration. This can be illustrated by the high plating efficiencies for tobacco protoplasts using a relatively low Ca^{++} medium (modified from White's salts) in the work of Shepard and Toten (1975). Von Arnold and Eriksson (1977) and Meyer and Abel (1975) claimed Mg^{++} ions could partially compensate for low levels of Ca^{++} ions. However the work of Binding (1974a) showed Mg^{++} and Ca^{++} optima for protoplast division to be independent of each other. In an experiment where $CaCl_2$ and $MgSO_4$ levels were varied independently the Mg^{++} optimum was 4 mM (984 mg/l $MgSO_4.7H_2O$) which is the V47 medium level. In another experiment where Ca $(NO_3)_2$, Mg $(NO_3)_2$, $CaCl_2$ and Mg SO_4 were all varied independently the optimum Mg^{++} concentration was 15 mM(3,700 mg/l $MgSO_4.7H_2O$) which is the level in V60 medium. Meyer and Abel (1975) warned that high levels of Mg^{++} were toxic to some batches of tobacco protoplasts.

Fe^{+++} and Zn^{++} ion concentrations were optimized for pea protoplast growth by Von Arnold and Eriksson (1977). Fe^{+++} was optimal at 50 μM and could be supplied by Fe-EDTA, $FeCl_3$ or Fe-citrate. Zn^{++} was optimal at 0.5-10 mg/l Zn-EDTA and could also be supplied by $ZnSO_4$. Wallin and Eriksson (1973) could only induce continued division of carrot protoplasts with chelated forms of Fe and Zn.

The level and nature of nitrogen is of considerable importance to protoplast culture. Fortuitously Frearson et al (1973) observed petunia protoplasts to divide in a medium composed of White's salts only when pretreated with 0.25M $NaNO_3$ (a fusing agent). They then developed a salt medium (FPC) with increased NO_3^- and NH_4^+ which allowed divisions of protoplasts not treated with $NaNO_3$. Others have successfully employed media with high NH_4^+ and NO_3^- levels to improve protoplast culture (Nagata and Takebe, 1971; Nagy and Maliga, 1976; Dudits et al, 1976b). Bhojwani et al (1977) increased cotton protoplast survival in culture by adding 250 mg/l NH_4NO_3 (and raising the $CaCl_2$ concentration) to the medium based on B5 salts which has a large amount of KNO_3 but a low NH_4^+ion concentration (Gamborg et al,1968). An optimum concentration of 1200 to 1600 mg/l of NH_4NO_3 was observed for pea protoplast division (Von Arnold and Eriksson,1977). Levels higher than 2000 mg/l were harmful. For potato leaf protoplasts NH_4^+ was entirely excluded because of its toxicity and nitrogen was supplied by NO_3^- and glutamine (Upadhya, 1975). Galun and Raveh (1975) improved tobacco protoplast culture in NT salts by greatly reducing NH_4^+ and NO_3^-ion concentrations and adding 300 mg/l casein hydrolysate.

Meyer and Abel (1975a) investigated the effects of various nitrogen sources on tobacco protoplasts. Wall formation and cleavage divisions were achieved with NH_4^+ (2-15 mM) or urea (10-100 mM of nitrogen) or combinations of NO_3^- and glutamine but not with NO_3^- alone or glutamine alone. It is obvious that nitrogen sources are critical but suitable forms and concentrations vary with species and perhaps even cultivars and isolation treatments. Sustained divisions of asparagus protoplasts were

only possible when 1000 mg/l (but not 200 mg/l) of glutamine (6.85 mM) was included in a medium already having very high levels of NH_4^+ and NO_3^- (Bui-Dang-Ha and Mackenzie, 1973). Similarly Gamborg et al (1975a) found 2-5 mM glutamine to be essential for consistent colony formation from pea shoot protoplasts using a medium where NO_3^- is the major nitrogen source. Glutamine (5 mM) also aided the culture of flax protoplasts (Gamborg and Shyluk, 1976).

The use of casein hydrolysate as a nitrogen source by Galun and Raveh (1975) has already been mentioned. Casein hydrolysate (N-Z amine) was also beneficial at concentrations of 150-1000 mg/l for the protoplasts of flax (Gamborg and Shyluk, 1976), bean (Pelcher et al, 1974), carrot (Dudits et al, 1976b). Brassica oleraceae (Gatenby and Cocking, 1977), rape (Kartha et al, 1974a), Vicia hajastana (Kao and Michayluk, 1975) and grape (Skene, 1975). In all of these instances the basal medium was B5 or a modification of it. Casein hydrolysate is composed mainly of aspartic acid, glutamic acid, serine, lysine, leucine, proline, methionine and glycine. Mitchell and Gildow (1975) were able to mimic the casein hydrolysate stimulation of Vicia faba callus growth with a mixture of these 8 amino acids. Casein hydrolysate did not prove to be of any advantage to the culture of the protoplasts of tobacco cultures in MS salts (Uchimiya and Murashige, 1976), nor of tobacco leaf in NT salts (Nagata and Takebe, 1971), nor of pea leaf in the basal medium of Von Arnold and Eriksson (1967)(Von Arnold and Eriksson, 1977). However these latter authors did find very low levels of amino acids to be beneficial to pea protoplasts culture. These levels were so low as to exclude

them as N sources and their stimulatory role may be described as that of vitamins. Glycine was essential at 2mg/l (about 10^5 fold the concentration of the other amino acids) independently of the others. Other authors have employed 1-4 mg/l glycine (Bui-Dang-Ha and Mackenzie, 1973; Durand et al, 1973; Potrykus, 1973; Binding, 1974a; Shepard and Toten, 1975; Uchimiya and Murashige, 1976; Skene, 1975; Frearson et al, 1973). However no thorough critical evaluation of the effects of glycine has been reported.

Individual amino acids other than glycine and glutamine have not been studied extensively in protoplast culture systems. However Skene (1975) observed a beneficial effect of serine (100 mg/l) on grape protoplast division. Upadhya (1975) included serine (0.1 mg/l) in his potato protoplast culture medium. Hydroxy-L-proline (0.1 mg/l) was included in the bean protoplast medium of Pelcher et al (1974) without evaluative comment. Indeed great care must be taken to interpret amino acid effects since they very often act co-operatively. In other words the effect of a single amino acid may be antagonised by the presence of others (Murashige, 1974).

A number of sugars have occasionally been added to protoplast media for roles other than being carbon sources. Von Arnold and Eriksson (1977) found that each of 1 mM xylose (150 mg/l), 1 mM arabinose (150 mg/l) and 1 mM glucose (180 mg/l) stimulated pea protoplast division while ribose and galactose did not. Ribose has nevertheless been included in a number of successful protoplast media at 125 to 5000 mg/l without reported assessment of its effect (Gamborg and Shyluk, 1976; Dudits et al,

1976b; Constabel _et al_, 1973; Pelcher _et al_, 1974; Kartha _et al_, 1974a; Skene, 1975). Kao and Michayluk (1975) did establish the inability of a mixture of sugars and sugar alcohols (ribose, xylose, fructose, mannose, rhamnose, cellobiose, sorbitol and mannitol each at 250 mg/l) to decrease the minimum _Vicia hajastana_ protoplast density required for growth. However the individual sugars were not tried and it is conceivable that one component's stimulatory effect is cancelled by another's toxicity. Certainly these sugars and sugar alcohols were present in the medium which could culture individual protoplasts in 4 ml of medium. Xylose was included at concentrations of 1.0 to 3.3 mM in protoplast media for carrot (Wallin and Eriksson, 1973), soybean and _Vicia hajastana_ (Kao _et al_, 1974), bean (Pelcher _et al_, 1974) and grape (Skene, 1975). Arabinose (1 mM) was also present in the first and last of these examples.

A number of vitamins were examined individually by Von Arnold and Eriksson (1977) for effects on pea protoplast survival and divisions. Nicotinic acid, pyridoxine, thiamine, and myo-inositol aided both survival and divisions optimally at 2, 1, 5 and 100 mg/l respectively. Folic acid (0.5 mg/l) also stimulated protoplast division. No stimulation could be attributed to biotin, calcium pantothenate or riboflavin in the range 0 to 10 mg/l. Meyer and Abel (1975a) also noted the ability of thiamine, folic acid and myo-inositol to stimulate tobacco protoplast growth. A large range of vitamins (14) was used in the very successful medium for _Vicia hajastana_ protoplasts but none were evaluated independently (Kao and Michayluk, 1975). Biotin (0.08 mg/l) clearly stimulated early divisions of oat protoplasts but sustained culture was not obtained (Brenneman and

and Galston, 1975). Vitamin E (dl-α-tocopherol acetate) stimulated soybean and ladino clover callus growth (Oswald et al, 1977) but remains to be tested with protoplasts. Although no comparative investigations are available one might expect vitamin requirements to be species-specific. The most frequently used vitamins in protoplast culture are thiamine, nicotinic acid, pyridoxine and myo-inositol. Thiamine is the most critical and universally required for plant tissue culture (Murashige, 1974; Gamborg et al, 1968). Myo-inositol may be particularly important for protoplast regeneration because it is implicated in wall synthesis (Jung et al, 1972).

A mixture of organic acids (sodium pyruvate, 20 mg/l, citric acid, 40 mg/l , malic acid, 40mg/l, and fumaric acid, 40 mg/l) was quite clearly advantageous to Vicia hajastana protoplast culture (Kao and Michayluk, 1975). These compounds decreased the minimum protoplast density able to grow by a factor of about 10 in three types of control media. Gamborg and Shyluk (1976) noted that 2 mM ammonium malate (302 mg/l) had no effect on flax protoplast divisions when 5 mM glutamine was present. However the effect observed by Kao and Michayluk (1975) warrants more detailed investigation in other protoplast systems.

The plating efficiency of Nicotiana sylvestris leaf protoplasts was significantly increased by the addition of 0.1 μg/l adenine (Banks and Evans, 1976). Formerly adenine had been tried in callus cultures in concentrations from 10^3 to 4×10^5 times greater than this with toxic results (Risser and White,1964) or shoot inducing results (Bui-Dang-Ha and Mackenzie, 1973; Murashige, 1974; Prabhudesai and Narayanaswamy, 1975). It is

interesting that large changes in concentrations can alter the mode of action of substances such as the adenine base or amino acids. The poorly defined media supplement, coconut milk, has been used to advantage in the culture of Vicia hajastana protoplasts (Kao and Michayluk, 1975), soybean protoplasts (Kao et al, 1974), haploid petunia protoplasts (Binding, 1974a) and carrot protoplasts (Dudits et al, 1976b). However coconut milk was not beneficial for pea protoplast culture (Constabel et al, 1973; Von Arnold and Eriksson, 1977) and was detrimental to oat protoplasts (Brenneman and Galston, 1975).

Exogenous phytohormones are invariably required for protoplast regeneration and division. The optimum types and concentrations varies with the species. This is amply demonstrated by the optimum auxin/ cytokinin balances reported for 6 species of Petunia (Power et al, 1976a). Hormone optima differences in various reports may also be caused by the use of different cultivars, differing isolation procedures or differences in other media components. Petunia hybrida protoplasts divided optimally with 8μM(1.5 mg/l) NAA in the work of Binding (1974a) but with 5 mg/l NAA in Power et al (1976a). Watts et al (1974) also observed the tobacco leaf age to effect the NAA requirement. In tobacco protoplast culture cytokinin was unnecessary in the work of Uchimiya and Murashige (1976) and uncritical according to Watts et al (1974). However Nagata and Takebe (1970), Takebe and Nagata (1973) and Meyer and Abel (1975a) found cytokinin to be essential. The types of auxins and cytokinins needed also varies. The auxin, 2,4-D, could not be replaced by NAA or IAA for pea protoplasts (Von Arnold and Eriksson, 1977).

Similarly 2,4-D could not be replaced by IAA or NAA, nor BA by kinetin or zeatin in *Antirrhinum* protoplast culture (Poirier-Hamon *et al*, 1974). However for tobacco protoplasts kinetin and BA and zeatin were interchangeable (Watts *et al*, 1974; Nagata and Takebe, 1970) and 2,4-D could be replaced by NAA or pCPA (Takebe and Nagata, 1973). It is not possible to predict the hormone requirements for protoplast culture from the corresponding cell culture requirements. For example suspension cultures of *Parthenocissus* crown gall grew independently of exogenous phytohormones, however their protoplasts did need auxin and cytokinin for division. This growth regulator dependence again disappeared once walls had regenerated and divisions began (Scowcroft *et al*, 1973). Also 27 µM 2,4-D, which was used to maintain sugarcane cultures, was totally inhibitory to divisions of the resulting protoplasts and 1 µM was better (Maretzki and Nickell, 1973).

Adequate aeration of protoplasts has been recognised as essential and achieved by using droplets or shallow layers of culture. However very little attention has been paid to the influence of the gaseous environment on protoplasts in culture. Gathercole *et al* (1976) demonstrated CO_2 to be essential for sycamore cell culture growth and found 1% to be optimal. Since normal air has only about 0.03% CO_2 it would be valuable to test the effects of CO_2 levels on protoplasts.

The pH is another parameter of culture which can drastically effect growth. Binding (1974a) observed a definite optimum at pH 5.65 (measured after 72 h when the % division was also assessed) for petunia protoplasts. This corresponded to

pH 5.6 before autoclaving and pH 5.9 immediately after autoclaving. To some extent the optimum initial pH will depend upon the degree to which the pH decreases during culture which in turn depends on the extent of buffering in the medium. In WO.6 medium (Meyer and Abel, 1975a) the pH dropped from 5.8 to 4.8 in one week of tobacco protoplast culture. However these authors did not find appreciable variation of culture results when the initial pH was varied between 4.5 and 6.0. Pea shoot apex protoplasts grew better at pH 6.2 than 5.5 in the media of Gamborg et al (1975a). Conversely flax protoplasts preferred pH 5.5-5.8 to pH 6.2 even in MES buffered media (Gamborg and Shyluk, 1976).

Lighting can be a critical parameter for protoplast culture. It has been claimed that darkness is essential during the initial culture of the protoplasts of cotton callus (Bhojwani, 1977), pea leaf (Constabel et al, 1973) and sugarcane spindle (Krishnamurthi, 1976). Such light sensitivities are probably species- or tissue-specific. This was demonstrated by Banks and Evans (1976) who found *Nicotiana otophora* L. leaf protoplasts to be highly susceptible to light intensities above 100 lux but *N. tabacum* L., *N. sylvestris* L., *N. tabacum* x *N. otophora* and *N. sylvestris* x *N. otophora* leaf protoplasts cultured well in intensities exceeding 700 lux. On the other hand Meyer and Abel (1975a) found 800 lux better than darkness and Nagata and Takebe (1971) found 2300 lux superior to both 700 and 5000 lux for tobacco leaf protoplast division. Recovery and division of flax protoplasts was favoured by diffuse light rather than darkness (Gamborg and Shyluk, 1976). The most thorough investigation of light effects has been by Binding (1974a) who examined the culture of green leaf haploid and diploid petunia protoplasts and etiolated haploid

protoplasts in light intensities from 0 to 8000 lux. Optimum for both types of green protoplasts was between 1000 and 2000 lux while the optimum for the non-green protoplasts was between 50 and 300 lux.

Temperature also can have a dramatic species-specific effect on growth. Zapata et al (1977) observed plating efficiency maxima for Lycopersicon esculentum and L.peruvianum protoplasts at 27°C and 29°C respectively. L.peruvianum protoplasts were unable to grow at 25° and 33°C, while L.esculentum protoplasts were totally inhibited at 24° and 31°C. Meyer and Abel (1974a) also observed an increase in growth of tobacco protoplasts when the temperature was raised from 25° to 27°-29°C. This parameter may have been responsible for the failure of hitherto uncultureable species of protoplasts.

The initial density of protoplasts has often been shown to critically effect culture probably due to cross-feeding effects with both beneficial metabolites and toxic substances. Plating efficiencies were optimized with protoplasts of diploid petunia at a density of $4\text{-}7 \times 10^3$/ml (Binding, 1974a), haploid petunia at 2×10^4/ml (Binding, 1974a), Petunia parodii at 5×10^4/ml (Haywood and Cocking, 1975), tobacco at 4×10^4/ml (Galun and Raveh, 1975), Atropa belladonna at $6\text{-}10 \times 10^4$/ml (Gosch et al, 1975a) and Citrus sinensis at $8\text{-}10 \times 10^4$/ml (Vardi et al, 1975). Optimal plating density can change depending on whether culture is in liquid or solid medium (Pearce and Cocking, 1973; Frearson et al, 1973). There are a number of reasons why it would be advantageous to be able to culture protoplasts at very low plating densities. Firstly it may enable rare mutants or somatic recombinants to grow in a

selective medium amongst a majority of non-growing cells. Secondly it may enable rare fusion heterokaryons to be individually picked out from parentals by micromanipulation and cultured in non-selective media.

One approach to enable the lowering of plating densities has been to use conditioned medium in which cells have previously been grown. The conditioning cells are removed and the medium resterilized. Osmolality may also be readjusted to suit the protoplasts. Binding (1974a) showed that the cut-off density for petunia protoplast growth could be lowered from $3.3x10^3$/ml to $3.3x 10^2$/ml by the use of conditioned medium. Barley and sugarcane protoplasts were only able to grow when conditioned media were used (Koblitz, 1976; Maretzki and Nickell, 1973). The potential of this approach is illustrated by the colony formation from a single isolated geranium protoplast in conditioned medium (Abo-El-Nil and Hildebrandt, 1976). An alternative approach has been to use non-growing feeder protoplasts to support low densities of viable protoplasts. The efficiency of colony formation was reduced by 50% in tobacco protoplasts by 750 R (Galun and Raveh, 1975) and in orange protoplasts by 3000 R (Vardi _et al_, 1975) of X irradiation. Raveh and Galun (1975) and Vardi and Raveh (1976) found higher Xray doses (about $5x10^3$ R) to completely eliminate colony formation. However these non-viable protoplasts could cross-fed and support colony formation from a low density of non-irradiated protoplasts plated in a layer above them. Tobacco protoplast densities as low as 5/ml and orange protoplast densities as low as 500/ml were successful.

A third approach has been to devise media sufficiently

enriched with the metabolites involved in cross-feeding. Kao and Michayluk (1975) developed a medium capable of culturing single *Vicia hajastana* protoplasts in a 4 ml thin layer to colonies and eventually cell cultures. The enriching additives included many vitamins, pyruvate, citric acid, malic acid, fumaric acid, a range of sugars and sugar alcohols, casein hydrolysate and coconut milk. With the loss of the cell wall, the protoplast may have lost some of its ability to retain important metabolites. In dense protoplast suspensions this diffusion of metabolites is compensated by the nearness of neighbours similarly diffusing these metabolites. Some of various growth enhancing effects discussed throughout this section may be mimicking and compensating for lost metabolites.

Once the protoplast regeneration process has been initiated it eventually becomes unnecessary to keep the cells in the high osmotic pressure environment. High osmolarity can in fact be inhibitory to sustained cell division. However the dilution of the osmotic agent should be gradual in order to minimize the shock. When the initial protoplast culture is in liquid medium it is most usual to gradually dilute the high osmotic culture with small volumes of low osmotic medium over a number of weeks (Kao *et al*, 1971; Kartha *et al*, 1974a; Thomas *et al*, 1976; Koblitz, 1975). Gatenby and Cocking (1977) transferred 1 ml aliquots of initial liquid *Brassica oleraceae* protoplast culture onto 2 ml of agar-solidified medium with a low osmolarity. Presumeably the diffusion of the mannitol into the agar was sufficiently gradual to allow the cells to adapt. When the initial culture is in solid medium a number of authors transferred agar blocks containing growing regenerates to new media with lower osmolarity. Sometimes such transfers are repeated up to 4 times across a series of media

with ever decreasing osmolarity (Gleba *et al*, 1974; Davey *et al*, 1974; Power *et al*, 1975). Due to the gradual diffusion of the components of the agar block into the new medium this technique avoids any discontinuous, quantum change in the osmotic environment.

Table 2-1 lists the species of protoplast which have been cultured either to plants or to cultures which can be perpetually subcultured. Of the 23 species which have proved capable of regeneration to at least shoots only 3, *Daucus carota*, *Citrus sinensis* and *Atropa belladonna*, could do this with protoplasts derived from cultured cells. In each of these 3 cases the cultures had only recently been isolated. All the other species of protoplasts were from *in vivo* tissues, mostly leaves. This may be significant and will be referred to again in 2-4. In addition to the species of Table 2-1 protoplasts of the mosses, *Physcomitrella patens* and *Polytrichum juniperinum*, can be regenerated to leafy gametophyte plants (Stumm *et al*, 1975; Gay, 1976).

TABLE 2-1 Protoplasts regenerated to sustained cultures or plant organs

PLANT SPECIES	PROTOPLAST SOURCE	FORM OF MAXIMUM REGENERATION	REFERENCE
Solanum tuberosum	leaf	callus + roots	Upadhya, 1975
Citrus sinensis	ovular callus	plants	Vardi *et al.*, 1975
Vitis vinifera	pericarp callus	callus	Skene, 1975
Datura innoxa	leaf	plants	Schieder, 1975
Antirrhinum majus	leaf	embryoids	Poirier-Hamon *et al.*, 1974
Phaseolus vulgaris	leaf	callus	Pelcher *et al.*, 1974
Paul's scarlet rose	culture	culture	Pearce and Cocking, 1973
Catharanthus roseus	callus	callus	Koblitz, 1975
Pharbitis nil	cotyledon	callus + roots	Messerschmidt, 1974
Lycopersicon esculentum	leaf	callus	Zapata *et al.*, 1977
L. peruvianum	leaf	callus + shoots	
Vicia hajastana	culture	callus	Kao and Michayluk, 1975
Glycine max	culture	callus	Kao *et al.*, 1971, 1973
Pelargonium	leaf	plants	Kameya, 1975b
Atropa belladonna	culture	plants	Gosch *et al.*, 1975a
Haplopappus gracilis	culture	culture	Kao *et al.*, 1971
Ranunculus sceleratus	leaf	plant	Dorion *et al.*, 1975
Vigna sinensis	leaf	callus + roots	Davey *et al.*, 1974
Cucumis sativus	leaf	callus + roots	Coutts and Wood, 1975, 1977
Brassica napus	haploid leaf	plant	Thomas *et al.*, 1976
	diploid leaf	plant	Kartha *et al.*, 1974
Brassica oleraceae	leaf	callus + roots	Gatenby and Cocking, 1977
Acer pseudoplatanus	culture	callus	Bright and Northcote, 1974
Nicotiana tabacum	leaf	plants	Takebe *et al.*, 1971
			Watts et al., 1974
			Meyer, 1974
			Gleba *et al.*, 1974
			Shepard, 1975
			Shepard and Totem, 1975
			Banks and Evans, 1976

N.tabacum	haploid leaf	plants	Ohyama and Nitsch, 1972
N.tabacum	culture	culture	Uchimiya and Murashige, 1974, 1976
N.sylvestris	leaf	plants	Nagy and Maliga, 1976 Banks and Evans, 1976
N.otophora *N.otophora* x *N.tabacum* *N.otophora* x *N.sylvestris*	leaf	plants	Banks and Evans, 1976
Petunia hybrida	leaf	plants	Potrykus, 1973 Frearson *et al.*, 1973 Durand *et al.*, 1973 Power *et al.*, 1976a Binding, 1974a
P.hybrida	haploid leaf	plants	Binding, 1974a Power *et al.*, 1976a
P.parodii	leaf	plants	Haywood and Power, 1975
P.inflata *P.violaceae* *P.axillaris* *P.hybrida* x *P.parodii*	leaf	plants	Power *et al.*, 1976a
Pisum sativum	leaf	callus	Constabel et al., 1973 Gamborg et al., 1975a Von Arnold and Eriksson, 1976, 1977
Daucus carota	culture	plants	Dudits *et al.*, 1976b Grambow *et al.*, 1972
D.carota	root	embryoids	Kameya and Uchimiya, 1972
Linum usitatissimum	hypocotyl	callus + roots	Gamborg and Shyluk, 1976
Hordeum vulgare	callus	callus	Koblitz, 1976
Saccharum officinarum	culture	culture	Maretzki and Nickell, 1973
Oryza sativa	leaf or callus	callus + roots	Deka and Sen, 1976
Bromus inermis	leaf	plant	Kao et al., 1973
Asparagus officinalis	cladodes	plant	Bui-Dang-Ha and Mackenzie, 1973 Bui-Dang-Ha *et al.*, 1975

2-1-4 Protoplast Fusion

Protoplast fusion can be divided into spontaneous and induced fusion. Spontaneous fusion of two or more protoplasts may occur during the isolation with enzymes when the plasmodesmatal connections between adjacent cells expand (Withers and Cocking, 1972; Power *et al*, 1971). Much higher frequencies of spontaneous fusion have been possible in immature tissues (leaf, cotyledon, endosperm) as opposed to the same tissue when mature (Usui *et al*, 1974). However spontaneous fusion can only result in homokaryons and can be of no help in the fusion of protoplasts of different genotypes. Therefore spontaneous fusion will not be discussed further.

Induced fusion is when two or more freely isolated protoplasts fuse in the presence of a fusion-inducing agent, also called a fusogen. It is only in the exceptional case of *Lilium* and *Trillium* meiotic protoplasts (isolated from microsporocytes) that high frequencies of fusion resulted from simply tapping them into contact in a monolayer (Ito, 1973a; Ito and Maeda, 1973). Generally isolated protoplasts neither aggregate nor fuse without alteration of their chemical environment. The first fusogen in somatic animal cell hybridization was inactivated Sendai virus (Bernhard, 1976). Sendai virus is still very widely used even though other myxoviruses can also be effective. Sendai virus failed to induce aggregation or fusion of plant protoplasts in the work of Bala Bawa and Torrey (1971) and of Keller *et al* (1973). However even in animal cell fusion different isolates and strains of Sendai virus can vary greatly in fusogenic activity (Koprowski and Croce, 1973). Withers (1973) tried two strains of Sendai

virus on plant protoplasts and found one to be totally inactive while the other caused aggregation and a damaging extent of fusion.

There has been contradictory evidence as to the mechanism of viral fusogenic action. Barbanti-Brodano et al (1971) found that lysolecithinase treatment of Sendai virus destroyed its fusing activity but not its ability to infect or agglutinate cells. They proposed that lysolecithin in the viral envelope was the fusogenic principle. However Parkes and Fox (1975) found no correlation at all between the levels of lysophosphatides of various strains of Newcastle disease virus and their ability to fuse mouse fibroblasts. Nevertheless lysolecithins themselves can cause animal cells to fuse especially when aggregation of cells is aided by dextran (Lucy, 1970; Poole et al, 1970; Koprowski and Croce, 1973). Lysolecithins are extremely toxic to cells causing bursting. This toxicity can be somewhat reduced by using lipid emulsions or by raising the pH to 8.0 (Koprowski and Croce, 1973). Lysolecithins also cause extensive lysis of plant protoplasts at at concentrations exceeding 1 μg/ml and did not induce fusion at lower concentrations (Withers, 1973; Keller et al, 1973; Bright and Northcote, 1974). Indeed protoplast agglutination and fusion by polyethylene glycol was reduced when the protoplasts were pretreated with 0.01-0.1 μg/ml lysolecithin (Constabel and Kao, 1974).

Glycerol mono-oleate is another surface-active lipid and can cause cell fusion with less devastating damage (Lucy et al,1971). Hybrid clones of hen erythrocytes x mouse fibroblasts have been isolated with this fusogen (Cramp and Lucy, 1974). Attempts to use glycerol mono-oleate with plant protoplasts have been unsucess-

ful (Withers, 1973; Bright and Northcote, 1974). Lucy (1970) and Poole et al (1970) have proposed a model to account for the effects of fusogens such as lysolecithin and glycerol mono-oleate. It was suggested that such wedge-shaped molecules may insert into the lipid bilayer causing an increase in lipid micelles within the membrane. When such "activated" membranes appose, the interdigitation of the micelles may lead to fusion as shown in Fig. 2-1.

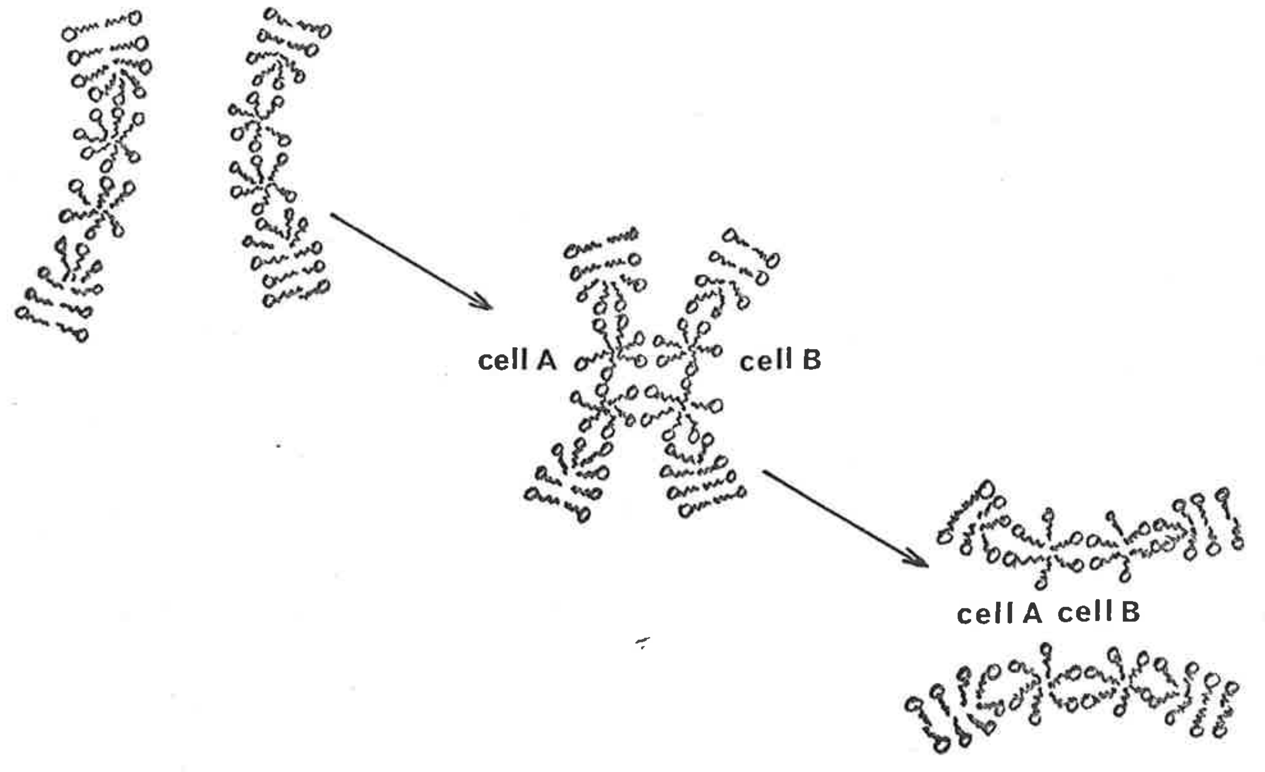

Fig. 2-1 Micelle Model of Membrane Fusion
(Lucy, 1970)

It is significant that dextran is required for lysolecithin- or glycerol mono-oleate-induced fusion. Dextran simply aggregates the cells making possible the apposition of membranes. Kameya (1973, 1975a) employed both high molecular weight gelatin and dextran sulphate to aggregate plant protoplasts. In both cases there was also evidence of ensuing fusion and in the case of dextran sulphate plants were regenerated from fused

protoplasts. Protoplast aggregation may also be induced by antisera and lectins and by phenylazoglycosides (see sections 4,5 and 6).

Whether or not lipid micelles within membranes play a role in fusion the model in fig. 2-1 is an oversimplification. Cell membranes contain proteins, glycoproteins and glycolipids some of which are substantially external, some internal and some traverse the membrane. Poste and Allison (1971) and Maroudas (1975) suggested that such intramembranous particles prevented close contact of membranes by their steric exclusion volume which involves not only their geometric rigidity but also thermodynamic exclusion resulting from the free energy and entropy of their variable configuration. Animal cells have been fused by treating them with lysosomal enzymes which digest the intramembranous particles. Potrykus (1973) and Binding (1974c) found lysozymes caused protoplast agglutination and fusion but only in non-ionic osmotica. Pretreatment of protoplasts with 0.01-0.1 µg/ml lysozyme reduced the level of agglutination in polyethylene glycol but increased the frequency of fusion heterokaryocytes (Constabel and Kao, 1974).

Ahkong *et al* (1975a) found that dimethyl sulphoxide and glycerol both induced fusion of dextran-agglutinated erythrocytes. Both of these substances caused aggregation of intramembranous particles leaving regions of uninterrupted lipid bilayer. Calcium was essential for this fusion. Lawson *et al* (1977) reported that lectins and antibodies (ferritin-conjugated) do not bind to regions of membrane in the process of fusing but are sometimes concentrated at the edges of fusing regions. This

implies a displacement and aggregation of glycoproteins prior to fusion. Mayo and Cocking (1969 in Cocking, 1971) also found that areas of plant protoplast membrane which had undergone pinocytosis (a fusion event) were devoid of glycoprotein. Ahkong et al (1975b) and Zakai et al (1977) showed that Ca^{++} induced erythrocyte fusion especially when ionophores (A-23187 and X537A) were present to facilitate the diffusion of the Ca^{++} into the cytoplasm. Evidence was also presented that the resulting high levels of cytoplasmic Ca^{++} caused the formation of protein-free regions of membrane. Spectrin proteins are proteins on the inner surface of membranes and when aggregated by internalised anti-spectrin antibodies there is a corresponding aggregation of transmembrane glycoproteins (Nicolson and Painter, 1973). Ahkong et al (1975b) suggested the cytoplasmic Ca^{++} may similarly aggregate the anionic spectrin molecules by electrostatic bridging leaving denuded stretches of lipid bilayer available for fusion.

Ca^{++} has almost universally been found essential for membrane fusion at least at low concentrations (Poste and Allison, 1971; Ahkong et al,1975a, b; Lucy, 1977). Even with polyethylene glycol (PEG)-induced fusion (see later) Ca^{++} has proved necessary (Wallin et al, 1974; Ferenczy et al, 1976; Maggio et al, 1976) although it may be replaced in part by Mg^{++}. It may be that fusogens in various ways simply increase the uptake of exogenous Ca^{++} into the cytoplasm. High levels of plant and liverwort protoplast fusion has been possible using high levels of Ca^{++} (as $CaCl_2$ or $Ca(NO_3)_2$) especially when the pH is elevated to 8-10.5 (Schieder, 1974a, b; Keller and Melchers, 1973; Binding, 1974c). Under these conditions the cytoplasmic levels of Ca^{++} might also

be elevated resulting in intramembranous particle aggregation.

A fusogen which has attracted much attention since 1974 is polyethylene glycol (PEG). Its use was developed almost simultaneously by Kao and Michaylak (1974) and Wallin, Glimelius and Eriksson (1974). Kao and Michayluk (1974) showed that appropriate PEG solutions caused protoplasts to adhere in small aggregates to a glass coverslip and when the PEG was gradually washed away fusion occurred. The MW of the PEG had to be greater than 400 for any adhesion and fusion, and was best when greater than 1000. While PEG 6000 was more effective than PEG 1540 on a mole basis, they were about equally as effective on a weight basis. High concentrations (0.16 and 0.33 M PEG 1540, or 0.074 and 0.037 M PEG 6000) were needed if adhesion was to be reasonably rapid (45 min). The % of heteroplasmic aggregates and of fused heterokaryocytes was greatly increased by raising the Ca^{++} content of the PEG treatment from 0.02 to 8.1 mM. They also found that K^{+} ions (146 mM) inhibited adhesion even in the presence of Ca^{++}. The solution used for washing away the PEG (the step where fusion appears to occur) contained 9.4 mM Ca^{++}.

The technique of Wallin et al (1974) involved suspension of protoplasts in 28% (0.187 M) PEG 1500 (1:1 mixture of protoplasts in culture medium and 56% aqueous PEG) in a total volume of 2 ml for 1 h. The aggregated protoplasts were diluted with 5 ml of culture medium for a further hour. Protoplast density affected % aggregation which increased to a plateau at about 1.5 x 10^{6} /ml. Fusion frequencies increased with temperature from 10 to 35C and with PEG concentration from 0% to a plateau at 28% (w/w) for dinucleates (although 35% gave more multinucleates).

They examined the effect of Ca^{++} ions on aggregation in 28% PEG 1500. Raising the Ca^{++} concentration from 0.1 to 1mM gave a dramatic increase of % aggregation. However Mg^{++} ions could almost exactly mimic the effect of Ca^{++}. This effect was dependent on pH. At pH 6 higher Ca^{++} levels were required for optimal aggregation. It was also suggested that membrane fusion may be occurring during the aggregation stage but that cytoplasmic mixing does not occur until the plasmolyzing effect of PEG is removed by dilution.

PEG has been used successfully to induce fusion without reducing viability in animal cells (Ahkong et al, 1975a; Maggio et al,1976), hen erythrocytes x yeast protoplasts (Ahkong et al, 1975c), human HeLa cells x plant protoplasts (Jones et al, 1976; Dudits et al, 1976c), fungal protoplasts (Ferenczy et al, 1975b, 1976) as well as many species of plant protoplasts (Fowke et al, 1975; Gosch et al, 1975b; Dudits et al, 1976a; Weber et al, 1976; Constabel et al, 1976). PEG has also proved useful for inducing the uptake of chloroplasts into plant protoplasts (Bonnett and Eriksson, 1974; Bonnett, 1976; Davey et al, 1976), chloroplasts into fungal protoplasts (Vasil and Giles, 1975), nitrogen-fixing algae into plant protoplasts (Burgoon and Botlino, 1976) and micro-organisms into plant protoplasts (Davey and Power, 1975). There are varying reports as to whether such particles are enveloped in a host membrane vesicle or not (Davey et al, 1976; Davey and Power, 1975; Bonnett, 1976; Vasil and Giles, 1975).

There may be some advantage in combining the PEG treatment with a high Ca^{++}/high pH environment to increase the frequency of fusion hybrids (Kao et al, 1974; Jones et al, 1976; Binding, 1976).

However Burgoon and Bottino (1976) denied that elution of PEG with high Ca^{++}/ high pH solutions improved algal uptake into protoplasts. The remarkably fortuitous aspect of fusion by PEG is that although the frequency of resulting dinucleates is high the frequency of multinucleates is low. This makes it well suited for somatic hybridization.

The mechanism for PEG-induced fusion is not clear. Its general formula is $HOCH_2(CH_2-O-CH_2)_nCH_2OH$ and the molecule has ordered helical regions alternating with disturbed regions. The ether linkages when exposed in the disturbed regions attract water molecules strongly by H-bonding. Thus the osmotic effect of PEG molecules will vary depending on the configuration of the molecules. Michel and Kaufmann (1973) demonstrated that the osmolarity of PEG 6000 solutions decreased linearly as temperature increased since higher temperatures appear to increase the relative proportion of coiled configuration. However in the presence of nutrient salts the helical regions were less extensive and the osmolarity greater. It has been estimated that a solution of 28% (w/w) PEG 1500 has an osmotic effect exceeding 2000 mOs (Wallin et al, 1974). Most protoplast osmotica have an osmolarity of about 500 mOs and therefore the PEG treatment causes a rapid shrinking of protoplasts. For example the carrot protoplasts used by Bonnett (1976) experienced a 35% decrease in surface area. This shinkage leaves a considerable amount of "spare" membrane (Wallin et al, 1974; Kao and Michayluk, 1974; Davey et al, 1976).

Ca^{++} has been implicated as essential for PEG-induced fusion at least in some cell systems (Ferenczy et al, 1975b, 1976; Maggio et al, 1976) and certainly stimulatory in others (see above).

It may be that PEG/Ca^{++} complexes act as bridges for agglutinating cells (due to the net negative potential of the cell surface)(Kao and Michayluk, 1974). Alternatively it is also conceivable that the disordered segments of a PEG molecule H-bond to the membranes of two or more adjacent cells. In addition, PEG may enable the transport of Ca^{++} into the cytoplasm. The cytoplasmic Ca^{++} and the osmotic shock of the PEG solution may together produce spare membrane devoid of intramembranous particles which fuses easily. The agglutination of cells and protoplasts may be explained by the drop in cell surface potential by several hundred millivolts even with 10^{-5} M PEG 6000 (Maggio _et al_, 1976). Ordinarily electrostatic repulsion prevents cells adhering by such close range interactions as H-bonding and hydrophobic bonds. In the absence of surface potential such forces will cause the adhesion of protoplasts which touch randomly.

All cells and protoplasts possess a net -ve transmembrane potential relative to the external solution. Graham and Bowling (1977) demonstrated that this potential difference consists of a metabolic component and a basal component. Using sunflower root cortical cells they showed that cutting the cells off from photosynthetic energy supply (by excision or incubation in darkness or ringing the bark) or using metabolic inhibitors decreased the transmembrane potential (PD) from about -150 to -60 mV. Sucrose added to excised roots restored the high PD. Bowling, Graham and Dunlop (pers. comm.) have also implicated an electrogenic ATPase/phosphate pump as the source of the metabolic component of PD. It is therefore significant that Kohn and Klibansky (1967) found that the pretreatment of animal cells with ATP rendered them

insensitive to fusion by Newcastle Disease virus. Giles (1973) also noted that etiolated leaf protoplasts were far more prone to spontaneous fusion than green leaf protoplasts. In the presence of ATP or photosynthetic energy the transmembrane potential will be higher and the cells and protoplasts less able to adhere. Thus aggregation and fusion of protoplasts should be aided by conditions which destroy the metabolic component of PD. Aggregation should be further stimulated by conditions which somehow annul the basal PD. To this end the following is important.

Plant protoplasts have been fused by suspension in solutions with high concentrations of Na^+ under slightly deplasmolysing conditions. Sodium nitrate has been used for fusion usually at 0.25 M (Power et al, 1970; Cocking, 1971; Withers and Cocking, 1972; Carlson et al, 1972; Chakrabarti, 1975). Power et al (1975) fused Petunia and Parthenocissus protoplasts by pelleting in 10.5% sucrose and 5.5% $NaNO_3$ (calculated osmolarity of 1.6) and obtained fusion products which could be cultured to callus. Kameya and Takahashi (1972) claimed fusion with Na^+ ions was more extensive when protoplasts had been isolated in K^+ salt solutions and then transferred to Na^+ salt solutions. Others have claimed that $NaNO_3$ can be replaced by NaCl (and seawater or artificial seawater), LiCl, Na NO_2, KNO_3 (Schenk and Hildebrandt, 1971; Eriksson, 1971; Kameya and Takahashi, 1972; Withers, 1973; Binding, 1974c). Some authors, however, have failed to observe significant induction of protoplast fusion in Na NO_3 (Keller et al, 1973; Potrykus, 1973; Melchers and Labib, 1974; Bright and Northcote, 1974).

An indication as to the mechanism of Na^+ ion-induced

aggregation of protoplasts came from the work of Grout and Coutts (1974). They used whole protoplast electrophoresis to investigate the effects of various exogenous substances on the electrophoretic mobilities and hence on the surface potentials of plant protoplasts. $NaNO_3$ reduced the mobility of tobacco protoplasts towards the cathode from 3.99 μm/sec. volt cm to zero mobility at a concentration of 0.1175 M. This is about the concentration of $NaNO_3$ which allegedly causes aggregation and fusion. It can be postulated that at this concentration even the basal transmembrane potential is annuled allowing protoplasts to approach to within distances where short range attractive forces (H-bonds, Van der Waals forces etc) can operate. Grout and Coutts (1974) also noted the ability of certain concentrations of polycations such as poly-L-ornithine, poly-L-lysine, protamine sulphate and DEAE dextran to reduce the electrophoretic mobility of protoplasts to zero. This phenomenon is likely to be significant for the uptake of viral particles into plant protoplasts which has been observed in the presence of poly-L-ornithine (Burgess et al, 1974; Huber et al, 1977; Barker and Harrison, 1977) and protamine sulphate (Beier and Bruening, 1976). Indeed Zhuravlev et al (1976) noted that tobacco mosaic virus uptake into tobacco protoplasts by poly-L-ornithine was suppressed by polyanions such as dextran sulphate or RNA.

However the work of Grout and Coutts (1974) is not entirely satisfactory because of a failure to control pH and to equilibrate osmolarity for the various concentrations of test substances. Pilet and Senn (1974) also used cell electrophoresis and reported a very large drop in electrophoretic mobility of *Valerianella* leaf

protoplasts with Ca^{++} ions. Melchers and Nagata (pers. comm. to Vasil, 1976) reported that plant protoplasts loose the negative PD during high Ca^{++}/pH 10.5/37°C treatment. As was mentioned earlier PEG 6000 treatments also greatly reduce the transmembrane potential of cells (Maggio et al, 1976). One cannot rule out the possibility that each of these fusogenic treatments functions, at least in part, by drastically reducing transmembrane potentials and thus allowing protoplasts to approach to distances where non-electrostatic bonds can operate.

It would seem valid to consider a protoplast suspension as a colloidal dispersion and to apply some of the rigorous mathematical concepts of sol agglomeration to protoplast adhesion and fusion. A negatively charged colloidal particle will be surrounded by a diffuse electric double layer in an ionic solution such that the concentration of cations and anions near the particle will be as shown in fig. 2-2.

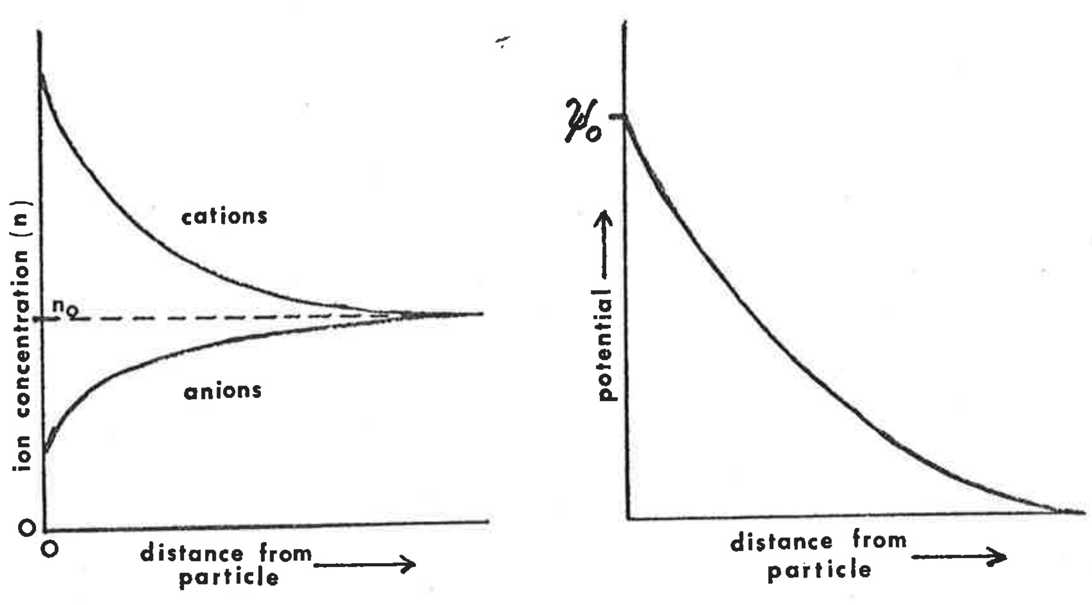

Fig. 2-2 Schematic representations of diffuse electric double layer (Van Olphan, 1963)

n_o = the bulk concentration of each ion

The "thickness" of the double layer is reduced by increasing bulk ion concentrations and by substituting polyvalent cations for monovalent cations. Two negative colloidal particles are subject to electrostatic repulsive forces and Van der Waals attractive forces. The predominating force will depend upon the "thickness" of the double layer as shown in fig. 2-3 (Van Olphen, 1963). When the double layer is extensive and repulsive forces predominate (Fig.2-3) the net effect is that an approaching particle requires a great deal of kinetic energy to pass the repulsive 'hump' and enter the sphere of Van der Waals forces. When the double layer is very compact (Fig.2-4) an approaching particle comes into the sphere of Van der Waals forces before there is any electrostatic repulsion and therefore the particles adhere.

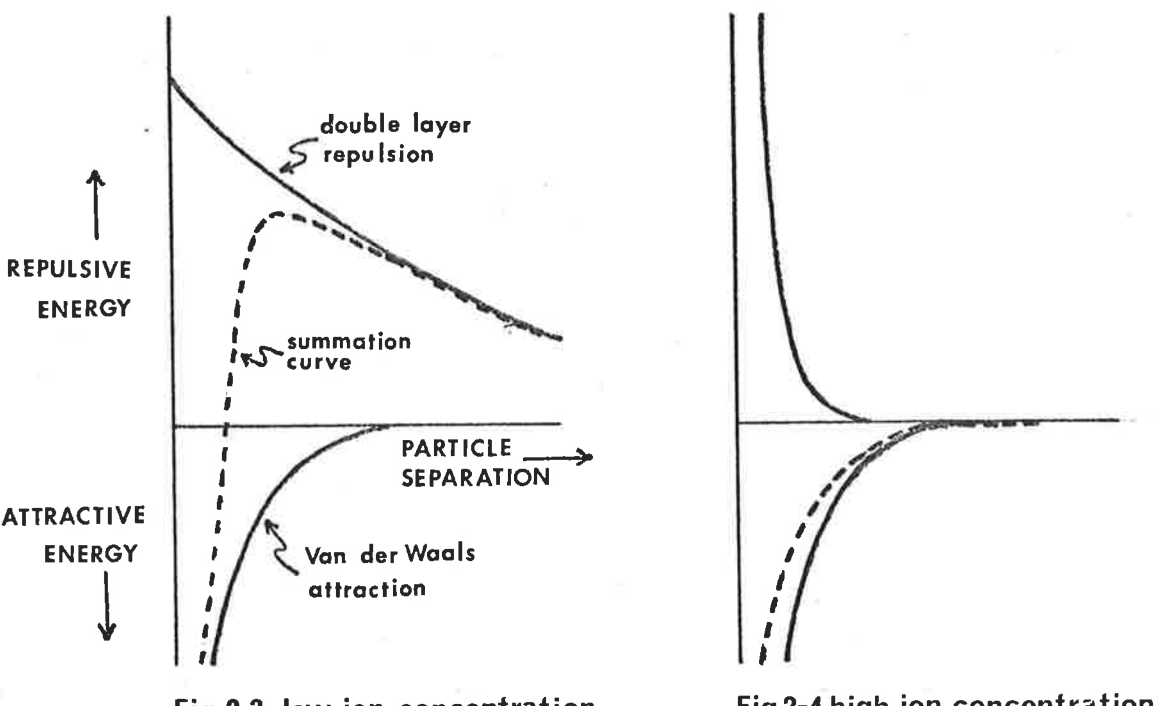

Fig. 2-3 and 2-4 Net interation energy as a function of particle separation when the double layer is "thick" (Fig. 2-3) or "thin" (Fig. 2-4) (Van Olphen, 1963)

The protoplast will also be surrounded by a diffuse electric double layer and two approaching protoplasts will be subject to electrostatic repulsion and H-bonding and Van der Waals attraction. It is conceivable that polymers such as PEG compress the double layer. Very high Na^{+} concentrations may compress the double layer sufficiently for adhesion. Certainly high Ca^{++} concentrations would greatly increase the likelihood of adhesion and fusion, especially at the elevated temperature (37°C) often used since here the protoplasts would have greater kinetic energy to overcome any residual repulsive "hump". One might also predict that trivalent cations such as La^{+++}, Eu^{+++} or Te^{+++} may be effective as protoplast fusogens.

[illegible]-2 GENETIC LOSS IN WIDE CROSSES

It was proposed in the General Introduction that protoplast hybrid selection based on auxotrophic mutant complementation or additivity of antimetabolite resistances would fail to select many partial hybrids. If there is loss of portions of one or both parental genomes then many of the resulting partial hybrids will fail to complement. The literature establishes the precedent according to which genetic loss can be expected in wide fusion crosses.

When cultured animal cells of different origins are fused the somatic hybrids very often lose chromosomes entirely or predominantly from one parent (Ephrussi, 1972; Merril and Stanbro, 1974). It seems that this loss occurs in an early, rapid stage (within the first few divisions) but also later, more gradually. There is some evidence that chromosome loss not only favours one parent but also occurs more frequently with particular chromosomes (Bernhard, 1976). There is ample documentation of the preferential loss of rat chromosomes from mouse/rat hybrids and human chromosomes from mouse/human, Chinese hamster/human and rat/human hybrids. Only very rarely is there reported a fusion hybrid which loses chromosomes preferentially in the opposite direction to such precedents (Bernhard, 1976). Ferenczy *et al* (1977) presented evidence for preferential loss of the *Aspergillus fumigatus* genome in protoplast fusion hybrids of *A. fumigatus* + *A. nidulans*.

Preferential elimination of chromosomes has also been observed in wide sexual crosses of plants. Gupta (1968, 1969) was able to isolate addition lines of single *Nicotiana plumbaginifolia* chromosomes in *N. tabacum* because of preferential elimination

in the hybrid. Haploid Hordeum vulgare could be obtained from H.vulgare x H.bulbosum (Subrahmanyam and Kasha, 1973; Bennet et al, 1976) and H.vulgare x Secale cereale (Fedak, 1977). Subrahmanyam (1977) and Islam and Sparrow (1974) also demonstrated preferential chromosome loss in crosses of other Hordeum species and constructed hierarchies of genome predominance. Triticum aestivum x Hordeum bulbosum (2n = 14 or 28) hybrids degenerated to wheat haploids (Barclay, 1975) and there was elimination of chromosomes in H.vulgare x T.aestivum crosses with some selectivity against H.vulgare chromosomes (Islam and Shepard, pers. comm.). There is also some evidence for haploids resulting from chromosome elimination in the following crosses: Nicotiana sylvestris x N.tabacum, Solanum x chauca Juz. et Buk. x S.tuberosum L., N.tabacum x N.langsdorfii, Tripsacum dactyloides L. x Euchlaena mexicana Schrad.(Kasha, 1974).

There is now also evidence of unidirectional chromosome loss in plant protoplast fusion hybrids. Power et al (1975) fused Petunia hybrida leaf protoplasts with those of Parthenocissus crown gall callus and obtained callus from the cultured fusion mixture after selective culture conditions designed to eliminate parental cells. Karyotypic analysis showed the selected calli contained Parthenocissus chromosomes only (Petunia chromosomes are morphologically quite distinct). However isoperoxidase banding patterns clearly indicated the presence of some Petunia genetic material (although conceivably only cytoplasmic genes). The most apparent explanation is that the selected calli did derive from fusion hybrids but there was preferential elimination of the bulk of the Petunia genome.

It is clear from this example and from others in animal somatic hybrids (Merril and Stanbro, 1974; Bernhard, 1976) that loss of all the morphologically identifiable chromosomes of one parent does not necessarily mean loss of all genes characteristic of that parent. The most obvious means for retaining some genes of a lost chromosome would be by somatic cross-over events or transgenomic translocations. Vig (1975) induced elevated somatic cross-over frequencies with caffeine. This effect was enhanced in the presence of 5-fluorodeoxyuridine or cytosine-β-D-arabinofuranoside. Such agents may be valuable in plant protoplast hybridization where gross genetic loss of one parent is anticipated.

The mechanism(s) of chromosome elimination are not clearly established. Subrahmanyam et al (1976) presented some evidence that H.bulbosum elimination is the result of restriction endonucleases of the other genome degrading the bulbosum chromosomes. Conversely Bennet et al (1976) claimed that bulbosum chromosomes are lost by their failure to congress at metaphase or to reach a pole and suggested this resulted from a failure to form protein attachments to the spindle. The loss of chromosomes may in part be caused by the timing of the various mitotic cycle phases being different in the two parents. Pairs such as Vicia faba and Pisum sativum, or Hordeum vulgare and Lycopersicon esculentum have mitotic phase times which match very well (Van't Hof, 1974). Such pairs may be suitable parents for somatic hybridization.

It has become possible recently in mammalian cell hybrids to control to some extent the direction of chromosome loss. By pretreating one parent prior to fusion with X - or γ-irradiation or bromodeoxyuridine the chromosomes of that parent will be lost to

a greater extent than in a control hybridisation (Pontecorvo, 1974, 1975; Bernhard, 1976). Laser irradiation of a particular chromosome in metaphase results in its release from the spindle and subsequent elimination (Bernhard, 1976). The antimycotic, griseofulvin, produces chromosome count scatter in cultured mammalian cells (Pontecorvo, 1975). Such techniques may eventually be useful in constructing desired karyotypes during plant protoplast hybridization.

2-3 SOMATIC HYBRIDIZATION

Using the technologies of protoplast isolation, fusion and culture, somatic fusion hybrids of plant protoplasts have been produced and cultured. The following is an examination of somatic hybrids which have grown to plants or at least to callus.

a) *Nicotiana glauca* + *N.langsdorfii*.

This fusion hybrid was first reported by Carlson *et al* (1972) and the same experiments also described by Smith (1974). Young leaves were used for protoplast isolation and fusion induced by pelleting the mixed suspension in NT medium (Nagata & Takebe, 1971) plus 0.25 M $NaNO_3$. A two-fold selection of hybrids was used. Firstly the fused protoplasts were plated in NT medium in which it had already been found that the protoplasts of neither parent could grow but those of the sexually-produced hybrid could grow. Secondly the calli which grew in NT were transferred to a hormone-less LS medium (Linsmaier and Skoog, 1965). It had already been established that the sexually-produced amphiploid hybrid is tumorous and its callus can grow in the absence of auxin and cytokinin. The calli of both parents require hormones for growth. All 33 isolates from the NT medium also grew on the LS medium and shoots were regenerated (and in some cases also roots). Where only shoots were regenerated these were grafted onto young *N.glauca* plants and three grown to maturity. The evidence for being true hybrids was: (i) leaf morphology intermediate between parents and similar to sexual amphiploid (ii) leaf trichomes also intermediate (iii) spontaneous tumors developed as in the sexual amphiploid (iv) karyotype was identical to the sexual amphiploid ($2n = 24 + 18 = 42$)(v) peroxidase isozyme pattern was the summation of the parental patterns (vi) flower morphology was intermediate

(vii) they were fertile and selfed to produce plants identical to amphiploids.

Kung *et al* (1975) tested the fraction 1 proteins (ribulose diphosphate carboxylase) of the selfed progeny of one of the somatic hybrids of the original Carlson *et al* (1972) experiments. The large subunits of these proteins are coded by chloroplast DNA while the small subunits are coded by nuclear DNA. Isoelectric focussing of the subunits demonstrated both *N.glauca* and *N.langsdorfii* nuclear genes but only *N.glauca* chloroplast genes to be present. Kao *et al* (1974) repeated this hybridization using PEG1540 and a saline eluent for fusion. The culturing conditions were different but involved a selection step on hormone-less medium. Within 1 month, 135 tumor-like calli had developed with morphologies very much like the sexual amphiploid and with tumorous shoots.

Somatic hybrids of *N.glauca* and *N.langsdorfii* were again produced with PEG1540 but with a high Ca^{++}/pH 10.5 eluting solution (Wetter and Kao, 1976). Plants were regenerated from 4 selected hybrid calli and again tumorisity, morphologies and colours testified to the hybrid nature of the plants. In addition electrophoretic isozyme patterns were examined for glutamate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase, aminopeptidase and esterase using calli of the parents, sexual amphiploid and 4 somatic hybrid lines. These patterns consistently demonstrated the somatic hybrids to be true hybrids. However the somatic hybrid patterns were not always the summation of the parental patterns possibly due to aneuploidy or lability of some of the isozymes with prolonged storage of the extracts.

Smith et al (1976) used PEG1540 and culture medium elution to once again produce protoplast fusion hybrids of N.glauca and N.langsdorfil. Of the 174 calli which survived the hormone-less selection, 23 plants reached maturity. Tumor formation indicated the hybrid nature of the plants. Plant morphologies were distinct from both parents but were not identical to those of the sexual amphiploid. These differences obviously resulted from the unusual chromosome numbers of the somatic hybrids. Unlike the sexual amphiploid and the somatic hybrids of the 1972 experiments which were 2n = 18 + 24 = 42, the somatic hybrids in this experiment were 2n = 56 to 64 (mean 59.3) with meiotic bivalent numbers ranging from 26 to 30 (mean 28.8). It was assumed these hybrids were triple fusions (2n = 18 + 24 + 24 = 66 or 2n = 18 + 18 + 24 = 60) some or all of which had subsequently lost chromosomes.

Kung (1977) examined the fraction I proteins of 20 of the 23 somatic hybrid plants produced by Smith et al (1976). The small subunits were mixtures of the parental types in all these plants indicating that they were true nuclear hybrids. The large subunits showed that 12 of the hybrids had langsdorfii plastids only, 7 had glauca plastids only, and 1 had a mixture of parental plastids. This cytoplasmic and nuclear hybrid was very abnormal.

b) Nicotiana tabacum + N.suaveolens.

Kung et al (1975) used poly-L-ornithine to induce chloroplast uptake from a plastid preparation of N.suaveolens into non-green leaf protoplasts of a variegated albino N.tabacum. One green plant was regenerated which had an abnormal morphology and a chromosome count which varied from 2n =61 to 66. N.tabacum

has 2n = 48 and N.suaveolens has 2n = 32. Examination of the subunits of fraction 1 proteins (ribulose diphosphate carboxylase) by isoelectric focussing showed this plant to have plastids of both parents and also nuclear genes of both parents. Apparently free nuclei had been present with the plastid preparation and had been taken up as well.

c) Nicotiana tabacum + N.tabacum.

Melchers and Labib (1974) and Melchers et al (1975) reported the production and selection of somatic hybrids of two complementing chlorophyll deficient, light-sensitive mutants of N.tabacum. The plants were produced as haploids (24 chromosomes) by another culture and designated v and s. Even as callus these mutants were light sensitive especially when the organic constituents of the NT medium were reduced to 1/5 or 1/10 the normal concentrations and when the oxygen partial pressure was higher. Fusion was induced using high Ca^{++}/pH 10.5/37°C conditions and the protoplasts grown for 4 or 5 weeks in normal NT medium. Selection was then applied by plating the calli in NT with reduced organics, no auxin and reduced cytokinin and grown in 8000-10000 lux warm white light at 28°C. Under these conditions s, v, s+s and v+v genotypes are pale yellow or, at best, light green. By contrast the complemented somatic hybrids s+v are deep green and regenerate shoots. Somatic hybrid plants were produced from at least 12 independent fusion events out of 2.2×10^6 calli.

The authenticity of these somatic hybrids could be established by the morphological distinctness from the s, s+s, v and v+v plants but resemblance to the sxv sexual hybrid. Spontaneous reversion of the mutant could be ruled out by the segregation of s+v into some v-like and s-like as well as green

offspring in very similar proportions to the offspring of the sxv sexual hybrid. Chromosome counts of s+v and sxv plants showed considerable variation and although this analysis was still in progress s+v counts included numbers 48, 46, 96, 90, 72 and 70.

Gleba et al (1975) gave evidence of another use of chlorophyll deficient mutants within N.tabacum to detect and select intraspecific somatic hybridization. In this case one of the mutants was a semidominant nuclear gene (called Su) which was lethal in the homozygous state well before the plant matures. The other mutation was in the chloroplast DNA and caused variegation of leaves. Leaf protoplasts were isolated (from young seedlings of Su Su and from the cytoplasmic mutant) and fused with PEG. The fusion mixture was cultured to callus and regenerated to plants. Of the 30 plants obtained 20 were yellow-green (indicating Su su nuclear genotype) and 7 of these 20 were also variegated (indicating the presence of the mutant plastid). These 20 were all polyploid and therefore were presumed to be somatic amphiploids. The other 10 were green (indicating su su nuclear genotype) and some of these were variegated. This is a case where selection was applied only against one parent and yet the somatic hybrids were not overwhelmed by the other parent.

Kameya (1975a) obtained protoplasts from the leaves of a haploid N.tabacum with 2 recessive mutations (ws_1 ws_2) and used 20% dextran sulphate + 2% KCl + 0.5% $CaCl_2$ + 0.5% $NaNO_3$ to induce aggregation and fusion when mixed with normal haploid protoplasts. Colonies were grown and 8 of these produced numerous green plants while 4 colonies produced albino plants. The plants from 7 of the 8 green colonies reached maturity and their offspring examined. The offspring of the plants from 5 out of the 7 green colonies were

all green and therefore their parent colonies may be assumed to have been diploid self- fusion products of the normal haploid protoplasts. The offspring of the plants from the other two colonies were mainly green but some were albino. One of these 2 colonies conformed to the 15:1 ratio expected of a diploid somatic hybrid (Ws_1 ws_1 Ws_2 ws_2), but the other did not conform as well. Plants from both these colonies were shown to be diploid.

d) Nicotiana tabacum + N.sylvestris.

Melchers (1977) briefly reported the production of somatic hybrid plants from N.tabacum (s mutant as in Melchers and Labib, 1974) and N.sylvestris (a chlorophyll deficient, light-sensitive mutant able to complement s). Presumably selection for hybrids was based on tolerance to high light intensities.

e) Nicotiana tabacum + Petunia hybrida

Melchers (1977) also briefly reported the production of a few calli which contained varying numbers of both Petunia and Nicotiana chromosomes. However no plants from such calli could be regenerated.

f) Nicotiana glauca + Glycine max.

Kao (1976) was able to employ the improved protoplast culture media (Kao and Michayluk, 1975) to grow N.glauca + G.max hybrid cells. Individual single hybrid protoplasts were recognised visually following fusion and micromanipulated into isolated culture. No selective conditions were required. More than 20 such heterokaryocytes were individually cultured to more than 10 million cells within 2 months. Karyotype analysis confirmed the presence of both sets of parental chromosomes.

g) Parthenocissus tricuspidata + Petunia hybrida.

Power et al (1975) used 10.2% sucrose + 5.5% $NaNO_3$ to fuse crown gall callus protoplasts of Parthenocissus tricuspidata Planch.

with Petunia hybrida leaf protoplasts. This work has already been discussed in section 2-2. The Petunia protoplasts grew well in NT medium but the resulting callus could not grow on MS medium (Murashige and Skoog, 1962) lacking auxin and cytokinin. Conversely Parthenocissus protoplasts could not divide in NT medium but its crown gall callus could grow in the absence of exogenous hormones. It was assumed that only fusion heterokaryons should be able to grow through both selection steps (initial plating in NT followed by transferring colonies to MS without hormones). Some calli did survive both selection steps. These contained Parthenocissus chromosomes but no morphologically-recognisable Petunia chromosomes. Therefore the selected calli were not cross-feeding chimeral mixtures. However isoperoxidase banding patterns quite clearly demonstrated a mixture of characteristic Petunia and Parthenocissus bands. It seems a reasonable hypothesis that somatic cross-over or translocation events resulted in some Petunia genes remaining despite the elimination of Petunia chromosomes.

h) Petunia hybrida + P.parodii

Power et al (1976b) demonstrated that P.hybrida leaf protoplasts were significantly more sensitive to actinomycin D than those of P.parodii. P.hybrida protoplasts could grow to callus on MS medium but P.parodii protoplasts could only form small colonies. It was assumed a fusion heterokaryocyte would inherit actinomycin D resistance and also the ability to form callus on MS. After 28 days in the selective medium (MS + 1.0mg/l actinomycin D) 10 calli of independent origin were recovered and 8 of these were regenerated to plants. For comparison tetraploid P.hybrida (2n = 28), tetraploid P.parodii (2n = 28) and the sexual amphiploid P.hybrida x P.parodii (2n = 28) were produced. Chromosome counts (2n = 14 + 14 = 28 or sometimes less, but 24 was minimum), flower

colour (purple as opposed to red for *P.hybrida* and white for *P.parodii*) and peduncle length consistently confirmed the difference of the somatic hybrids from the parental plants (diploid or tetraploid) and their resemblance to the sexual amphiploid. In addition isoperoxidase electrophoresis showed the somatic hybrids to have patterns which were the summation of the parental patterns plus an additional hybrid band. This hybrid band was also present in the sexual amphiploid pattern but not in that of the mixture of parental extracts.

Recently Power *et al* (1977) reaffirmed the ability to select somatic hybrids of *P.hybrida* + *P.parodii* using media in which neither parent grows. The medium MS + 5mg/l 2, 4-D + 0.5mg/l BA + 0.75 mg/l actinomycin D was found to have the highest selective efficiency. The chromosome counts of the 176 somatic hybrid plants produced varied from 24 to 28. Cocking *et al* (1977) also selected 2 calli which were regenerated to flowering hybrid plants (28 chromosomes). The selection in this case was partly nutritional and partly visual. The *P.parodii* leaf protoplasts could only grow to small green colonies in MS + 2mg/l1 NAA + 0.5 mg/l BA. The *P.hybrida* culture protoplasts (from an albino seedling) grew to white callus. Only the fusion hybrids could grow to green callus.

i) *Sphaerocarpos donnellii* Fabr + *S.donnellii*

S.donnellii is a dioecious liverwort. The male plants have 7 autosomes plus a small Y sex chromosome. The female plant has 7 autosomes plus a large heterochromatic X chromosome. Schieder (1974a) obtained a nicotinic acid auxotroph which was green (nic_2) and a glucose auxotroph which was a light green mutant (pal_2). Leaf protoplasts of female nic_2 and male pal_2 plants

were fused using high Ca^{++}/pH9 conditions and when grown in minimal medium one somatic hybrid plant was regenerated. This plant was green and nicotinic acid autotrophic and possessed 14 autosomes plus an X and a Y chromosome.

In addition to these examples of somatic hybrid development to at least the callus stage there are numerous examples of somatic heterokaryocytes being observed to divide only a few times. The majority of these reports have come from the Prairie Regional Laboratory, Saskatoon, Saskatchewan. Table 2-2 lists these heterokaryocytes. In almost all cases one parent is a cultured cell protoplast and the other a leaf protoplast so that the fusion hybrid can be visually identified since cytoplasmic mixing is usually not rapid. The exception is _N.tabacum_ + _Senecio vulgaris_ L. where both parents are leaf protoplasts but one parent is differentiated by prestaining it in a 0.01% neutral red solution for 10 min. The extensive use of soybean suspension culture protoplasts results from the fact that soybean protoplasts divide readily and appear to be able to induce division of the alien nucleus even though this alien genome when not fused with soybean is unable to divide (Kao and Michayluk, 1974; Kao et al, 1974; Constabel _et al_, 1976).

Some of the intergeneric fusion products of Table 2-2 may have continued into sustained division. But the visual distinction between the parental protoplasts disappeared after a few divisions and in the absence of selection the hybrids were lost. There is very little data available on the nature of nuclear fusion in these hybrids. Most observations suggest that the two genomes exert regulatory influence on each other and divide synchronously

Table 2-2 Protoplast heterokaryocytes undergoing divisions.

Parental species		Fusogen	Maximum number of divisions	Reference
Culture	Leaf			
Glycine max	Hordeum vulgare	C	5	1,2,12
"	Zea mays	D	6	1,3,12
"	Brassica napus	A	3	1,4
"	Pisum sativum	C	6	1,3,5,12
"	Medicago sativa	B	3	3,6
"	Melilotus sp.	B	3	3,6,7
"	Cicer arietinum	?	?	3
"	Nicotiana tabacum	A	3	8
"	N.langsdorfii	A	3	8
"	N.rustica	A	3	8
"	Colchicum autumnale	A	3	8
"	Caragana arborescens	B	3	6
"	Vicia hajastana	C	?	9,12
Vicia hajastana	Pisum sativum	D	6	2,12
"	Vicia villosa	D	?	9,12
"	Daucus carota	?	?	9
"	Hordeum vulgare	?	?	9
Daucus carota	Pisum sativum	?	?	1
"	N. tabacum	E	4	10
"	Angelica archangelica	?	?	3
"	Hordeum vulgare	A	10	11
Melilotus sp.	Brassica napus	?	?	1
N.tabacum (leaf)	Senecio vulgaris	E	4	10

A = PEG (M.W.1540) + Ca^{++} (elution with culture medium)
B = PEG (M.W.1540) + Ca^{++} (elution with pH9 solution)
C = PEG (M.W.1540) + Ca^{++} (elution with high Ca^{++}/pH10.5)
D = PEG (M.W.1540) + Ca^{++} (elution with high NaCl solution)
E = 20% DEXTRAN SULFATE (M.W. 500000)

1. Gamborg *et al* (1975b)
2. Kao and Michayluk (1974)
3. Gamborg *et al* (1974)
4. Kartha *et al* (1974b)
5. Constabel *et al* (1975a)
6. Constabel *et al* (1975b)
7. Fowke *et al* (1976)
8. Constabel *et al* (1976)
9. Constabel and Kao (1976)
10. Kameya (1975a)
11. Dudits *et al* (1976a)
12. Kao *et al* (1974)

(Miller *et al*, 1971; Ito and Maeda, 1974, Constabel *et al*, 1975a; Kao *et al*, 1974; Power *et al*, 1971). As a result of synchronous division using a common spindle the nuclei fuse. There have also been some observations suggesting the fusion of interphase muclei. Dudits *et al* (1976a) published photographs convincingly indicating the union of premitotic nuclei of barley and carrot which stained differentially with modified carbol fuchsin.

In many of the soybean fusion hybrids in Table 2-2 the culture conditions allowed growth of soybean and heterokaryocyte protoplasts but not of the other donor protoplasts. Selection of the hybrids would therefore only require the soybean protoplasts to be placed in a selective disadvantage to the hybrid. Constabel *et al* (1975b) attempted to use canavanine-sensitivity for this purpose. Canavanine is a toxic analogue of arginine and is synthesised by sweet clover (*Melilotus* sp.), alfalfa (*Medicago sativa*) and *Caragana arborescens* whose cultures and protoplasts are tolerant to 10^{-3}M canavanine. Soybean (*Glycine max*) protoplasts are inhibited by 10^{-4}M and 10^{-3}M canavanine. However the fusion hybrids proved to be just as sensitive to canavanine as the soybean parent. This illustrates a basic problem with using antimetabolite-resistance for hybrid selection — the hybrid may inherit the sensitivities rather than the resistances of the parents. It is possible that suitably dominant resistance genes will be found and used advantageously.

Cocking *et al* (1974) suggested that natural resistances for certain drugs may obviate the need to isolate mutants for use in hybrid selection. These authors examined the effects of a

range of amino acid analogues and other toxic substances at various concentrations on the growth of petunia and tobacco protoplasts. Significant differences in response between the two species were observed with azatryptophan, homoarginine, abscisic acid, ethanol and 4-Cl-phenoxyisobutyric acid. Power *et al* (1976b) used a naturally occuring differential sensitivity to actinomycin D as one of the selective devices in the production of hybrid *Petunia hybrida* + *P.parodii* plants as was discussed above. Here apparently it was the extra resistance of *P.parodii* rather than the sensitivity of *P.hybrida* which was inherited.

2-4 PROGRESS AND PROBLEMS WITH MUTANT CELL LINES

The most common approach to hybrid cell selection is on the basis of genetic complementation of nutritional mutants. It has already been pointed out that selection based on the existence of particular genes in the hybrid cells will lose the majority of partial hybrids. In addition to this fundamental flaw in the approach there are difficulties in the production and use of mutant cell lines. Ferenczy *et al* (1975a, 1975b, 1976, 1977) have successfully employed adenine, lysine and methionine auxotrophs of *Aspergillus* and *Penicillium* fungi in complementation selection of protoplast fusion hybrids. Attempts to obtain and use auxotrophs of cultured plant cells have not been as successful. Using BUdR or FUdR incorporation into growing cells and subsequent killing with exposure to light, presumptive auxotrophs (unable to grow on minimal media) were isolated (Carlson, 1970; Zyrd, 1976; Aviv and Galun, 1977). However these mutants were invariably either "leaky" (able to grow slowly or after a delay on unsupplemented media) or not auxotrophic at all when tested in isolation (Carlson, 1970; Aviv and Galun, 1977). There seems to be a basic difficulty in obtaining amino-acid or nucleotide base auxotrophs in the cells of green plants which could be because of the existence of alternative biosynthetic pathways when the major one is blocked.

Even if stable, non-leaky auxotrophs can be obtained there remains the problem of cross-feeding. When two differing auxotrophs are in mixed culture on minimal medium the missing metabolites may diffuse through the medium from the sufficient to the deficient cells allowing both parents to grow as well as any

complemented hybrids (Carlson *et al*, 1972). Such cross-feeding would not occur if the two mutants were auxotrophic for the same metabolite. The hybrid would still benefit from genetic complementation provided different biosynthetic enzymes were affected by the two parental mutations. Even here care would be needed to choose mutants whose affected enzymes could not translocate from cell to cell.

The HAT selection technique has been of considerable use in somatic animal cell fusion (Rao and Johnson, 1972). One parent is deficient in thymidine kinase (TK) and the other deficient in hypoxanthine guanine phosphoribosyltransferase (HGPRT). These mutations prevent the use of preformed, exogenous nucleotide bases. The HAT selection medium contains hypoxanthine, aminopterin and thymidine. Aminopterin blocks *de novo* synthesis of nucleotides. Only the fusion hybrids are complemented and able to use the exogenous bases (hypoxanthine and thymidine) for growth. Both parents are blocked. An additional advantage of this system is that TK-deficient cells are resistant to BUdR and HGPRT-deficient cells are resistent to 8-azaguanine and therefore such mutants have been isolated with relative ease from mammalian cultures.

Some attempts have been made to isolate similar mutants from plant cell cultures. BUdR-resistant lines have been obtained in sycamore (Bright and Northcote,1974), soybean (Ohyama, 1974) and tobacco (Maliga *et al*, 1973b). At least in the latter case the resistance persisted through plant regeneration and re-establishment of callus and appeared to be a simple semi-dominant nuclear gene mutation (Marton and Maliga, 1975). The

resistant sycamore and soybean lines were also at least partially sensistive to Hypoxanthine-Aminopterin-Thymidine (HAT) selective medium (Bright and Northcote, 1974; Ohyama, 1974). Azaguanine-resistant sycamore and tobacco cultures have also been isolated (Lescure, 1973; Bright and Northcote, 1975). There have been no reported attempts to fuse and select for somatic hybrids of an azaguanine-resistant and a BUdR-resistant plant cell line.

The work of Constabel et al (1975b)(discussed in section 2-3) demonstrated the danger that antimetabolite sensitivities rather than tolerances may be inerited by hybrids. This example does not entirely negate the potential usefulness of the additive-antimetabolite-resistance strategy but it does serve as a warning against taking additivity for granted. Table 2-3 lists some resistance mutants. Despite the stability and heritability of some of these, there is no report of the resistance characteristics of fusion hybrids between any two of them.

Another serious drawback to a hybrid selection system based upon mutant cell lines is the often observed loss of morphogenic ability and karyotype aberrations associated with cultured plant cells. If both cell lines intended for use as parents in somatic hybridization have lost their totipotency then their fusion hybrids will also fail to form plants. A great many authors attest the widespread occurrence of changes in chromosome numbers when plant cells are cultured. Some cultures stabilize with a chromosome count different to the parental tissue (Miller et al, 1971; Singh et al, 1975; Singh and Harvey, 1975b; Guo, 1972). Many cultures develope a broad spread of counts on either side of the "normal" number. Boyer and Shannon (1974) examined 3 year

TABLE 2-3 Antimetabolite-resistant plant cell lines

Culture species	Antimetabolite	Stability	Reference
sycamore	BUdR	B	Bright and Northcote, 1974
tobacco	BUdR	B A	Lescure, 1973 Maliga *et al*, 1973b Marton and Maliga, 1975
soybean	BUdR		Ohyama, 1974
carrot	5-fluorouracil	A	Sung, 1976
tobacco	azaguanine	B	Lescure, 1973
sycamore	azaguanine	B	Bright and Northcote, 1975
tobacco	streptomycin	A	Maliga *et al*, 1973a
tobacco	streptomycin		Umiel and Goldner, 1976
carrot	cycloheximide	A	Sung, 1976
tobacco	cycloheximide	C	Maliga *et al*, 1976
Stylosanthes tobacco rice	kanamycin sulphanilamide allyl alcohol canavanine chlorate		Langridge and Scowcroft, 1977
tobacco	chlorate	A	Müller, 1976
tobacco	methionine sulphoxide	A	Carlson, 1973a
rice	S-(β-aminoethyl)-cysteine	B	Chaleff and Carlson, 1975
tobacco	S-(β-aminoethyl)-cysteine	B	Widholm, 1976b
tobacco carrot	p-fluorophenylalanine	B	Palmer and Widholm, 1975
sycamore	p-fluorophenylalanine	B	Gathercole and Street, 1976
Nicotiana sylvestris	ethionine		Zenk, 1974
carrot	ethionine	B	Widholm, 1976
tobacco	δ-hydroxylysine	B	Widholm, 1976
carrot	hydroxyproline	B	Widholm, 1976
carrot	thioproline	B	Widholm, 1976
carrot	5-methyltryptophan	B	Widholm, 1974
clover	2,4-D or 2,4,5-T or 2,4-DB		Oswald *et al*, 1977
tobacco *Capsicum* L.	NaCl	B	Dix and Street, 1975
maize (T cytoplasm)	*Helminthosporium maydis* T toxin	B	Gengenbach and Green, 1975

A = resistance stable even when plants regenerated and cultures reformed.

B = resistance stable for a number of subcultures in absence of the antimetabolite.

C = resistance is transient.

old callus of maize endosperm (3n=30) and found cells with from 21 to over 200 chromosomes. A large variation of chromosome numbers have also been reported in the cultures of wheat (Shimada *et al*, 1969; Kao *et al*, 1970b; Shimada, 1971; Okamoto *et al*, 1973; Asami *et al*, 1975), tobacco (Matthews and Vasil, 1976; Sacristan and Melchers, 1969), sugarcane (Heinz and Mee, 1969; Liu and Chen, 1976) *Vicia faba* (Yamane, 1975; Larkin, unpublished) and onion (Yamane, 1975; Nandi *et al*, 1977).

The mechanisms responsible for such variations may include endomitoses, multipolar mitoses and spindle failure (lagging chromosomes)(Bayliss, 1973; Sunderland, 1973). The auxin 2,4-D has been implicated as responsible for the extent of these aberrations (Ronchi *et al*, 1976) but has been exonerated by the work of others (Bayliss, 1975; Singh, 1975; Singh and Harvey, 1975a). Singh (1976) found kinetin and yeast extract increased polyploidy and aneuploidy in *Haplopappus* cultures.

In addition to chromosome number changes there are also structure aberrations. Dicentrics, telocentrics, fragments, rings and anaphase bridges have been reported in cultured plant cells (Okamoto *et al*, 1973; Sunderland, 1973; Singh, 1975; Boyer and Shannon, 1974; Nandi *et al*,1977). Chromosome number and structure alterations are indicative of changes resulting in the loss of morphogenic capacity as a culture ages (Barba abd Nickell, 1969; Meyer-Teuter and Reinert, 1973; Rashid and Street, 1974; Smith and Street, 1974; Haddon and Northcote, 1975; Negrutiu *et al*, 1975).

SECTION 3 PURIFICATION AND VIABILITY DETERMINATIONS OF PLANT PROTOPLASTS*

P.J. Larkin   Planta (Berl.) 128 : 213-216 (1976)

SUMMARY

A method is described for purifying plant protoplasts from cellular and subcellular debris. The procedure utilizes a density buffer containing 9.6% sodium metrizoate and 5.6% Ficoll. The use of fluorescein diacetate for assessing the viability of plant protoplasts is also reported.

INTRODUCTION

Plant protoplasts are now used in many plant sciences including somatic cell genetics, plant pathology, photosynthesis, cell wall biosynthesis and membrane physiology. In many studies it is a great advantage to have a protoplast preparation uncontaminated by microorganisms, subcellular debris (especially chloroplasts), vascular elements, undigested cells, and broken protoplasts. Additionally it is imperative to have at one's disposal reliable techniques for determining protoplast viability. The first step in purifying protoplasts is the removal of large pieces of undigested tissue by filtering through sieves or muslin.

Existing further steps include:

a)flotation on dense sucrose solutions (Gregory and Cocking, 1965; Power and Cocking, 1970; Evans *et al.*,1972; Grout and Coutts, 1974; Davey *et al.*, 1974; Cocking *et al.*, 1974);

b)flotation on Ficoll solutions (Schenk and Hildebrandt, 1971);

c)repeated centrifugation and resuspension (Bui-Dang-Ha and Mackenzie, 1973; Kartha *et al.*, 1974a; Pelcher *et al.*, 1974);

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* Abbreviation : FDA = fluorescein diacetate.

d)repeated sedimentation (without centrifugation) and resuspension (Bawa and Torrey, 1971; Eriksson, 1971; Kameya and Uchimiya, 1972).

All of these techniques give inconsistent results and often only partial purification (Kanai and Edwards, 1973; Watts et al., 1974).

Kanai and Edwards (1973) developed a density buffer which when mixed with 0.13 volumes of crude protoplast suspension and centrifuged at 300 g for 6 min partitioned into two phases. The debris and broken protoplasts were left suspended in the lower phase while the intact protoplasts collected at the interface. The density buffer contained polyethylene glycol (M.W 6,000), dextran (M.W 40,000), sodium phosphate and sorbitol. Despite the requirement to subject protoplasts to pH 8.0 and despite the sensitivity of the technique to the brand of dextran, this method is a vast improvement on former methods.

In his attempts to obtain pure lymphocyte preparations from blood, Boyum (1964, 1968) investigated a number of density buffers. The density buffers contained dextran, Ficoll (polysucrose), or methylcellulose. When blood was layered on top of one of these solutions the erythrocytes clumped and sedimented. The lymphocytes remained at the interface. There have been a number of modifications of the two-phase lymphocyte preparation technique (Thorsby and Bratlie, 1970; Ting and Morris, 1971; du Bois et al., 1973; Brown and Greaves, 1974).

A commercial density buffer called Lymphoprep (Nyegaard A/S Oslo, Norway) is composed of 9.6% (w/v) sodium metrizoate and

5.6% (w/v) Ficoll with a specific gravity of 1.077 ± 0.001 g/ml. The use of this product for purifying protoplasts is the subject of part of this paper.

Some fields of protoplast research have an ultimate, long-term demonstration of the viability of the protoplasts. However it is desirable to have a fast, short-term method for assessing their health. The literature records a number of methods with this end in view:

a)observation of cyclosis as an indicator of active metabolism (Raj and Herr, 1971; Pelcher et al., 1974);

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

c)size variation of intact protoplasts with osmotic changes (Kanai and Edwards, 1973);

d)oxygen uptake measured by an oxygen electrode, indicating respiratory metabolism (Taiz and Jones, 1971);

e)photosynthetic activity (Kanai and Edwards, 1973).

Cyclosis can be very difficult to observe, especially in mesophyll protoplasts in which there is a peripheral layer of chloroplasts. Size variation with osmotic changes is painstaking and allows only a few protoplasts to be assessed. These two methods and dye exclusion can only be applied to individual protoplasts. Oxygen uptake and photosynthetic activity can only be applied to whole suspensions.

This paper reports the use of fluorescein diacetate (FDA) as a protoplast viability stain. It has formerly been used with pollen (Heslop-Harrison and Heslop-Harrison, 1970), cultured animal cells (Rotman and Papermaster, 1966; Cercek et al., 1973a, b), human lymphocytes (Martel et al., 1974), yeast cells (Cercek

and Cercek, 1973a, b) and suspension cultured plant cells (Widholm, 1972). Intact cells fluoresce as fluorescein (an enzymic product of FDA) accumulates inside the membrane.

MATERIALS AND METHODS

Plant Materials and Protoplast Production.

All plants were grown in soil/peat mixtures and well supplied with NH_4^+ and NO_3^- nitrogen. The exceptions were *Brachycome lineariloba* (n=4), which was grown in sandy soil with little water, and *Zea mays* which was germinated in water for five days.

Tobacco, petunia and *Lathyrus sativus* leaves were sampled just prior to flowering and the fully expanded leaves stripped of their lower epidermis. The mature *Brachycome* shoots (2 cm high), the maize coleoptiles, petunia petals and the tulip shoots were lightly diced with a scalpel blade.

All tissues were preplasmolysed for 1-2 h in an osmoticum containing (mg/l): $MgSO_4.7H_2O$(250),$CaCl_2.2H_2O$(159),KNO_3(27.2), $Fe_2(SO_4)_3.6H_2O$(2.5),KI(0.16), $CuSO_4.5H_2O$ (0.025), 9% mannitol adjusted to pH 5.9.

Table 3-1 shows the composition of the four enzyme mixtures used. Table 3-2 shows which solutions were used on each cell source. All incubations were at 25°C and continued for 10-14 h without shaking. Protoplasts could be isolated from each tissue listed in Table 3-2.

The supply of nitrogen fertilizer to the growing plants was found to be essential for consistent tobacco and petunia protoplast production. Others have also made this observation (Watts

TABLE 3-1. Enzyme solutions for protoplast isolation

Enzyme	Concentration (%)			
	A[a]	B[a]	C[a]	D[a]
Cellulase (Onazuka P1500)	3.0	2.0	1.0	2.5
Cellulase (Calbiochem)	-	-	0.25	-
Driselase	-	0.5	0.5	0.25
Rhozyme	-	0.25	-	0.1
Macerozyme	0.25	0.25	0.25	0.25

[a] Solution made up in the osmoticum and the pH adjusted to 5.9

TABLE 3-2 Cell materials and enzyme solutions

Species	Tissue	Enzyme solution
Nicotiana tabacum cv. White Burley	leaf	A
Nicotiana tabacum cv. Hicks	leaf	A
Petunia hybrida cv. Confetti	leaf	A
	petal	D
Brachycome lineariloba (n=4)	shoot	B
Lathyrus sativus	leaf	B
Zea mays	coleoptile	C
Tulip	shoot	D

et al.,1974; Shepard and Totten, 1975).

Protoplast Purification.

After filtration through muslin, 0.5-3.0 volumes of crude protoplast suspension were layered on top of 1 volume of lymphoprep in a centrifuge tube. Spinning at 50-200 g for about 10 min allowed the protoplasts to be removed from the interface with a pasteur pipette.

Viability Determination.

Fluorescein diacetate (FDA) was stored in an acetone stock solution (5mg/ml) at $0^{o}C$. It was added to the protoplast suspensions to give a final concentration of 0.01%. After 5 min at room temperature the protoplasts were examined for fluorescence using a Zeiss fluorescent microscope. The exciter filter BG12 and barrier filter 47 were suitable (giving transmissions of 330-500 nm and 460 nm respectively).

RESULTS

A sample of the crude protoplast suspensions was incubated with FDA. Preparations varied in having 50-98% of the protoplasts fluorescing brightly. Mesophyll protoplasts were considered viable if they had a yellow/green fluorescence. They were deemed non-viable if they did not fluoresce or if red fluorescence (due to the chlorophyll) was not dominated by the fluorescein (yellow/green) fluorescence. In most preparations there was a high incidence of subcellular debris.

After purification with lymphoprep and incubation with FDA very little debris remained and often there was an increased percentage of viable protoplasts. In one experiment petunia

protoplasts at a density of 10^5/ml were 65% viable prior to purification. There was a 75% recovery of protoplasts following the lymphoprep purification (and accompanying manipulations). Seventy-five percent of the recovered and highly purified protoplasts were deemed viable. Kanai and Edwards (1973) achieved recoveries of no better than 50% in their two-phase purification method.

Figs. 3-1 and 3-2 demonstrate the differentiation of viable and non-viable protoplasts. Although the fluorescein accumulated while the incubation with FDA continued, there was a slow leakage once the protoplasts were washed free of excess FDA. The rate of exit of fluorescein molecules is much lower for intact membranes than for dead membranes but it is nevertheless significant (Cercek _et al_., 1973b; Rotman and Papermaster, 1966). Within 15 min the fluorescence had decreased notably.

Fig. 3-3 shows the green band of tobacco mesophyll protoplasts at the interface between the density buffer (lymphoprep) and enzyme solution. All six types of protoplasts behaved similarly.

The pellets were resuspended and examined microscopically. In the case of mesophyll protoplasts the pellets contained largely chloroplasts, vascular elements and walled cells. Negligible debris or walled cells could be found in the interfacial protoplast band.

Protoplasts suspended in the brown enzyme solutions or in 0.5% Trypan blue dye were used to demonstrate the negligible mixing of the two phases. After centrifugation over lymphoprep

Fig. 3-1 *Petunia* mesophyll protoplasts in the presence of fluorescein diacetate but illuminated with light (x 500).

Fig. 3-2 Same field as Fig. 3-1 but illuminated with U.V. light. Only one protoplast is viable and fluoresces (x 500).

Fig. 3-3 Purification of tobacco mesophyll protoplasts. The protoplasts are at the interface between the lymphoprep (lower phase) and enzyme solution (upper phase). The debris is at the bottom of the centrifuge tube.

3-1
3-2
3-3

the brown or blue colouration remained entirely in the top layer with the protoplasts at the sharp boundary.

DISCUSSION

The use of lymphoprep arrears to be a particularly effective and easy method for purifying plant protoplasts. It may prove a valuable technique for all fields of research involving protoplasts.

Lymphocyte purifications with density buffers related to lymphoprep, rely on the clumping of red blood cells at the interface by the agglutinating activity of Ficoll and the subsequent sedimentation of the clumps. Dextran and methylcellulose also show this activity (Boyum, 1964, 1968). When used to purify protoplasts it is not yet clear whether the debris and walled cells are agglutinated or whether they are sufficiently dense to sediment through the density buffer without agglutination.

Fluorescein diacetate has shown itself to be a useful viability stain for plant protoplasts. It is suitable at the individual protoplast level and also at the protoplast population level. The latter is possible by washing the protoplasts, resuspending and measuring fluorescence spectrophotometrically. Using this stain the lymphoprep method of protoplast purification appears to give a high yield of cleaned protoplasts with a slightly increased proportion of viability.

FDA is non-fluorescing, non-polar and freely permeable across intact plasmalemma. Fluorescein is a fluorescent polar product of esterase activity on FDA and is not freely permeable across intact plasmalemma. Therefore fluorescein accumulates

in viable cells but not in "dead" cells. Investigations are continuing to determine the extent to which FDA staining correlates to the plating efficiency of the protoplasts.

Acknowledgements

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SECTION 4

4-1 MODEL 1 FOR HYBRID SELECTION USING ANTISERA

Fig. 4-1 presents a model for separating hybrid protoplasts from parental protoplasts. The model assumes there will be sufficient antigenic differences between the surfaces of different species of protoplast so that specific (cross-absorbed) antisera can be prepared. It also assumes that the fusion hybrid will have a surface with a mixture of the specific antigens. Self-fusion products would be antigenically similar to unfused parental protoplasts. The 2-step agglutination procedure would eliminate first one and then the other parent by rinsing away unagglutinated cells. This model is general inasmuch as the same scheme would be used for any chosen parents. However it is specific in requiring different antisera for each new prospective parent. To test the model it first had to be shown that rabbit antisera (raised by protoplast injection) could in fact agglutinate protoplasts of that species.

MODEL 1

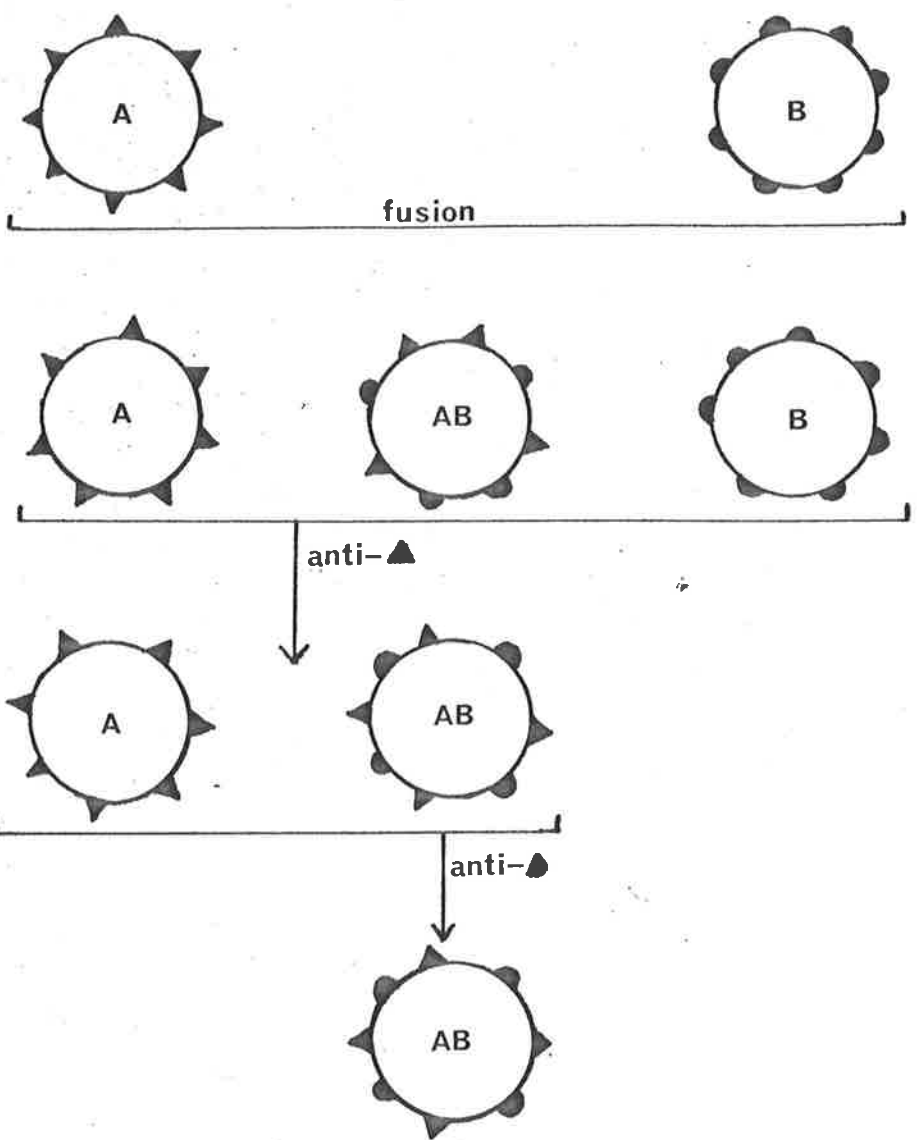

Fig 4-1

anti-▶ = specific anti-▶ serum

anti-◗ = specific anti-◗ serum

4-2 PLANT PROTOPLAST AGGLUTINATION AND MEMBRANE-BOUND β-LECTINS

LARKIN, P.J. J. Cell Science 26 : 31-46 (1977)

SUMMARY

Plant protoplast agglutination caused by normal and immune serum and by artificial carbohydrate antigens is described. Evidence is presented that the new class of lectins, called all-β lectins by previous authors, are present on protoplast membranes and responsible for both forms of agglutination. Some non-specific serum component and the artifical antigens are the 2 respective passive 'bridge' molecules between agglutinating protoplasts.

INTRODUCTION

Both Hartmann, Kao, Gamborg & Miller (1973) and Strobel & Hess (1974) observed the interaction of rabbit sera with plant protoplasts. They concluded that the resulting agglutination was mediated by antibodies. Serum-mediated agglutination has attracted some attention as a potential means of inducing fusion. The work reported here was initiated in an attempt to use agglutination to distinguish and separate different protoplasts in mixed suspension. In fact the findings suggest that the agglutination observed is not produced by the binding of antibodies to protoplast membrane antigens. Rather, the binding molecules appear to be on the protoplast membrane and the agglutination results from the binding of these to ligands on multivalent serum macromolecules.

The binding molecules on the protoplast membrane are all-β lectins (as defined by Jermyn & Yeow (1975)) and they bind to molecules bearing accessible β-D-glycosyl residues. Hereafter the all-β lectins will be referred to simply as β-lectins.

MATERIALS AND METHODS

Protoplast isolation

Tissues were surface-sterilized with 70% ethanol for 2min. and sometimes also with 6% sodium hypochlorite for 10min. *Nicotiana tabacum*, *Petunia hybrida*, *Vicia faba*, *Brassica napus* and *Brachycome lineariloba* leaves were stripped of lower epidermis. Cereal leaves and seedling hypocotyls were sliced longitudinally with a scalpel. All tissues were preplasmolysed in one of the osmotica of Table 4-1 for 20-60min. and placed in enzyme solution as indicated in Table 4-2. Crude protoplast suspensions were washed in enzyme-free osmotica and sometimes also purified by centrifuging over a layer of density buffer (Larkin, 1976, Section 3). The final rinse was always in osmoticum adjusted to pH 7.2 (usually V47ml or OSI). The viability of protoplasts was assessed using the fluorescein diacetate (FDA) technique (Larkin, 1976, Section 3). Those results reported are of viable preparations only.

Sera

Four rabbits were used in an attempt to produce anti-tobacco protoplast antisera. All were prebled to give control sera designated PB1, PB2, PB3 and PB4. The first 2 rabbits were challenged intravenously with approximately 10^7 *Nicotiana tabacum* cv. Hicks mesophyll protoplasts on each of 3 consecutive weeks. The injection medium of Hartmann *et al.* (1973) was

TABLE 4-1 Osmotica composition

Constituents	Concentration (mg/ml unless otherwise shown)				
	OSI	OSII	OSIV	OSV	V47ml
Mannitol	0.5M	0.25M	0.25M	0.2M	
Sorbitol		0.25M			
Sucrose			0.25M	0.2M	0.05M
NaCl					0.25M
$CaCl_2.2H_2O$	150	875	875	150	
$CaH_4(PO_4)_2.H_2O$		125	125		
$MgSO_4.7H_2O$	250			250	
KNO_3	100			100	
KH_2PO_4	27.2			27.2	
$Fe_2(SO_4)_3.6H_2O$	2.5			2.5	
KI	0.16			0.16	
$CuSO_4.5H_2O$	0.025			0.025	
polyvinyl-pyrrolidone				1%	
V47 salts (Binding, 1974)					V47 salts

TABLE 4-2 Enzyme solutions

Constituents (%)	PLANT TISSUES					
	Nicotiana, *Petunia*, *Brachycome*, *Vicia* mesophyll	*Bromus* mesophyll, *Zea mays* coleoptile	Cereal mesophyll	Carrot cell culture	Tulip shoot, Flax hypocotyl	Rapeseed mesophyll
Calbiochem Cellulysin	2-3	1	2.5-3.0	2.5-4.0	2.5	0.5
Calbiochem Macerase	0.25-1.0	0.25	0.25-0.5	0-0.25	0.25	0.5
Sigma pectinase				0-1.0		
Driselase	0-1.0	0.5	0.25-1.0	0.25-1.0	0.25	0.5
Rhozyme HP150	0-0.25		0.25	0.05-1.0	0.1	0.5
Calbiochem fungal cellulase		0-0.5				
Osmoticum	OSI, OSII, OSIV, OSV, or V47ml	OSI	OSI,OSII	OSI	OSI	OSII
pH	5.5-6.0	5.9	5.9-6.0	6.0	5.9	6.2

used (0.85% NaCl, 0.1% $CaCl_2.2H_2O$, 0.5% glucose, 0.5% mannitol). These 2 rabbits were bled 1 week after the last injection (sera TB1 and TB2).

The other 2 rabbits were challenged with approximately 10^7 tobacco mesophyll protoplasts on each of 3 weeks. The first injection was intraperitoneal with Freund's complete adjuvant. Another subcutaneous injection followed 2 weeks later. After another week they were bled (sera TB3 and TB4). Rabbit 4 was also bled 2 weeks after TB4 and the serum designated TB6. Rabbits 2 and 3 were also bled 3.5 months after TB2 and TB3 and the sera designated TB7 and TB8 respectively.

Normal unimmunized sheep, cats and guinea pigs were bled for serum. Samples of serum from hairy-nose wombat (*Lasiorhinus latifrons*), swamp wallaby (*Wallabia bicolor*) ring-tail possum (*Pseudocheirus peregrinus*), and western-grey kangaroo (*Macropus fulginosus*) were supplied by Dr. R.M. Hope (Genetics Department, Adelaide University).

Unless otherwise stated all sera were incubated at 56°C for 30 min to inactive complement.

Ammonium sulphate serum precipitation

The following procedure was followed to determine whether the serum molecule responsible for mediating in protoplast agglutination would precipitate with the globulin fraction. Cat serum (2ml) was added to 1.64ml of saturated $(NH_4)_2SO_4$ solution (pH 7.0) giving 45% saturation. This was left at 4°C for 4-5h and then centrifuged for 10 min at 300 rev/min to give a white precipitate and straw-coloured supernatant. Both the

precipitate (redissolved in saline) and the supernatant were dialysed against saline at 4°C for 6.5 h and then against OSI (pH 5.9) over-night and then against OSI (pH 7.2) for 1 h. They were stored frozen.

Yariv antigens and β-lectins

The artificial carbohydrate antigens here referred to as Yariv antigens were first synthesized by Yariv, Rapport & Graf (1962). The structure of these red-coloured phenylazo glycosides can be seen in Fig. 4-2. The β-D-glucosyl, β-L-glucosyl and α-D-galactosyl Yariv antigens (here-after called β-D-GLU, β-L-GLU and α-D-GAL respectively were synthesized and generously supplied by Dr. M.A. Jermyn (C.S.I.R.O. Division of Protein Chemistry, Parkville). Dr. Jermyn also purified and donated the sample of *Phaseolus lunatus* β-lectin (refer to Jermyn & Yeow, 1975). The identity of the Yariv antigens was confirmed by spectral scan data by comparison with Yariv *et al*.(1962).

Agglutination experiments

One drop of test solution was mixed with one drop of protoplast suspension (approximately 10^6 protoplasts/ml) in a sterile plastic Petri dish (usually 5 drops per dish). These were incubated for 20-40 min with occasional rocking at 20-27°C and examined microscopically for agglutination and protoplast integrity. All dilutions were in an appropriate osmoticum at pH 7.2.

Tissue extracts and gel diffusion plates

Cells were broken in a mortar and extracted with a minimal volume of extractant (0.02 M Gomori phosphate buffer, 1% NaCl, 0.01% NaN_3, pH 7.2). Such extracts were stored at 2-3°C.

Fig 4-2 | 1,3,5-Tri-(p-β-D-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene

or

(β-D-GLU)$_3$

Suspension culture broth was used directly. Gel diffusion plates were made with 0.6% purified agar in extractant. Yariv antigen solutions used in double diffusion experiments were either 0.5 or 1.0 mg/ml. Plates were incubated at 27°C for 24-48 h before examining for precipitin lines.

RESULTS

Interaction of protoplasts with immune and normal serum

Rabbits were challenged with tobacco mesophyll protoplasts by the injection regimes described. A protoplast agglutinating immunogenic response was expected (particularly on the basis of Hartmann *et al*. 1973). However the control sera (pre-bleeds) had titres for agglutinating activity at least as high as the testbleeds. Table 4-3 shows the results of titre determinations conducted concurrently with the one preparation of tobacco protoplasts. It is apparent that in no case did the antigenic challenge cause a significant increase in agglutinating activity. On the contrary in rabbits 1 and 4 there was a drop in titre from 320 to 40 and 80 respectively.

In other preliminary experiments the rabbit sera were shown to have strong agglutinating activity against wheat (*Triticum aestivum* L.) and oat (*Avena sativa* L.) protoplasts. Using wheat, PB2 and TB2 both had titres of about 100. Using oats, PB4 and TB4 had titres of 150 and 100 respectively. This latter result suggested that exposing rabbits to protoplasts may cause a drop in protoplast agglutinating titre even with respect to a different species of protoplast.

It should not necessarily be concluded that normal rabbit serum has a greater capacity to agglutinate tobacco protoplasts

TABLE 4-3 Agglutinating titres of anti-tobacco protoplast rabbit sera.

	RABBIT SERUM	DESCRIPTION	SERUM DILUTIONS							ANTI-TOBACCO TITRE
			1/20	1/40	3/160	1/80	1/160	1/320	1/640	
RABBIT 1	PB1	prebleed	++++[c]	++++	++++	++++	++++	±	-	320
	TB1	anti-tobacco I.V.[a]	++++	++	-	-	-	-	-	40
RABBIT 2	PB2	prebleed	++++	++++	++++	++++	++	-	-	160
	TB2	anti-tobacco I.V.	++++	++++	++++	++++	+++	+	-	320
	TB7	3½ months after TB2	++++	++++	+++	++	+	-	-	160
RABBIT 3	PB3	prebleed	++++	++++	+++	+++	++	-	-	160
	TB3	anti-tobacco I.P.-I.M.[b]	++++	+++	++	++	+	-	-	160
	TB8	3½ months after TB3	++++	+++	+++	+++	+	-	-	160
RABBIT 4	PB4	prebleed	++++	++++	++++	++++	+++	+	-	320
	TB4	anti-tobacco	++++	+++	+	+	-	-	-	80
	TB6	2 weeks after TB4	++++	++++	+	+	-	-	-	80

[a] I.V. = intravenous injection of tobacco protoplasts

[b] I.P.-I.M. - intraperitoneal-intramuscular injection of tobacco protoplasts (See Materials and Methods)

[c] ++++ = 75-100% agglutination; +++ = 50-75%; ++ = 25-50%; + = <25%
- = no agglutination.

than wheat or oat protoplasts. Indeed in other experiments the reverse trend occurs. Different osmotica and particularly differences in the physiological condition of the cells make it inadvisable to compare titres from different experiments.

The agglutination observed involved close adpression but rarely was there difficulty distinguishing one protoplast from the next in an aggregated clump. Mesophyll protoplasts in particular retained even their spherical shape while in large tight masses (Fig. 4-3a,b). No attempt was made to detect fusion, however it was suspected in low percentage especially in non-green protoplasts.

If the hypothesis was to be retained that antibodies were responsible for the observed agglutination then unimmunized rabbits contained high titres of such anti-bodies and these could not be further increased by normal means. Normal sera were collected from a range of animals and tested for activity (Table 4-4). All the combinations of animal and plant species tested were positive at least under certain conditions. The *Petunia*/rabbit, tulip/rabbit, and tulip/cat combinations were negative in some experiments even though the cells were FDA-viable. Preliminary attempts have failed to find the experimental factors responsible for the occasional negative results.

Two samples of cat serum (neat) were absorbed 3 times with protoplasts; the first sample with 10^8 tobacco mesophyll protoplasts per ml at 25°C for 30-60 min on each of the 3 occasions: the second similarly with *Petunia* mesophyll protoplasts. The results (Table 4-5)reveal that absorption of activity against tobacco also depletes activity against *Petunia* and vice versa. The same

Fig. 4-3. *Brassica napus* mesophyll protoplasts.(a) no agglutination with OS I (pH 7.2);(b) agglutination with the rabbit control serum, PB2 (1/8 dilution in OS I pH 7.2). x 150.

Fig. 4-4. *Nicotiana tabacum* cv. Hicks mesophyll protoplasts.
(a) agglutination with TB6 (1/8 dilution in OS I pH 7.2);
(b) agglutination with β-D-GLU (0.5 mg/ml in V47ml pH 7.2).x 150.

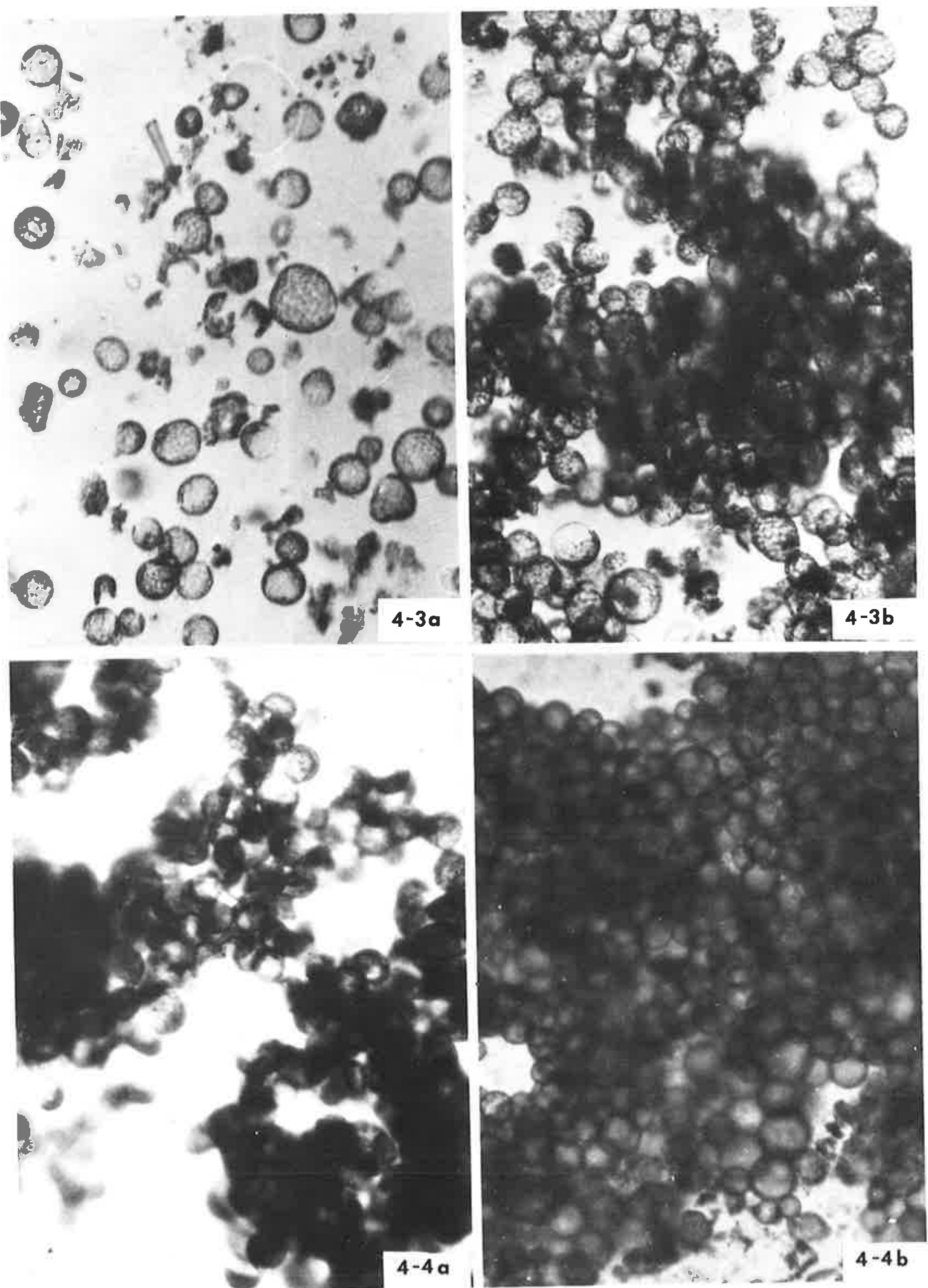
4-3a
4-3b
4-4a
4-4b

TABLE 4-4 The interaction of protoplasts with animal sera and β-D-GLU Yariv antigen

SOURCE OF PLANT PROTOPLASTS		ANIMAL SPECIES[a]									β-D-GLU
SPECIES	TISSUE	RABBIT	CAT	KANGAROO	WALLABY	POSSUM	WOMBAT	SHEEP	GOAT	GUINEA PIG	YARIV[b] ANTIGEN
Nicotiana tabacum	mesophyll	+	+	+	+	+	+	+	+	+	+
N. tomentosa	mesophyll	+	+	·	·	·	·	·	·	·	+
Petunia hybrida	mesophyll	*	+	+	+	+	+	+	+	+	+
	cell culture	*	+	·	·	·	·	·	·	·	+
	petals	*	+	·	·	·	·	·	·	·	+
Brassica napus	mesophyll	+	+	·	·	·	·	·	·	·	·
Daucus carota	cell culture	+	+	·	·	·	·	·	·	·	+
Brachycome lineariloba	hypocotyl	+	·	·	·	·	·	·	·	·	·
Vicia faba	mesophyll	+	+	·	·	·	·	·	·	·	+
Linum usitatissimum	hypocotyl	·	·	·	·	·	·	·	·	·	+
Bromus inermis	mesophyll	+	·	+	·	·	·	+	·	·	+
Triticum aestivum	mesophyll	+	+	·	·	·	·	·	·	·	+
Zea mays	hypocotyl	+	+	·	·	·	·	·	·	·	+
Tulipa	shoots	*	*	·	·	·	·	·	·	·	+
Avena sativa	mesophyll	+	·	·	·	·	·	·	·	·	+
Triticale	mesophyll	·	·	·	·	·	·	·	·	·	+
Hordeum vulgare	mesophyll	·	·	·	·	·	·	·	·	·	+
Sorghum vulgare	mesophyll	·	·	·	·	·	·	·	·	·	+

[a] 1/10 dilution sera [b] concentration 0·125 mg/ml

+ = agglutination

* = varies between + and - with experimental conditions.

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TABLE 4-5 The Effect of Absorption of Cat Serum with Tobacco and Petunia Protoplasts.

TREATMENT OF CAT SERUM	FINAL DILUTION	OSMOTICUM	MESOPHYLL PROTOPLASTS	
			Tobacco	Petunia
absorbed 3x with tobacco protoplasts	1/8	OSI(pH 7.2)	-	-
		V47ml (pH 7.2)	-	-
absorbed 3x with petunia protoplasts	1/8	OSI(pH 7.2)	-	-
		V47ml (pH 7.2)	-	-
unabsorbed cat serum	1/8	OSI(pH 7.2)	+++	+++
		V47ml (pH 7.2)	+++	+++
osmotica		OSI(pH 7.2)	-	-
		V47ml (pH 7.2)	-	-

- = no agglutination

+++ = 50-75% agglutination

population of serum molecules appears to be responsible for agglutination of the 2 species of protoplast.

Interaction of protoplasts with Yariv antigens

A reasonable alternative explanation for serum-induced agglutination could be that there is an active binding molecule in the protoplast membrane and a non-specific serum molecule with multivalent 'antigenicity' to the binding molecule. The serum molecules would act as 'bridges' between protoplasts causing them to aggregate. The work of Jermyn & Yeow (1975) and Clarke, Knox & Jermyn (1975) suggested that β-lectins may be the membrane-bound binding molecules.

Samples of Yariv antigens were obtained and tested on plant protoplasts in osmotica at pH 7.2. Agglutination with β-D-GLU was rapid and extensive and occurs with all plant species tested (Table 4-4). It was similar in appearance to serum-mediated agglutination (Fig. 4-4a,b). The control Yariv antigens, α-D-GAL and β-L-GLU did not cause any agglutination (Table 4-6). α-D-GLU could not be used as a control because of its very low water-solubility. These results indicate that the membrane lectin can discern conformational differences in the glycoside including anomeric differences (α- or β-linkage) and enantiomer differences (D or L conformation). The ability to differentiate the mirror image glycosides of β-D-GLU and β-L-GLU is striking evidence that the phenomenon is not a non-specific chemical effect. Table 4-7 shows that β-D-GLU is still active at 0.5×10^{-3} mg/ml.

Using FDA as a viability stain, neither β-D-GLU nor serum reduced the percentage of green fluorescing protoplasts when applied at the usual concentrations. It is of interest to know

TABLE 4-6 The specificity of the Yariv antigen interaction with protoplasts.

YARIV ANTIGEN	FINAL CONCENTRATION (mg/ml)	MESOPHYLL PROTOPLASTS		
		tobacco	petunia	oats
β-D-GLU	0.1	++++	+++	+++
	0.05	++++	+++	
	0.005	++++	+++	
β-L-GLU	0.1	-	-	-
	0.05	-	-	
	0.005	-	-	
α-D-GAL	0.1	-	-	-
	0.05	-	-	
	0.005	-	-	
osmoticum V47ml (7.2)		-	-	-

TABLE 4-7 Determination of the potency of β-D-GLU activity

Final concentration of β-D-GLU in V47ml (pH 7.2)(mg/ml)	PLANT PROTOPLASTS	
	tobacco	petunia
0.5	+++	+++
0.5×10^{-1}	+++	+++
0.1×10^{-1}	+++	+++
0.5×10^{-2}	+++	+++
0.1×10^{-2}	+++	+++
0.5×10^{-3}	++	+
0.5×10^{-4}	-	-
0.5×10^{-5}	-	-
0	-	-

- = no agglutination
+++ = 50-75% agglutination
++++ = 75-100% agglutination

whether the β-lectins need to be mobile within the membrane before agglutination is possible. Glutaraldehyde fixation completely inhibits green fluorescence with FDA and is thought to prevent movement of macromolecules within the membrane. Protoplasts fixed in glutaraldehyde (3% in V47m1 pH 7.2) had some tendency to spontaneously aggregate. Neither serum nor β-D-GLU was able to agglutinate fixed protoplasts beyond the spontaneous degree of agglutination. Both β-D-GLU and serum appeared to clump FDA-viable protoplasts in preference to non-viable protoplasts (fig. 4-5a,b).

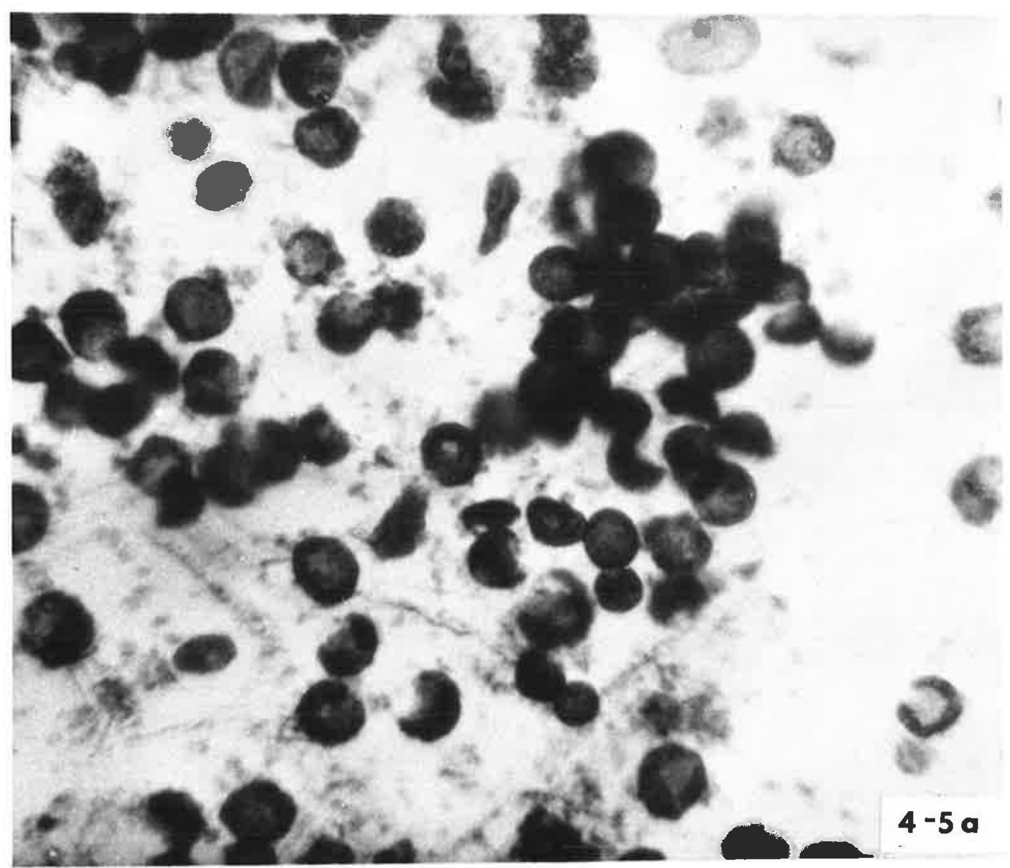
Characteristics of the serum factor(s)

A crude globulin fraction was precipitated from cat serum with 45% saturation ammonium sulphate and both the precipitate (redissolved) and supernatant were dialysed against OS 1 (pH 7.2). The activity precipitated and was retained by the cellulose dialysis membrane (Table 4-8)(Fig. 4-6a-c). In another experiment dialysis of whole serum was also shown to retain activity and increase it slightly.

Cat serum was also heated at 56, 70 or 95°C for 30 min and after cooling tested for activity (Table 4-9): 70°C for 30 min was sufficient to destroy activity.

Electrophoretic fractionation of serum is a convenient method for distinguishing certain groups of serum components. However, preliminary attempts with whole or electrophoresed serum have failed to show any observable interaction in a gel matrix with purified β-lectin. Therefore this technique has not yet helped to characterize the serum factor.

Fig. 4-5. Nicotiana tabacum cv, Hicks mesophyll protoplasts. A mixture of live and dead protoplasts agglutinating with TB5 serum (1/8 dilution in OS I pH 7.2) in the presence of 0.01% fluorescein diacetate. (a) visible light illumination showing some agglutination; (b) ultraviolet illumination of the same field as in (a). Only viable (i.e. fluorescing) protoplasts agglutinate. x 150.


4-5 a

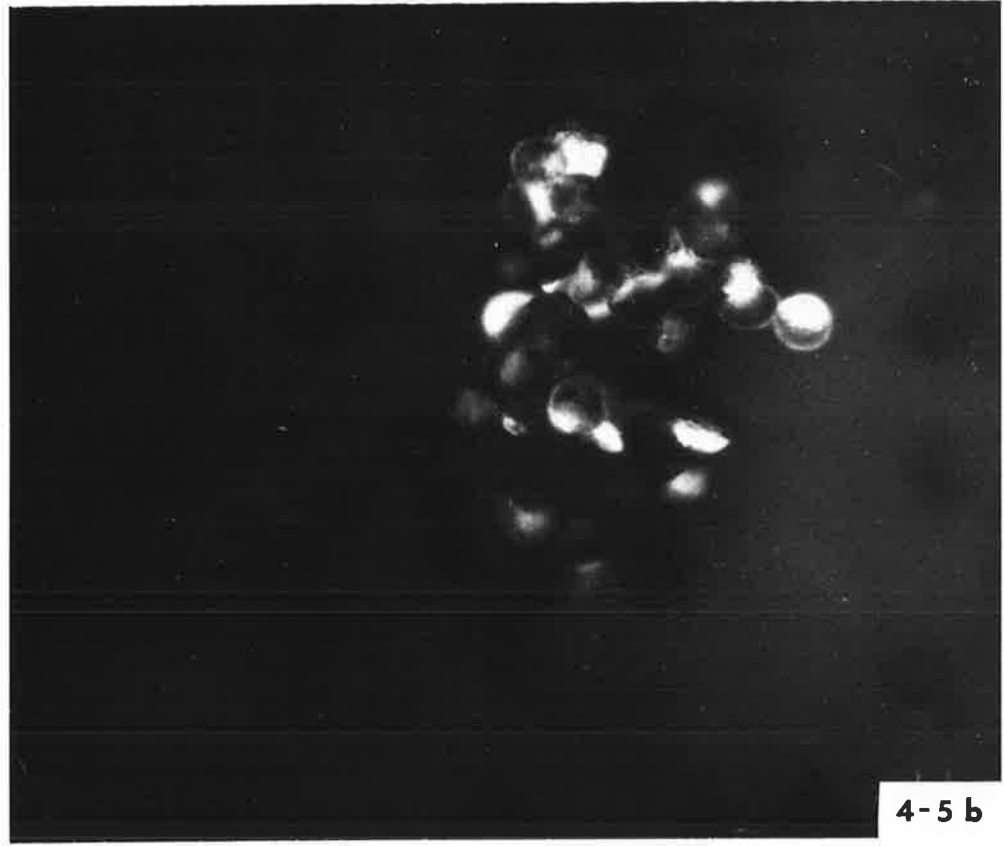

4-5 b

TABLE 4-8 The Effect of $(NH_4)_2SO_4$ precipitation of cat serum

	Final dilution	PLANT PROTOPLASTS	
		tobacco	petunia
OSI (pH 7.2)		-	-
CAT serum	1/4	++++	+++
	1/10	+++	+++
CAT serum PRECIPITATE dialysed to OSI (pH 7.2)	1/4	++++	+++
	1/10	+++	+++
CAT serum SUPERNATANT dialysed to OSI (pH 7.2)	1/4	-	-
	1/10	-	-

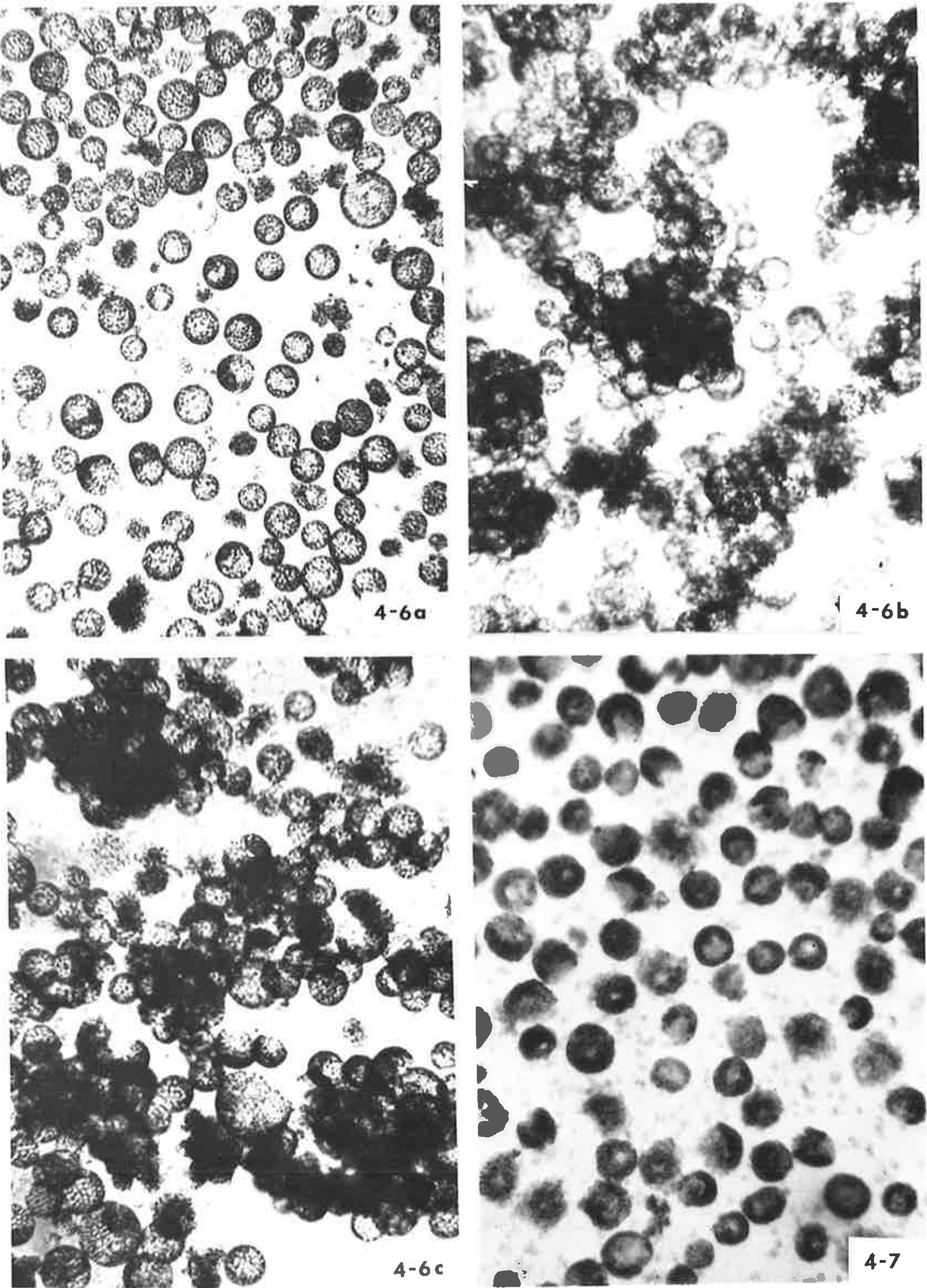
- = no agglutination
+++ = 50-75% agglutination
++++ = 75-100% agglutination

TABLE 4-9 Heat inactivation of cat serum activity

Cat serum treatment	Final dilution in OSI (7.2)	PROTOPLASTS		
		tobacco mesophyll	petunia mesophyll	petunia cell culture
56°C, 30 mins	1/10	+++	+++	++
70°C, 30 mins	1/10	-	-	-
95°C, 30 mins	1/10	-	-	-

Fig. 4-6. Nicotiana tabacum cv. Hicks mesophyll protoplasts. (a) no agglutination with the supernatant of cat serum precipitated with 45% saturation $(NH_4)_2SO_4$. The supernatant was made up to volume, dialysed against OS I (pH 7.2), and used at a final dilution of 1/10. (b) agglutination with the precipitate (as above) made to volume, dialysed against OS I (pH 7.2) and used at a final dilution of 1/10. (c) agglutination with whole cat serum (1/10 in OS I pH 7.2). x 150

Fig. 4-7. Nicotiana tabacum cv. Hicks mesophyll protoplasts lysed by fresh guinea-pig serum. x 150.


4-6a
4-6b
4-6c
4-7

Complement is the term used for a series of serum components, some of which are heat labile, which are required to lyse cells. Antigen-antibody complex formation is the usual mediating event in the activation of complement. Fresh sera which have not been heated to 56°C for 30 min (a routine procedure for inactivating serum complement) were sometimes found to cause protoplast lysis (Fig. 4-7). It is not known whether complement or some other labile factors are responsible for this lysis. Non-antibody mediated complement activation is known to occur in a number of ways; by the aggregation of γ-globulin with 63°C, 10 min heating or tetrazotized benzidine cross-linking or ultrasonification (Humphrey & White, 1970; Roitt, 1971); certain polyanions and polycations can cause complement activation (Baker _et al_ 1976; Fiedel, Rent, Myrhman & Gewurz, 1976); erthyrocytes treated with colloidal salicin or tannin are readily lysed by complement (Boyd, 1966); bacterial endotoxin lipopolysaccharide is a more effective complement activator than antigen-antibody complexes (Gewurz, 1971). It is suggested that some serum glycoprotein of the globulin fraction is aggregating on the surface of protoplasts due to β-lectin activity. This may non-specifically trigger complement reactions.

Inhibition of protoplast agglutination

No simple sugar or glycoside was found able to inhibit protoplast agglutination as induced by either serum or β-D-GLU. Those tried included 0.3 M glucose, cellobiose, lactose, methyl-α-D-glucoside, methyl-β-D-glucoside, methyl-β-D-galactoside. Heparin was also not inhibitory. Also tested were the 2 phenolic glycosides, salicin (O-hydroxymethylphenyl-β-D-glucopyranoside) and

p-β-D-glucosyloxyphenylazo-4-hydroxybenzene (a one-armed Yariv antigen synthesized by Dr. Jermyn and referred to as MONO). Salicin was an effective, though not complete, inhibitor of both serum- and β-D-GLU-induced agglutination when present at a concentration near saturation (0.143 M salicin + 0.357 M glucose, pH 7.2). MONO was not inhibitory at 0.25 mg/ml (1mM) which is near saturation. The MONO may have been inhibitory if concentrations approaching the salicin concentration had been possible.

The crude phenolic extract from the leaves of *Echeveria gibbiflora* DC. was prepared as the aqueous phase of a hot ethanol extract partitioned between chloroform and water. When this was osmotically balanced with mannitol and adjusted to pH 7.2 it completely inhibited both β-D-GLU- and serum-mediated protoplast agglutination.

Gel double-diffusion experiments

Red precipitin lines were readily observed between wells of β-D-GLU and extracts of suspension cultures of *Phaseolus vulgaris*. Even the cell-free broth of this suspension culture contained significant activity. No precipitin lines formed between the *P. vulgaris* extracts and wells of β-L-GLU or α-D-GAL. The purified β-lectin of *Phaseolus lunatus* (1 mg/ml) behaves similarly but with a slightly sharper and more intense precipitin arc.

Extracts of suspensions of tobacco and *Petunia* protoplasts, once they had been evaporatively concentrated, showed precipitin activity against β-D-GLU but not β-L-GLU or α-D-GAL. The precipitin line was grey-pink and fused with lines from *P. lunatus* purified β-lectin. After 3 weeks storage of the extracts they produced 2 distinct lines in double-diffusion gels. One was

blue-grey and one red and the latter fused with P. lunatus β-lectin precipitin. The identity and explanation of the grey line is not known; however, the experiments do demonstrate some β-lectin activity associated with mesophyll protoplasts. The fusion of precipitin lines from β-lectins of different species was observed by Jermyn & Yeow (1975) and is an indication of very similar interactions.

DISCUSSION

It is noteworthy that β-lectins are present in all protoplasts tried including wheat and oats which were 2 of the species whose β-lectins had not been detected by the gel diffusion method (Jermyn & Yeow, 1975). Protoplast agglutination is apparently more sensitive (responding to as little as 0.5×10^{-3} mg/ml β-D-GLU) however it has the disadvantage that not all plant species and tissues yield protoplasts. The overall impression is that β-lectins are probably universally distributed in plants, suggesting some important, highly conserved function.

The serum factor responsible for agglutination has not been identified. It is globulin by $(NH_4)_2SO_4$ precipitation behaviour and likely to be one of the major serum components since it exists in each of 9 mammalian species (both placental and marsupial). Clarke et al.(1975) employed the fact that both Con A and PHA bind specifically to normal serum glycoproteins. It would not be suprising that β-lectins are also able to bind to certain normal serum glycoproteins. Provided such molecules were multivalent for the 'antigenic determinant', they then could be the bridging molecules between agglutinating protoplasts.

The results presented do not necessarily suggest that no

immunological response was made to the protoplasts by the rabbits. However, the antibodies, if induced, either were not effective at agglutination (such as if only IgG antibodies develop) or their effect was completely masked by the β-lectin activity. The decrease in titre sometimes observed may be due to the injected protoplasts reacting with and temporarily reducing the concentration of the non-specific agglutination-mediating molecules.

Hartmann *et al.*(1973) found specific rabbit antiserum preparations to agglutinate soybean (*Glycine max* L.) and brome grass (*Bromus inermis* Leyss.) and *Vicia hajastana* Grossh. cultured cell protoplasts. They claim control sera caused no protoplast adhesion. These results are difficult to explain in terms of the results presented in this paper. Indeed brome grass protoplasts (though of leaf mesophyll origin) were found to agglutinate with rabbit, sheep and kangaroo prebleeds. Some doubt can be cast on the proposed antibody mediation of agglutination by the fact that anti-soybean serum was more active against *Vicia* protoplasts than soybean protoplasts. The degree of cross-reactivity was very high. They argue that antibodies are responsible because sheep anti-rabbit globulin serum caused very rapid agglutination of protoplasts already exposed to specific antisera. However this observation may be explained in one of the following ways: (a) The membrane-bound β-lectins bind certain serum glyco-proteins of the globulin fraction. The sheep anti-rabbit globulin antibodies complex with the β-lectin-bound globulins causing a rapid agglutination. Or (b) both the rabbit and sheep sera contribute glycoproteins to which the membrane-bound β-lectins bind. Results (not published) in fact show

sheep prebleeds may have a higher titre of agglutination-mediating activity than do rabbit prebleeds.

Strobel & Hess (1974) demonstrated agglutination of sugar-cane protoplasts by rabbit serum. The antisera were raised against membrane-bound proteins (those responsible for the binding of the specific toxin of the sugar-cane pathogen, *Helminthosporium sacchari*). They too claimed that control sera caused no agglutination. It is interesting that this antiserum when tritiated did not radioactively label sugar cane protoplasts significantly more than did tritiated control serum. However, the label was significantly greater for free cells and crude preparations of the binding protein. It may be that other serum glycoproteins (all of which would be tritiated) bind to the protoplast β-lectins in sufficient quantity to make the extra contribution from specific tritiated antibody negligible.

There is a growing awareness of the important biological role of recognition events based on carbohydrate specificity (Albersheim & Anderson-Prouty, 1975; Hughes, 1975; Jermyn, 1975; Knox *et al*. 1976; Winzler, 1970). Cell surface glycoproteins and glycolipids are sufficiently diverse and widespread to be likely contenders for a role as recognition codes. The carbohydrate moieties protrude from the cell surface making themselves well situated for such a role in recognition phenomena.

The classical lectins do not seem likely to be 'recognition molecules' because they are generally localized both to cotyledons and, where examined, to cytoplasm. The β-lectins are universally distributed and locate to cell membranes, walls

andinter-cellular spaces (Clarke *et al*.1975). These therefore may serve as a more appropriate model in attempts to understand cell recognition and cell-to-cell contact phenomena.

The assay systems so far used to investigate β-lectins fail to demonstrate the degree of specificity that would be required. However, when in their true physiological location, they may well have binding characteristics far more involved than in the *in vitro* assays. The Yariv antigens and salicin both have monosaccharides β-linked to phenolic groups and can be bound by β-lectins. *In vivo* the functional form of binding may require a series of sugar residues in a particular sequence and spatial conformation.

The preoccupation with hemagglutination as an assay for lectin activity has probably led to many lectins being overlooked. Yariv antigens represent a new form of assay and investigation whose further use will undoubtedly extend our understanding of the biological significance of carbohydrate-binding molecules.

Acknowledgement is made of Professor C.J. Driscoll's valuable advice and encouragement. I should also like to thank Dr. M.A. Jermyn for the gifts of Yariv antigens and purified β—lectin and Dr. G. Jackson (University of New South Wales) for his help with immunological techniques.

4-3 CONCLUSIONS FOR MODEL 1

Model 1 is untenable in its present form because animal sera were able to agglutinate protoplasts in a way apparently not dependent on the antibody specificities present. The β-lectins present on protoplast surfaces were responsible for this non-specific agglutination. It is unclear which of the serum components are acting as ligands for the β-lectins. It may be possible to use specific antisera for protoplast surfaces provided that either (i) the non-specific serum factor can be removed without removing antibody activity, or (ii) the β-lectin/serum factor interaction can be blocked (see section 8). The break-down of Model 1 nevertheless led to the identification of β-lectins on protoplasts and the further investigation of the properties of these molecules lead to other models for hybrid protoplast selection.

SECTION 5

5-1 MODEL 2 FOR HYBRID SELECTION USING β-LECTIN DIFFERENCES

Following the recognition of β-lectins on protoplast surfaces it was thought possible that different plant species may have β-lectins which respond differentially to Yariv antigens with various glycoside moieties. If such differential responses could be found then the model presented in fig 5-1 may be valid at least for particular pairs of plant species. In order to evaluate this model it was necessary to obtain and examine the effect of a wide range of Yariv antigens on a wide range of plant protoplasts. The results which follow record that endeavour.

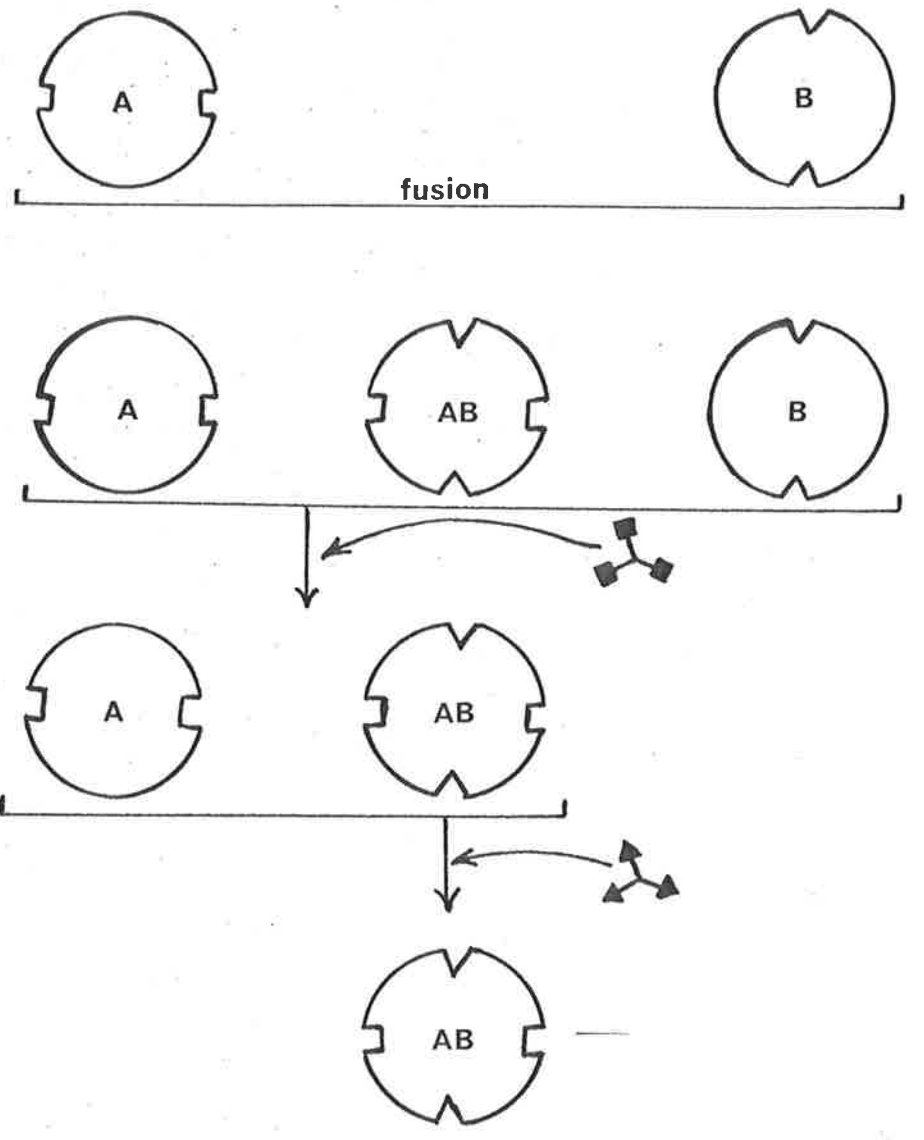


Fig 5-1

= Yariv antigen with ■ glycosyl units

= Yariv antigen with ▶ glycosyl units

5-2 PLANT PROTOPLAST AGGLUTINATION BY ARTIFICIAL CARBOHYDRATE ANTIGENS

LARKIN, P.J. J. Cell Science (in press)

SUMMARY

The existence of β-lectins on protoplast surfaces is confirmed by the agglutination of protoplasts by those Yariv antigens that have sugar specificities which also interact with isolated β-lectins. Agglutination by β-maltosyl but not by β-D-mannosyl Yariv antigens is used to identify some of the structural features required of the ligand for β-lectin binding. Inhibition of agglutination by phenolic glycosides and the effect of protoplast fixation are also investigated.

INTRODUCTION

In a preceeding report (Larkin, 1977, Section 4-2) both normal mammalian sera and β-glucosyl Yariv antigen (a triple-liganded synthetic β-D-glucosyl antigen) interacted with protoplasts causing agglutination. A new class of plant lectin called all-β lectin (first described by Jermyn and Yeow, 1975) was inferred to be present on protoplast surfaces and to be responsible for both types of agglutination (Larkin, 1977, Section 4-2).

In this paper the above observations are extended to include a larger range of Yariv antigens. The specificities required to induce agglutination are shown to conform to the specificities required for all-β lectin binding (Jermyn and Yeow,

1975) and for arabinogalactan-protein binding (Anderson, Clarke, Jermyn, Knox and Stone, 1977). Yariv antigen/protoplast interactions and agglutination inhibition studies are used to draw inferences concerning the structural characteristics required of a receptor molecule before β-lectin can bind. The effect of glutaraldehyde fixation on protoplast agglutination is also examined.

MATERIALS AND METHODS

Protoplast isolation

Plants were grown and protoplasts isolated as described previously (Larkin, 1976, 1977, Sections 3, 4-2). The osmoticum used throughout these experiments was either OSI or V47ml, which have mannitol or NaCl, respectively, as the major osmotic stabilizer (Larkin, 1977, Section 4-2). Viability was determined using the fluorescein diacetate technique (Larkin, 1976, Section 3).

Yariv antigens

Yariv antigens are red-coloured phenylazo-glycosides with three glycoside moieties on each molecule (Fig.5-2). These were a generous gift of Dr. M.A. Jermyn (C.S.I.R.O. Division of Protein Chemistry). Yariv, Rapport and Graf (1962) and Jermyn and Yeow (1975) give details of their synthesis. The following abbreviations will be used to refer to the various Yariv antigens: $(\beta\text{-D-GLU})_3$ for the Yariv antigen with three β-D-glucosyl units; similarly $(\beta\text{-D-GAL})_3$ has β-D-galactosyl units, $(\beta\text{-MAL})_3$ has β-maltoside units, $(\beta\text{-D-XYL})_3$ has β-D-xyloside units, $(\beta\text{-LAC})_3$ has β-lactosyl units, $(\beta\text{-CELL})_3$ has β-cellobiosyl units, (α-L-RHA) has α-L-rhamnosyl units, $(\beta\text{-D-MAN})_3$ has β-D-mannosyl units,

Fig 5-2 1,3,5-Tri-(p-β-D-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene

or

(β-D-GLU)$_3$

(α-D-NAcGAL)$_3$ has α-D-N-acetylgalactosaminyl units; (α-NAcGLU)$_2$ (β-D-GLU)$_1$ for the Yariv antigen with a β-D-glucosyl unit on one arm and α-N-acetyl-glucosaminyl units on the other two arms; (β-D-GLU)$_2$(NO_2)$_1$ has two β-D-glucosyl units and the third arm has only a -NO_2 unit in place of a glycoside; (β-D-GLU) is ρ-β-D-glucosyloxyphenylazo-4-hydroxylbenzene. Other designations follow this pattern.

Agglutination tests

The test for agglutination involved incubation in drops of test solution plus protoplast suspension in plastic Petri-dishes for 20-40min. with occasional rocking at 22-27°C. The protoplast suspensions and test solutions were always at pH 7.2 and the osmolality about 0.5 osmols. OSI was the most frequent osmoticum. Particular care was taken in the inhibition experiments to maintain a constant osmolality even as the concentration of test glycoside varied. This was achieved by altering the concentration of mannitol. The test drops were examined microscopically and rated for agglutination on a subjective scale.

RESULTS AND DISCUSSION

Agglutination by Yariv antigens

Table 5-1 summarizes the results of agglutination in the presence of different Yariv antigens. Figs. 5-3, 5-4, 5-5, 5-6 exemplify the nature of the cell interactions observed. In some preparations the leaf protoplasts of Nicotiana tomentosa, N.alata and N.miersii had unusually large diameters (100μm). When this was the case (β-D-XYL)$_3$ was unable to mediate agglutination. In other preparations (even from the same plants

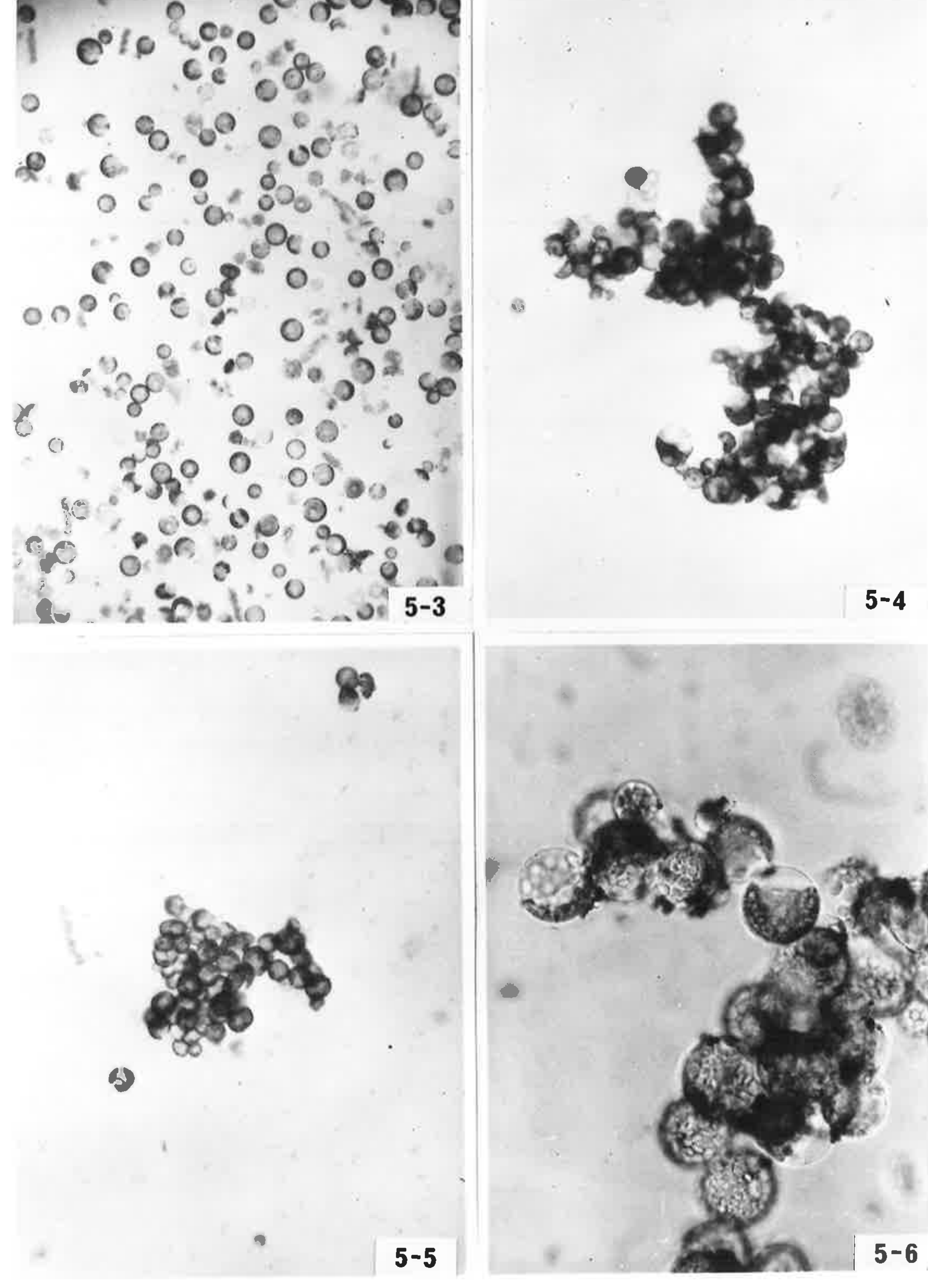
Table 5-1. *The interaction of protoplasts with Yariv antigens*

Yariv antigen	Final conc., mg/ml	Protoplast species: *Nicotiana tobacum* leaf	*Nicotiana tomentosa* leaf	*Nicotiana miersii* leaf	*Nicotiana alata* leaf	*Petunia hybrida* leaf	*Vicia faba* leaf	*Triticum aestivum* leaf	*Avena sativa* leaf	*Bromus inermis* leaf	*Daucus carota* culture	Other species† leaf
(β-D-GLU)$_3$	0·05	+	+	+	+	+	+	+	+	+	+	+
(β-L-GLU)$_3$	0·05	−	.	.	.	−	.	.	−	.	.	.
(β-D-GAL)$_3$	0·05	+	+	+	+	+	+	+	+	+	.	.
(α-D-GAL)$_3$	0·05	−	−	.	.	−	.	.	−	−	.	.
(β-MAL)$_3$	0·05	+	+	.	.	+	+	+	.	.	.	.
(β-D-XYL)$_3$	0·05	+	*	*	*	+	+	.	.	.	.	.
(β-L-XYL)$_3$	0·05	−	.	.	.	−	.	.	.	.	.	.
(β-LAC)$_3$	0·05	+	+	.	.	+	+	.	.	.	.	.
(β-D-MAN)$_3$	0·05	−	−	−	.	−	−	.	−	−	.	.
(α-D-NAcGAL)$_3$	0·05	−	−	.	.	−	−	.	.	.	.	.
(α-L-RHA)$_3$	0·05	−	.	.	.	−	.	.	.	.	.	.
(β-CELL)$_3$	0·05	+	+	.	.	+	+	+	.	.	.	.
(β-D-GLU)$_1$ (α-NAcGLU)$_2$	0·05	−	.	.	.	−	.	.	−	−	.	.
(β-D-GLU)$_1$ (α-D-GAL)$_2$	0·05	−	.	.	.	−	.	−	−	−	.	.
(β-D-GLU)$_2$ (α-D-GAL)$_1$	0·05	+	+	.	.	.	+	.	.	.	.	.
(β-D-GLU)$_2$ (NO_2)$_1$	0·05	+	.	.	.	.	+	.	.	.	.	.
(β-D-GLU)$_1$ (NO_2)$_2$	0·05	−	.	.	.	.	−	.	.	.	.	.
(β-D-GLU)$_1$	0·25	−	−	.	.	−	.	.	.	.	.	.

† *Hordeum vulgare*, *Sorghum vulgare*, *Triticale*, *Zea mays*, *Linum usitatissimum*, *Tulipa* sp., *Brachycome* sp., *Brassica napus*.
* When protoplasts $< 50\ \mu m$ diameter, + = 25–100% agglutination; when protoplasts $> 100\ \mu m$ diameter, − = no agglutination.

Figs. 5-3, 5-4, 5-5, 5-6 *Triticum aestivum* mesophyll protoplasts.

Fig. 5-3	Control in OSI (pH 7.2)	x 150
Fig. 5-4	Agglutination in 0.05mg/ml $(\beta\text{-CELL})_3$.	x 150
Fig. 5-5	Agglutination in 0.05mg/ml$(\beta\text{-MAL})_3$.	x 150
Fig. 5-6	Agglutination in 0.05mg/ml$(\beta\text{-D-GAL})_3$.	x 600


5-3
5-4
5-5
5-6

2 or 3 weeks later) the diameters were similar to those of other species (50μm) and agglutination with (β-D-XYL)$_3$ was normal.

It is interesting that the large diameter protoplasts could still be agglutinated by the other β-D-glycosyl Yariv antigens. It may be that the highly distended state causes a conformational restraint on the membrane-located β-lectin which interferes with (β-D-XYL)$_3$ binding. The osmolality and pH were unchanged between the experiments in which the average diameters varied. The interactions shown in Table 5-1 are consistent with the hypothesis in Larkin (1977, Section 4-2) that β-lectins exist in protoplast surfaces. In particular, agglutination occurred only in the presence of the Yariv antigens which Jermyn & Yeow (1975) found interacted with β-lectins and which Anderson _et al._ (1977) found interacted with arabinogalactan-proteins. These are namely (β-D-GLU)$_3$, (β-D-GAL)$_3$, (β-LAC)$_3$, (β- CELL)$_3$, (β-MAL)$_3$ and (β-D-XYL)$_3$.

A general conclusion is that activity is dependent on a β-D-glycosyl moiety. Yariv antigens with β-L, α-D- or α-L-glycosyl moieties do not interact with the β-lectins and hence do not mediate protoplast agglutination. An exception to this generalisation is (β-D-MAN)$_3$ which did not cause agglutination. Dr. Jermyn (pers. comm.) has also remarked on the inactivity of (β-D-MAN)$_3$ with isolated β-lectins.

Fig. 5-7 shows a comparison of the structures of β-D-mannoside, β-D-glucoside, β-D-galactoside and β-D-xyloside. The configuration at C_4 varies amongst the active structures and therefore may be inferred to be unimportant. The existence

β-D-mannoside

β-D-glucoside

β-D-galactoside

β-D-xyloside

Fig 5-7

of a 6th carbon is obviously not necessary since (β-D-XYL)$_3$ is bound. The configuration at C_3 is the same in all 4 structures and therefore there is no clue as to the importance of this carbon to β-lectin binding. The configuration of C_2 is the same in all the acceptable structures and opposite in β-D-mannoside. This infers that C_2 configuration and C_1 configuration (conferring α- and β- linkage) are important in β- lectin binding.

Another inference arising from Table 5-1 is that in the case of Yariv antigens with disaccharide moieties only the residue closest to the phenolic structure need be in acceptable form. (β-LAC)$_3$ has 4-O-β-D-galactopyranosyl-β-D-glucopyranoside moieties, (β-CELL)$_3$ has 4-O-β-D-glucopyranosyl-β-D-glucopyranoside and both of these are agglutinating. This is to be expected since both residues of these disaccharides are in acceptable configuration. However (β-MAL)$_3$ with 4-O-α-D-glucopyranosyl-β-D-glucopyranoside moieties is also agglutinating. Only the residue closest to the phenolic is in β-linkage and yet it is apparently just as effective a ligand for β-lectins as (β- CELL)$_3$ and (β-LAC)$_3$.

It was further demonstrated by the hybrid Yariv antigens (where the three arms are not identical) that agglutination was only possible where at least two of the arms carried an acceptable glycoside. This was to be expected since two protoplasts will adhere when the surface lectins are binding to the one molecule. The failure of (β-D-GLU)$_1$(α-NAcGLU)$_2$ and (β-D-GLU)$_1$(α-D-GAL)$_2$ and (β-D-GLU)$_1$(NO_2)$_2$ to cause protoplast agglutination may indicate that these molecules are not aggregating spontaneously

into complexes which would be multi-liganded with suitable glycosides.

Glutaraldehyde fixation and protoplast agglutination

It was reported earlier (Larkin, 1977, Section 4-2) that fixation prevents serum - or (β-D-GLU)$_3$ - mediated agglutination. This was re-investigated and extended. Bromus inermis leaf protoplasts were used to test the effect of glutaraldehyde fixation. A sample of protoplasts was suspended in 3% glutaraldehyde in V47ml (pH 7.2) for 2hr before thoroughly washing in osmoticum. This sample had no FDA-fluorescing protoplasts as compared to 90% fluorescing protoplasts in an unfixed control suspension. There was a low level of spontaneous aggregation of the fixed protoplasts which were not responsive to significant further agglutination by either Yariv antigens or animal sera (Table 5-2). Glimelius, Wallin and Eriksson (1974) also observed some spontaneous aggregation of glutaraldehyde fixed carrot protoplasts and a subsequent reduction of Con A-induced agglutinability.

Animal cell fixation has often been reported to reduce plant lectin agglutination (Nicolson, 1974; Rutishauser and Sachs, 1975) though not always (Marquardt and Gordon, 1975). This effect may be the result of a loss of lectin receptor mobility in the fixed animal cells resulting in a lack of clustering of sites which in turn may be necessary for strong adhesion of cells at contact points. This principle may apply to protoplasts in that membrane-located β-lectins may also need to cluster at contact points to facilitate agglutination. Alternatively fixation may simply reduce the deformability of

TABLE 5-2. Glutaraldehyde fixation and protoplast agglutination

Test treatment	*Bromus inermis* leaf protoplasts	
	glutaraldehyde fixed 0% FDA viable	untreated 90% FDA viable
V47ml (pH 7.2)	+	—
0.05mg/ml $(\beta\text{-D-GLU})_3$	+	++++
0.05mg/ml $(\beta\text{-D-GAL})_3$	++	++++
0.05mg/ml $(\alpha\text{-D-GAL})_3$	+	—
0.05mg/ml $(\beta\text{-D-MAN})_3$	+	—
1/10 normal RABBIT serum	++	++++
1/10 normal KANGAROO serum	++	++++
1/6 normal SHEEP serum	++	++++

++++ = 75-100% agglutination
++ = 25-50%
+ = 25%
- = negligible

protoplasts and thus reduce cell-to-cell surface contact which is essential for aggregation. This has also been suggested as an explanation for fixed animal cell behaviour (Nicolson, 1974; Gibson et al., 1975; Van Blitterswijik et al., 1976).

Inhibition of agglutination by glycosides

A number of simple sugars and glycosides were tested for the ability to inhibit agglutination mediated by serum or β-D-glycosyl Yariv antigens (Table 5-3). The substances were tested at varying concentrations and in each case the sum of the concentrations of mannitol and test-substance was 500mM and the pH was maintained at 7.2. Even at 500mM the non-phenolic glycosides showed no detectable inhibition. In addition to those of Table 5-3, lactose and cellobiose were not inhibitory up to 150mM.

Some of the glycosides were much less soluble and this restraint prevented thiodigalactoside and ρ-nitrophenyl-β-D-glucoside being tested beyond 25mM and 12.5mM respectively. They were not inhibitory up to these concentrations. However 50% reduction in agglutination was caused by three phenolic-β-D-glycosides, namely salicin (140mM), ρ-nitrophenyl-β-D-glucoside (10mM), and indoxyl-β-D-glucoside (25mM). The structures of these glycosides are shown in Fig. 5-8. These glycosides did not reduce the viability of the protoplasts.

The Yariv antigens themselves are of course phenolic glycosides. It is interesting that the structural requirements for binding by β-lectins not only involve the sugar moiety but also the nature of the phenolic moiety to which it is linked.

TABLE 5-3 INHIBITION OF PROTOPLAST AGGLUTINATION BY SIMPLE GLYCOSIDES

glycoside	minimum conc. causing 50% reduction in agglutination (mM)	agglutinating agent employed	protoplasts employed
glucose	> 500	(0.05 mg/ml (β-D-GLU)$_3$ (1/8 serum	(carrot culture (tobacco leaf
methyl-β-D-glucoside	> 500	(0.05 mg/ml (β-D-GLU)$_3$ (1/8 serum	(carrot culture (tobacco leaf
methyl-β-D-galactoside	> 500	(0.05 mg/ml (β-D-GLU)$_3$ (1/8 serum	(carrot culture (tobacco leaf
methyl-α-D-glucoside	> 500	(0.05 mg/ml (β-D-GLU)$_3$ (1/8 serum	(carrot culture (tobacco leaf
O-hydroxymethylphenyl-β-D-glucoside (salicin)	140	(0.05 mg/ml (β-D-GLU)$_3$ (1/40 serum	(*N. tabacum* leaf (*N. tomentosa* leaf
thiodigalactoside	> 25	(0.05 and 0.005 mg/ml (β-D-GLU)$_3$ (0.005 mg/ml (β-D-GAL)$_3$ (1/20 serum	tobacco leaf
p-nitrophenyl-α-D-glucoside	> 12.5	(0.05 and 0.005 mg/ml (β-D-GLU)$_3$ (0.005 mg/ml (β-D-GAL)$_3$ (1/20 serum)	tobacco leaf
p-nitrophenyl-β-D-glucoside	10	(0.05 and 0.005 mg/ml (β-D-GLU)$_3$ (0.005 mg/ml (β-D-GAL)$_3$ (1/20 serum	tobacco leaf
indoxyl-β-D-glucoside	25	(0.05 and 0.005 mg/ml (β-D-GLU)$_3$ (0.005 mg/ml (β-D-GAL)$_3$ (1/20 serum	tobacco leaf

Salicin
(*o*-hydroxylmethylphenyl-β-D-glucoside)

p-nitrophenyl-β-D-glucoside

indoxyl-β-D-glucoside

Fig 5-8

Too little data is as yet available to be able to define the necessary form of the phenolic structure.

CONCLUSIONS

Protoplast agglutination in the presence of a range of Yariv antigens has helped to define more fully the peculiar specificities of β-lectins located on protoplast surfaces. The preferred ligand for β-lectin-binding is a phenolic glycoside with the sugar residue closest to the phenolic being in D-conformation and in β-linkage. Carbon 1 configuration defines β-linkage and it is important that in addition the hydroxyl of Carbon 2 be in trans-orientation relative to the linkage of Carbon 1.

There have been a number of recent reports of lectins isolated from hypocotyl cell walls and the membranes of plasmalemma, Golgi apparatus, endoplasmic reticulum and mitochondria (Kauss and Glaser, 1974; Bowles and Kauss, 1975, 1976; and Kauss and Bowles, 1976; Bowles, Schnarrenberger and Kauss, 1976). After isolation these lectins were assayed by their ability to agglutinate trypsinized rabbit erythrocytes and their specificities investigated by inhibition studies. That these can be grouped with classical lectins is suggested by their ability to agglutinate erythrocytes strongly, and by the fact that they are inhibited by non-phenolic glycosides. Indeed the lectin from the inner mitochondrial membrane of Ricinus communis is likely to be identical to the classical castor bean lectin (Bowles et al,1976).

It is not yet clear whether the lectins described by these authors as being extractable from isolated plasmalemma exist

together with β-lectins on protoplast surfaces. A number of classical lectins have been shown to be able to interact with Yariv antigens of appropriate sugar specificities (Larkin, 1977, Section 6-2). Since the membrane-extracted classical lectins were almost invariably inhibited by α-D-galactosides it was conceivable, if they also existed on protoplasts, that (α-D-GAL)$_3$ may cause agglutination. Such agglutination was not observed with (α-D-GAL)$_3$ nor any other α-glycosyl Yariv antigen (Table 5-1). It may be that the lectins described by these authors are bound to the membranes by their own binding activity. This possibility was suggested by the fact that the addition of lactose to the extractant greatly improved the yield of activity from R. communis mitochondrial membranes since lactose is also a strong inhibitor of the lectin (Bowles et al. 1976). If this is the case and these lectins are bound to the protoplast plasmalemma by their own binding sites then obviously, unlike the β-lectins, they would not be able to react with exogenous substrates such as Yariv antigens. Alternatively the lectins described by Bowles and Kauss (1975, 1976) and Kauss and Bowles (1976) may be lost from the protoplasts during the enzyme incubation.

It remains to be seen whether lectin activities are artefacts of assay or whether they are meaningful representations of the true roles of these molecules. The β-lectins are apparently universally distributed in plants and located at cell surfaces. It is enticing to speculate that they may be part of the recognition systems for pollen compatibility, pathogenicity and symbiosis.

The physiological role may be assumed to involve

interactions with phenolic glycosides many of which exist in plant tissues (Harborne, Mabry and Mabry, 1975; Harborne, 1967; Ribéreau-Gayon, 1972). D-conformation sugars are almost invariably in β-linkage in plant phenolics. Initial investigations with crude phenolic extractions from various plant tissues have shown that some are indeed able to inhibit β-lectin directed protoplast agglutination.

5-3 CONCLUSIONS FOR MODEL 2

No satisfactory difference could be found between the agglutination response of the protoplasts of any two species to any Yariv antigen. It can be concluded that the β-lectins of different species are remarkably similar in their activity characteristics. This resemblance is also reflected in their chemical composition (Jermyn, pers. comm.). The possibility can not be ruled out that substrates other than Yariv antigens may evoke differential responses. However in the absence of such alternatives to Yariv antigens model 2 will be deemed invalid as a mechanism for selecting protoplast fusion hybrids.

SECTION 6

6-1 MODEL 3 FOR HYBRID SELECTION USING LECTINS

Classical plant lectins (phytohemagglutinins) have been used to sort mixed animal cell types into subpopulations. Such selective assortment is based on the differences in surface glycosidic moieties and the glycoside-specificities of the exogenous lectins. The most widely studied examples are of certain lectins able to differentiate erythrocytes of different ABO and MN blood types (Toms and Western, 1971; Lis and Sharon, 1973; Nicolson, 1974; Liener, 1976). Mouse thymocytes could be separated from mouse erythrocytes by selective adhesion to nylon fibres conjugated with a low density of Con A lectin (Lis and Sharon, 1977). T lymphocytes were separated from B lymphocytes by preferential agglutination with soybean lectin (Reisner *et al*, 1976) or by selective retention on Sepharose beads conjugated with *Helix* lectin. Kinzel *et al* (1976, 1977) used large Sepharose 2B beads conjugated with *Lens culinaris* lectin to bind HeLa and SV3T3 malignant cells. These cells could be released by incubating with an appropriate sugar hapten and were still viable. Other lectins have also displayed a high selectivity for transformed (malignant) cells relative to non-transformed cells (Lis and Sharon, 1973).

Model 3 (fig 6-1) assumes that protoplasts, at least of some different species, will have differences in their surface glycosides to such an extent that lectins will be able to discriminate between them. Fusion hybrids should be agglutinated by both specific lectins. In order to test this model a series of lectins were obtained and tested for agglutination of protoplasts of a range of plant species.

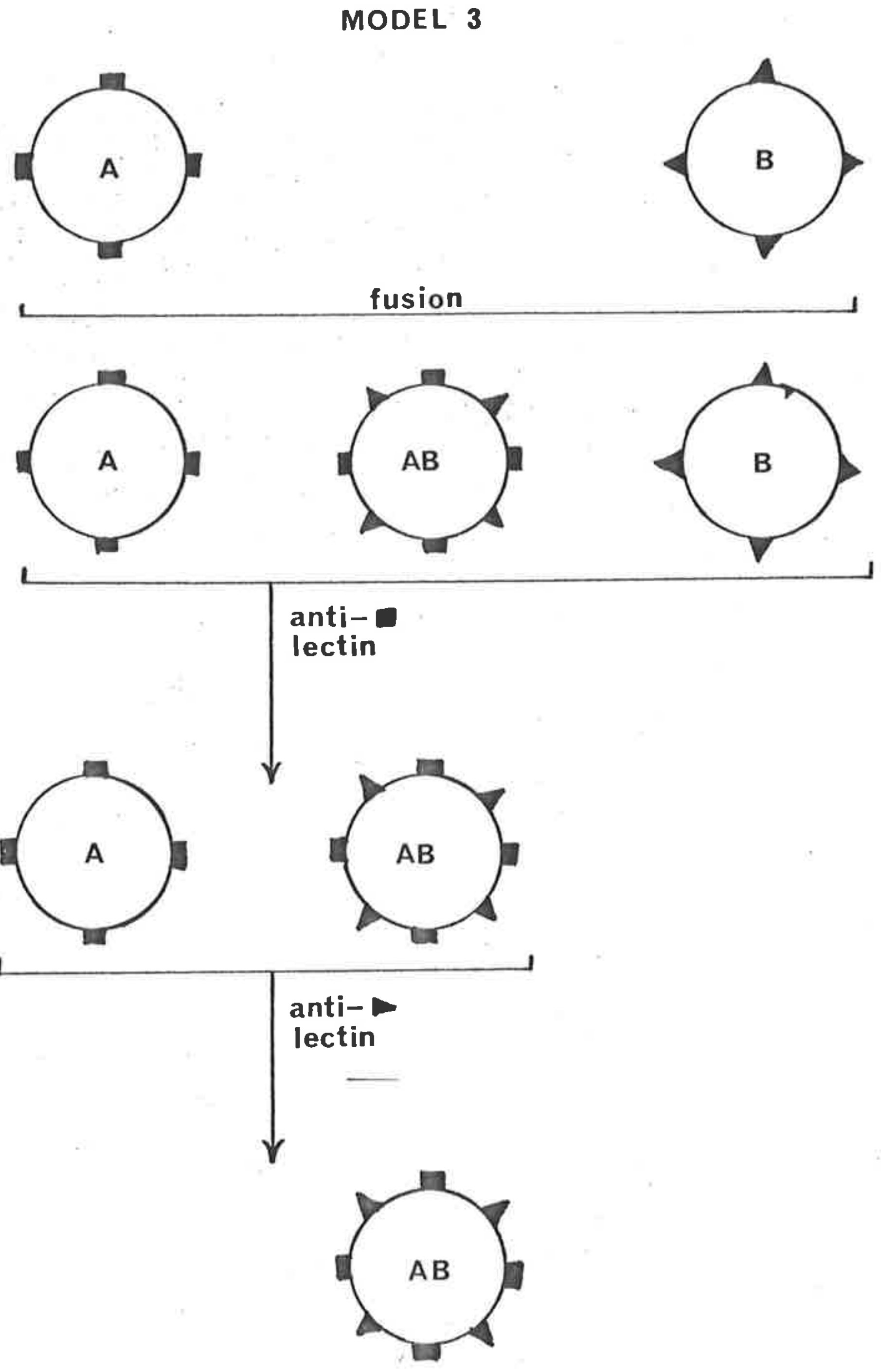


Fig 6-1

6-2 PLANT PROTOPLAST AGGLUTINATION BY CLASSICAL LECTINS

LARKIN, P.J. Plant Physiology (submitted)

ABSTRACT

Concanavalin A, soybean lectin, castor bean lectin and peanut lectin were able to agglutinate protoplasts prepared from a wide variety of plant species. The seven other lectins tried were unable to agglutinate those protoplasts tested. Protoplasts prepared from 11 species were used. The specificity profiles of 10 of the lectin preparations were constructed using gel-diffusion interactions with a series of Yariv artificial carbohydrate antigens and to a lesser extent by agglutination inhibition studies. No simple correlation existed between the specificities of the lectins and their ability to agglutinate protoplasts.

INTRODUCTION

Interest in plant lectins began with the study of their inter-actions with animal systems. It is ironical that investigation of the effects of plant lectins on plant cell systems is much more recent. *Phaseolus vulgaris* lectin (PHA) had a mitogenic effect on tomato callus cells (Levenko and Kiforak, 1975) and barley and pea root tip cells (Singh *et al*. 1975). Soybean lectin (SBA) was mitogenic for soybean callus cells (Howard *et al*. 1976). PHA and Concanavalin A (Con A) both stimulated *Lilium* pollen germination (Southworth, 1975). Golynskaya *et al*. (1976)

found hemagglutinins in Primula stigmata which also effected pollen tube growth. Pretreatment of Gladiolus stigmata with Con A prevented compatible pollen tubes from penetrating (Knox et al. 1976). There is also considerable evidence that the specificity of host-symbiont inter-actions is determined by lectin binding (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974; Dazzo and Hubbell, 1975; Wolpert and Albersheim, 1976; DeVay and Adler, 1976; Planqué and Kijne, 1977).

Gamborg and Miller (1973) suggested that lectins may be useful for protoplast aggregation which in turn would facilitate fusion. Since then Glimelius, Wallin and Erikson (1974) reported the agglutination of carrot culture cell protoplasts by Con A. They observed inhibition of the agglutination by methyl-α-D-glucoside and a great reduction of agglutination using glutaraldehyde-fixed protoplasts. Low temperature ($4^{o}C$) limited agglutination and higher cell densities favoured higher agglutination percentages. Burgess and Linstead (1976) confirmed the ability of Con A to interact with protoplast surfaces. Using Con A conjugated with colloidal gold they demonstrated both specific binding (inhibited by 0.1M α-methyl mannoside) to plasmalemma of Vitis vinifera culture protoplasts and tobacco leaf protoplasts, and also non-specific binding to amorphous material near the plasmalemma. The ability of plant lectins to interact with plasmalemma may be of significance to their physiological role. Ruesink (1975) and Williamson, Fowke, Constabel and Gamborg (1975 and 1976) have also used labelled Con A as a protoplast membrane marker.

There has been no reported examination of any of the many other classical plant lectins for their effects on protoplasts. This report details some observations with Con A as well as 10 other

lectins on some of 11 species of protoplast. The specificites of the lectins were examined with double gel diffusion using Yariv antigens (artifical carbohydrate antigens of defined glycosides) and to a lesser extent by inhibition studies.

The lectins examined here are designated "classical" by contrast to carbohydrate-binding molecules which are not inhibited by simple sugars such as the β-lectins described by Jermyn and Yeow (1975) which are found on protoplast surfaces (Larkin, 1977, Section 4-2).

MATERIALS AND METHODS

Protoplast isolation

Protoplasts were prepared from the various species with the procedures, enzymes and osmotica as detailed in Larkin (1977, Section 4-2). The most usual enzyme incubation osmoticum was OSI and the cleaned protoplasts were always finally suspended in OSI (pH 7.2) before use. Viability was determined by the fluorescein diacetate technique (Larkin, 1976, Section 3) and only viable preparations were used.

Lectins

Soybean lectin (SBA) and wheat germ lectin (WGA) were obtained from Pharmacia Fine Chemicals (Sweden) where they had been purified by affinity chromatography. Concanavalin A (Con A), *Ulex europaeus* (Anti-H) lectin, *Dolichos biflorus* lectin and *Phaseolus vulgaris* cv. red kidney lectin (PHA-M) were obtained from Calbiochem. PHA-M was prepared as a 40-80% saturation $(NH_4)_2SO_4$ cut of the initial seed extract. Caster bean Type 11 lectin (CBA-II) and *Bandeiraea simplicifolia* lectin were obtained from Sigma Chemical Company and both were reported as consisting

of one major elecrophoretic protein band with trace bands.

Peanuts (200g, uncooked) were milled, defatted with petroleum ether, and air-dried. A suspension in 0.9% NaCl was stirred for 3hr. and the insoluble matter removed twice by centrifugation. $(NH_4)_2SO_4$ was added to 60% saturation and the centrifuged pellet redissolved in 110ml 0.9% NaCl and dialysed against 0.9% NaCl for 24 hr. with one change of saline. Lotan, Skutelsky, Danon and Sharon (1975) used this preparative procedure plus affinity chromatography to isolate anti-T lectin from peanuts. One sample was also dialysed against OSI(pH 7.2).

Vicia faba beans (40g) were milled, defatted with petroleum ether, and dried in air. This was suspended and stirred for 1hr. in 200ml 0.9% NaCl. The centrifuged sediment was discarded. The supernatant was fractionated by $(NH_4)_2SO_4$ precipitation into the 0-48%, 48-65%, 65-80% and 80-100% saturation precipitates. These centrifuged pellets were redissolved in 40,20, 20 and 25ml 0.9% NaCl respectively. Each fraction was dialysed against 0.9% NaCl for 66 hr. with one change of saline. On the basis of preliminary lectin specificity determinations, by gel diffusion with Yariv antigens, it was decided to use only the 65-80 and 80-100% fractions for further experimentation. The other two fractions appeared to contain no types of lectin activities not represented by the latter fractions but also had some α-D-galactosidase activity (indicated by the grey precipitin formed with α-D-galactosyl Yariv antigen). A sample of each of the 65-80 and 80-100% fractions was dialysed against OSI(pH 7.2).

All lectin solutions used with protoplasts were made up in OSI(pH 7.2) osmoticum to avoid protoplast damage.

Agglutination tests

Agglutination was assessed by incubation in drops of the test lectin solutions with protoplasts in plastic Petri dishes for 20-40min. with occasional rocking at 22-27°C. Both lectins and protoplasts were in OSI(pH 7.2) osmoticum. Particular care was taken in the inhibition experiments to ensure the osmolality was kept constant. As the concentration of test glycoside or sugar was varied, the concentration of mannitol (the major component of OSI) was varied to maintain a total concentration of sugars of 0.5M. Agglutination was assessed on a subjective scale using a light microscope .

Yariv antigens

Yariv antigens are artificial carbohydrate antigens having three defined glycosidic moieties attached to a red phenylazo molecule (Fig.6-2). Synthesis details are recorded in Variv, Rapport and Graf (1962) and Jermyn and Yeow (1975). They were a gift from Dr. M.A. Jermyn who synthesized them. The following abbreviations will be used to refer to the various Yariv antigens: $(\beta\text{-D-GLU})_3$= the Yariv antigen with three β-D-glucosyl residues; similarly $(\beta\text{-D-MAN})_3$ has three β-D-mannosyl residues; $(\beta\text{-MAL})_3$ has three β-maltosyl residues; $(\beta\text{-LAC})_3$ has three β-lactosyl residues; $(\beta\text{-CELL})_3$ has three β-cellobiosyl residues; $(\alpha\text{-L-FUC})_3$ has three α-L-fucosyl residues; $(\alpha\text{-D-GAL})_3$ has three α-D-galactosyl residues; $(\alpha\text{-NAcGLU})_2(\beta\text{-D-GLU})_1$ has two α-N-acetyglucosaminyl and one β-D-glucosyl residue. Other Yariv antigen designations conform to this pattern.

Gel-diffusion plates

The gel used contained 1% NaCl, 0.03M Sorensen's phosphate

Fig 6-2 1,3,5-Tri-(p-β-D-glucosyloxyphenylazo)-2,4,6-trihydroxybenzene

or

(β-D-GLU)$_3$

buffer (pH 7), 0.02% NaN_3 and 1% purified agar. Wells were approx. 5mm apart and after cutting were sealed with melted agar. Lectins were placed in the wells at a concentration of 1mg/ml (except Bandeiraea simplicifolia lectin at 0.1mg/ml). Yariv antigens were used at 0.1mg/ml. The plates were incubated for 24-48hr. at 27^{0}C and examined for red precipitin lines. Each lectin/Yariv antigen combination was tested at least in triplicate.

RESULTS

Protoplast agglutination

Table 6-1 records the agglutinability of 10 species of plant protoplasts by lectins from 10 sources. As well as the results of Table 1, Dolichos biflorus lectin (0.5mg/ml) failed to agglutinate Zea mays and Vicia faba leaf protoplasts and Daucus carota culture protoplasts. Only Con A, SBA, CBA-II and peanut extract agglutinated protoplasts. Figs. 6-3, 6-4, 6-5, 6-6 illustrate the agglutination reaction. The only lectin to show any specificity with respect to different protoplasts was CBA-II. This lectin agglutinated all the species attempted except some preparations of Nicotiana tomentosa protoplasts. In some preparations these had unusually large diameters (>100µm) and when this was so, CBA-II could not agglutinate them. Con A, SBA and peanut extract could agglutinate these large protoplasts. Other N.tomentosa preparations (even from the same plant two weeks later) had more normal diameters (<50µm) and these could be agglutinated by CBA-II. It is not known why the diameters varied so dramatically in different preparations.

Table 6-2 shows the potency of SBA for agglutinating N. tabacum leaf protoplasts. The endpoint of agglutination was 0.05mg/ml.

TABLE **6-1** *The interaction of plant protoplasts with classical lectins*

lectin	final conc. (mg/ml)	protoplasts									
		Nicotiana tabacum leaf	*Nicotiana tomentosa* leaf	*Nicotiana miersii* leaf	*Nicotiana alata* leaf	*Petunia hybrida* leaf	*Vicia faba* leaf	*Daucus carota* culture	*Triticum aestivum* leaf	*Avena sativa* leaf	*Bromus inermis* leaf
SBA	0.5	++++ ‡	++++	n.a.	n.a.	++++	++++	+++	++++	++++	++++
Con A	0.5	++++	+++	n.a.	n.a.	++++	+++	+++	+++	++++	n.a.
WGA	0.5	-	-	-	n.a.	-	-	-	n.a.	-	n.a.
Ulex lectin	0.5	-	-	-	n.a.	-	-	n.a.	n.a.	-	n.a.
PHA-M	0.5	-	-	n.a.	n.a.	-	-	n.a.	n.a.	-	n.a.
CBA-II	0.5	++++	*	n.a.	+++	++++	+++	+++	++++	n.a.	n.a.
Bandeiraea lectin	0.05	-	-	-	n.a.	-	-	n.a.	n.a.	n.a.	n.a.
Vicia extract 1†	½ dilution	-	-	-	n.a.	-	-	n.a.	n.a.	n.a.	n.a.
Vicia extract 2†	½ dilution	-	-	-	n.a.	-	-	n.a.	n.a.	n.a.	n.a.
Arachis extract	½ dilution	++++	++++	++++	n.a.	++++	+++	n.a.	+++	n.a.	n.a.

† *Vicia faba* seed extracts 1 and 2 are the 65-80% and 80-100% $(NH_4)_2SO_4$ saturation fractions respectively.

‡ ++++ = 75-100% agglutination
+++ = 50-75%
- = negligible
n.a. = not ascertained

* = +++ when protoplasts<50μm diam.
- " " >100μm "

Figs. 6-3, 6-4, 6-5, 6-6 *Triticum aestivum* mesophyll protoplasts.

Fig. 6-3	Control in OSI (pH 7.2)	x 150
Fig. 6-4	Agglutination in 0.5mg/ml SBA	x 200
Fig. 6-5	Agglutination in 0.5mg/ml CBA-II	x 150
Fig. 6-6	Agglutination in 1/2 dilution peanut extract	x 600

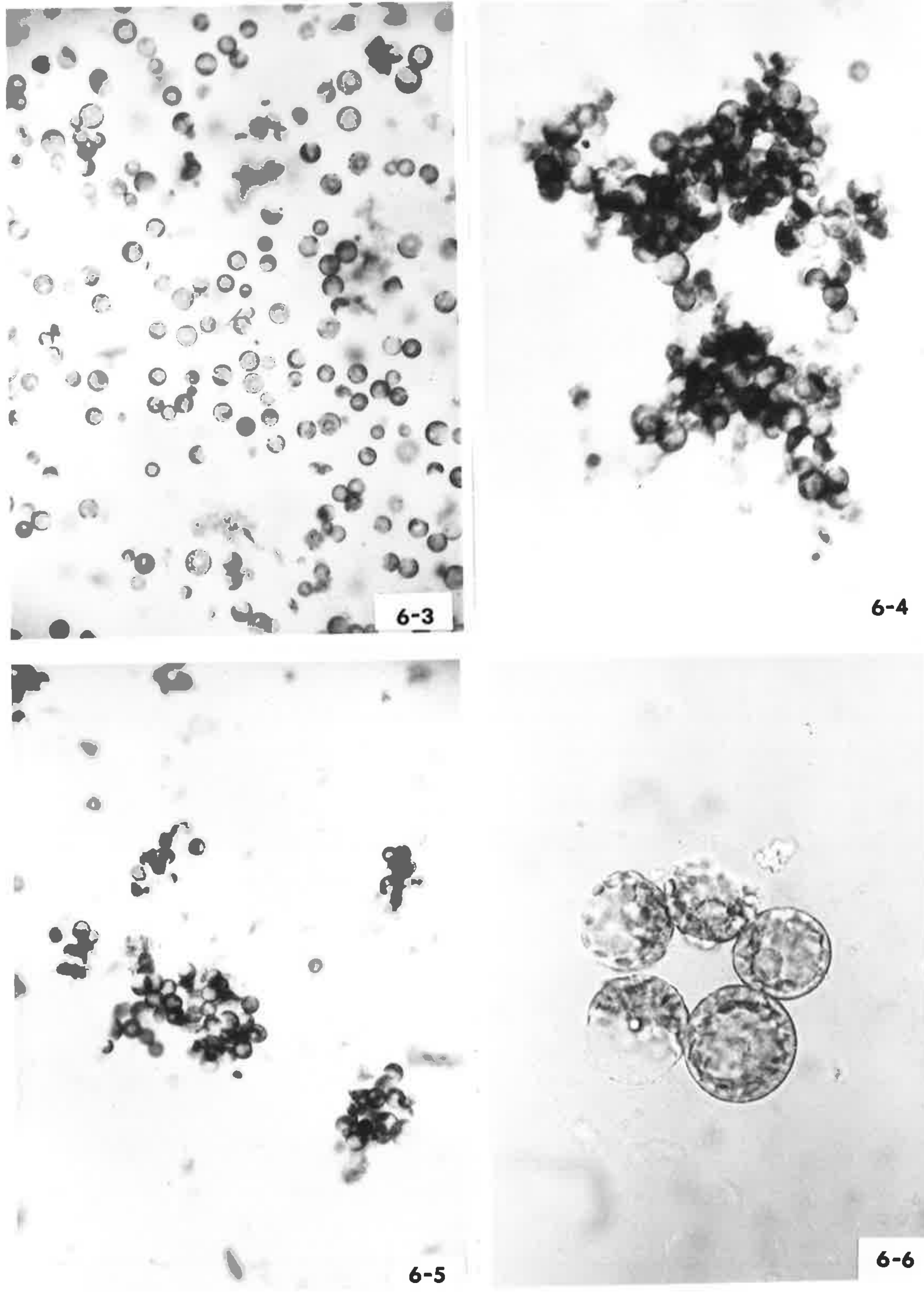

6-3
6-4
6-5
6-6

TABLE 6-2 Potency of SBA-induced agglutination

SBA concentration (mg/ml)	0.5	0.1	0.05	0.01	0.005
N.tabacum leaf protoplasts ($5x10^6$/ml)	++++*	+++	+	-	-

*++++ = 75-100% agglutination;
+++ = 50-75%;
\+ = 25%;
\- = negligible agglutination

The effect of fixation on agglutinability was also investigated. A sample of Bromus inermis leaf protoplasts was fixed in 3% glutaraldehyde for 2hr. These fixed protoplasts had 0% viability as judged by the fluorescein diacetate (FDA) method (Larkin, 1976, Section 3). A low degree of spontaneous aggregation (approx. 10%) existed in the fixed suspension and 0.5 mg/ml SBA could not significantly increase this level of agglutination. The unfixed control suspension (approx. 90% FDA viable) agglutinated in 0.5mg/ml SBA to 75-100% aggregation.

Specificities of the lectins

The specificities of the various lectins, with the exception of Dolichos biflorus lectin, were determined by double gel-diffusion studies using the triple-liganded artificial carbohydrate antigens called Yariv antigens. Gleeson and Jermyn (1977) used this technique to confirm the characteristic specificities of Con A. Red precipitin lines between wells of Yariv antigen and lectin were taken to indicate an ability of the

lectin to bind to the carbohydrate structure. The results of the gel-diffusion studies to determine the specificities of the lectins are shown in Table 6-3. The scale of reaction intensity was subjective and merely gives an indication of the ranking of reactions.

Ulex europaeus and Bandeiraea simplicifolia lectins both failed to react with any of the Yariv antigens in this study. SBA formed only very diffuse lines with $(\alpha\text{- D-GAL})_3$ and $(\alpha\text{-D-GAL})_2(\beta\text{-D-GLU})_1$ and on some plates even these were difficult to detect. Nevertheless the same solutions of SBA used in these experiments was able to strongly agglutinate protoplasts.

The precipitin profiles of all the remaining lectins were different although there was much overlap. The two V.faba extract fractions, the peanut extract and PHA-M had profiles which suggested the possibility that a number of lectins of differing specificities were present in the preparations.

Inhibition studies were used as a second method to investigate lectin specificities. Table 6-4 shows the effect of methyl-α-D-glucoside and methyl-β-D-galactoside on tobacco protoplast agglutination by the effective lectins. Methyl-α-D-glucoside was strongly inhibitory to Con A-induced agglutination and methyl-β-D-galactoside was strongly inhibitory to SBA-induced agglutination. Methyl -β-D-galactoside was to a lesser degree also inhibitory to peanut-, Con A- and CBA-II- induced agglutination.

DISCUSSION

In this report only 4 out of 11 classical lectins were

TABLE **6-3** *Gel-diffusion interactions between lectins and Yariv antigens*

		YARIV ANTIGENS											
		(β-D-GLU)$_3$	(β-L-GLU)$_3$	(α-NAcGLU)$_2$ (β-D-GLU)$_1$	(β-CELL)$_3$	(β-MAL)$_3$	(β-D-MAN)$_3$	(β-LAC)$_3$	(β-D-GAL)$_3$	(α-D-GAL)$_2$ (β-D-GLU)$_1$	(α-D-GAL)$_3$	(α-L-FUC)$_3$	(β-D-XYL)$_3$
AGGLUTINATING LECTINS	Con A	++*	-	++	-	-	+++	-	-	-	-	-	-
	SBA	-	-	-	-	-	-	-	-	±	±	-	-
	CBA-II	-	-	-	-	-	-	-	+	-	-	-	-
	Peanut (0-60% cut)	++	-	-	++	+	-	+	++	+	+	-	-
NON-AGGLUTINATING LECTINS	WGA	-	+++	+++	-	++	-	-	-	-	-	-	-
	Ulex lectin	-	-	-	-	-	-	-	-	-	-	-	-
	Bandeiraea lectin	-	-	-	-	-	-	-	-	-	-	-	-
	PHA-M	+	-	++	±	-	-	-	+	-	-	-	-
	Vicia (65-80% cut)	+	-	++	+	+	-	+	++	-	-	-	-
	Vicia (80-100% cut)	++	-	-	+	+	-	+	++	-	-	-	++

* - = no precipitation reaction
± = very faint precipitation reaction
+, ++, +++ = order of increasing intensity of precipitation reaction

TABLE 6-4 Glycoside inhibition of tobacco leaf protoplast agglutination.

GLYCOSIDE	Conc.† (mM)	LECTINS			
		Con A (0.5mg/ml)	Peanut lectin (½ dilution)	CBA-II (0.5mg/ml)	SBA (0.5mg/ml)
methyl-α-D-glucoside	0	+++*	++++	++++	++++
	10	-	++++	++++	++++
	30	-	++++	++++	+++
	60	-	++++	++++	+++
	100	-	++++	++++	+++
methyl-β-D-galactoside	0	+++	++++	++++	++++
	10	+++	++	++++	-
	30	++	++	+++	-
	60	+	++	++	-
	100	+	+	-	-

† The mannitol concentration of the OSI osmoticum was varied to compensate for the concentration of the added test glycoside. The protoplast density was approximately 5 x 10^6/ml.

*++++ = 75-100% agglutination; +++ = 50-75%; ++ = 25-50%; + = 25% - = no agglutination.

found to cause protoplast agglutination. These 4 lectins could agglutinate all the species of protoplast with which they were tested. The failure of CBA-II to agglutinate the large diameter *N. tomentosa* protoplasts may be a result of the CBA-II receptor concentration falling below the critical value required for agglutination. When the protoplasts are smaller and less distended the receptor concentration (relative to cell surface area) will be higher. The receptors for Con A, SBA and peanut lectin(s), may be sufficiently concentrated to be unaffected by the increase in diameter.

Generally the plant lectins failed to differentiate between protoplasts of different species using agglutination as the only criterion. This may be because their ability to bind to plasmalemma is independent of their physiological role or because their role is not species-specific. It remains to be shown which, if any, of the various effects of lectins on plant systems correctly reflect their true functions. The effects observed may all be artefactual.

It is interesting that *Ulex europaeus* lectin and *Bandeiraea simplicifolia* lectin did not react with any of these Yariv antigens, even those with glycosyl units that the literature records as being suitable. For example data in Sharon and Lis (1972), Nicolson (1974) and Liener (1976) suggests $(\alpha\text{-L-FUC})_3$ or $(\alpha\text{-NAcGLU})_2(\beta\text{-D-GLU})_1$ might have reacted with *Ulex europaeus* lectin. Liener (1976), Lönngren and Goldstein (1976) and Ross *et al.* (1976) suggested $(\alpha\text{-D-GAL})_3$ and $(\beta\text{-D-GAL})_3$ might have reacted with *Bandeiraea simplicifolia* lectin.

The specificities found for the lectins studied are broadly

in agreement with those reported by other authors (Lis and Sharon, 1973; Nicolson, 1974; Liener, 1976). There are however some exceptions. Peanut extract lectin(s) reacted with β-D-glucoside, β-maltoside, β-cellobioside, β-D-xyloside as well as β-D-galactoside, α-D-galactoside and β-lactoside. The latter three specificities were expected from Pereira et al. (1976) and Lotan et al.(1975) but not the former four specificities. These authors were able to purify a galactose-specific lectin by affinity chromatography from an extract produced as in this report. This suggests that the mixed specificities of the peanut extract are due to the presence of different lectins rather than a simple lectin of broad specificity.

The strong reaction of WGA with β-L-glucoside but not β-D-glucoside is interesting and apparently not reported before. PHA-M showed an affinity for α-N-acetylglucosamine and β-D-glucoside as well as β-D-galactoside. The latter specificity was in agreement with Lis and Sharon (1973) and Nicolson (1974) but not the former two specificities. Perera and Frumin (1966) observed D-glucose and maltose inhibition of V.faba extract lectin. In addition to these specificities reactions were also observed with β-lactosyl, β-cellobiosyl and β-D-galactosyl Yariv antigens. The 65-80% fraction also reacted with $(\alpha\text{-NAcGLU})_2(\beta\text{-D-GLU})_1$ and the 80-100% fraction reacted with $(\beta\text{-D-XYL})_3$.

The use of Yariv antigens in gel-diffusion plates to probe lectin specificities has the advantage of being a direct observation of the interactions. Inhibition studies are indirect in that one is observing the ability of a sugar to disturb the activity. One major advantage of inhibition methods is that they more easily give

quantitative data allowing the ranking of the intensity of binding to various sugar moieties.

A survey of the specificity profiles of the lectins did not reveal any correlations between specificities and agglutinating activity. It may be that the protoplasts did have suitable binding sites for the non-agglutinating lectins but that those sites were insufficiently mobile or concentrated for these lectins. Another lectin, using the same receptors, may be sufficiently large or multimeric to compensate for the low receptor concentration or mobility. Alternatively the ideal substrates may be considerably more complex than inhibition studies and Yariv antigen-binding suggest.

ACKNOWLEDGEMENTS

I wish to acknowledge Professor C.J. Driscoll's continuing interest and advice. Dr. M.A. Jermyn is also thanked for the gifts of Yariv antigens.

6-3 CONCLUSIONS FOR MODEL 3

The lectins examined either failed to agglutinate any of the protoplast species or else agglutinated all species without distinction. It is possible that other lectins may be found able to differentiate between certain species. It is also possible that significant surface differences could be unmasked by pretreating one or both parental protoplast suspensions with enzymes. For example trysin, papain, bromelin, ficin and neuraminidase have been used to pretreat some animal cells to enhance their agglutinability (Pereira *et al*, 1976; Toms and Western, 1971; Lis and Sharon, 1977; Gordon *et al*, 1972). Nevertheless the results presented here imply that the glycosidic composition of protoplast surfaces does not vary sufficiently to allow selection on this basis. Hence model 3 was deemed invalid.

SECTION 7

7-1 MODEL 4 FOR HYBRID SELECTION

The results of section 6 showed that different species of protoplast did not differ sufficiently in the glycosidic composition of their surfaces to allow cell sorting on that basis. However it may be possible to treat parental protoplasts in such a way as to create glycosidic surface differences. In section 5-2 it was shown that hybrid Yariv antigens, on which only one of the 3 arms of the molecule has a suitable β-D-glycoside, do not agglutinate protoplasts. Model 4 (fig 7-1) presupposes that the failure of such hybrid Yariv antigens to agglutinate protoplasts was simply because they were only univalent for a suitable glycoside. In addition this model presupposes that these hybrid Yariv antigens can and do bind to the surface β-lectins although they are unable to bridge between two opposing protoplasts.

Given that these presuppositions are valid, model 4 proposes the use of 2 such hybrid Yariv antigens to coat the parental protoplast plasmalemma differentially with α-linked glycosides. Classical lectins could then be used in a 2-step procedure to select the fusion hybrids which will have both types of molecular coating. The lectins chosen may have to be from those which do not agglutinate untreated protoplasts as well as having appropriate sugar specificities. The greatest attraction of this model is its universal applicability. If two appropriate Yariv antigens and two appropriate lectins can be found they could be used without modification with any two parental genotypes.

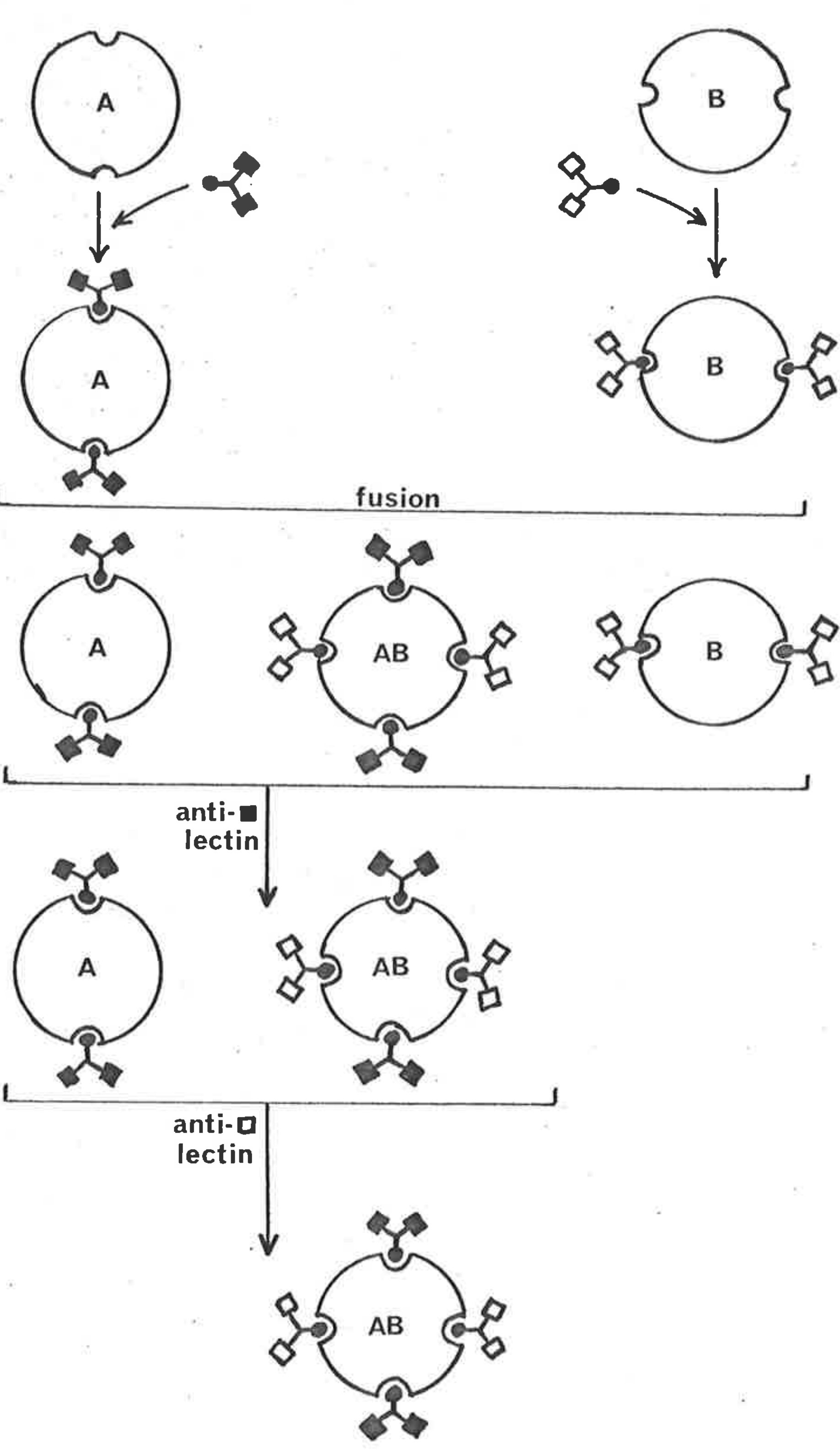


Fig 7-1

7-2 EVALUATION AND CONCLUSIONS FOR MODEL 4

According to table 6-3 the wheat germ lectin (WGA) would appear to be an appropriate lectin for use in model 4 in conjunction with $(\alpha\text{-NAcGLU})_2$ $(\beta\text{-D-GLU})_1$. In gel-diffusion plates WGA did not react with $(\beta\text{-D-GLU})_3$ but interacted strongly with $(\alpha\text{-NAcGLU})_2(\beta\text{-D-GLU})_1$. Therefore WGA can be assumed to bind to α-N-acetyl-glucosaminyl moieties. In addition to this WGA does not agglutinate untreated protoplasts. Protoplasts were prepared as described previously (section 4-2) and preincubated with hybrid Yariv antigens for 60-90 min in the concentrations indicated (tables 7-1 and 7-2). After washing once or twice by centrifugation the protoplasts were tested for agglutinability with lectins according to the methods of section 6-2.

The results of a number of experiments conducted at different times with independent protoplast preparations are summarized in table 7-1. The pretreatment of one sample with $(\alpha\text{-D-GAL})_2(\beta\text{-D-GLU})_1$ and a second sample with $(\alpha\text{-NAcGLU})_2$ $(\beta\text{-D-GLU})_1$ did not enable any of the lectins, including WGA, to differentiate between the two samples of protoplast. Moreover the failure of WGA to agglutinate protoplasts previously saturated with $(\alpha\text{-NAcGLU})_2$ $(\beta\text{-D-GLU})_1$ suggested that the hybrid Yariv antigen was not binding to the surface β-lectins with sufficient stability to survive the washing of the protoplasts. This conclusion was further supported by the observation that protoplasts pretreated with hybrid Yariv antigens were no less able to agglutinate in $(\beta\text{-D-GLU})_3$, $(\beta\text{-D-GAL})_3$, rabbit serum or sheep serum (table 7-2). In other words the hybrid Yariv antigens were not "blocking" the β-lectin sites so that they were no longer available for binding to suitable agglutination agents.

TABLE 7-1 Lectin agglutination and hybrid Yariv antigen-pretreated protoplasts

	Leaf Protoplast species	*Avena sativa*		*Petunia hybrida*		*Nicotiana tabacum*	
	Pretreatment agent (0.1 or 0.5 mg/ml)	$(\alpha\text{-D-GAL})_2$ $(\beta\text{-D-GLU})_1$	$(\alpha\text{-NAcGLU})_2$ $(\beta\text{-D-GLU})_1$	$(\alpha\text{-D-GAL})_2$ $(\beta\text{-D-GLU})_1$	$(\alpha\text{-NAcGLU})_2$ $(\beta\text{-D-GLU})_1$	$(\alpha\text{-D-GAL})_2$ $(\beta\text{-D-GLU})_1$	$(\alpha\text{-NAcGLU})_2$ $(\beta\text{-D-GLU})_1$
LECTINS	SBA (mg/ml) 0.5	++++	++++	+++	+++	++++	++++
	SBA (mg/ml) 0.1					++	+
	SBA (mg/ml) 0.05					+	+
	SBA (mg/ml) 0.01					–	–
	SBA (mg/ml) 0.005					–	–
	Con A (0.5 mg/ml)	++++	++++	+++	++++		
	Peanut lectin (½ dilution)					++++	++++
	CBA-II (0.5 mg/ml)			++++	++++		
	Vicia (65–80% cut) (½ dilution)					–	–
	Vicia (80–100% cut) (½ dilution)					–	–
	Bandeiraea lectin (0.05 mg/ml)					–	–

PHA-M (0.5 mg/ml)	–	–	–	–	–	–
Ulex lectin (0.05 mg/ml)	–	–	–	–	–	–
WGA (0.5 mg/ml)	–	–	–	–	–	–

++++ = 75–100% agglutination
+++ = 50–75%
++ = 25–50%
\+ = 25%
– = negligible agglutination

TABLE 7-2 Hybrid Yariv antigens do not block β-lectin sites

Date		3/8/76		7/10/76			9/2/77		
Leaf protoplast species		*Nicotiana tabacum*		*Bromus inermis*			*Nicotiana tabacum*		
Pretreatment agent (mg/ml)		$(\alpha\text{-D-GAL})_2$ $(\beta\text{-D-GLU})_1$ 0.5	V47 ml (pH 7.2)	$(\alpha\text{-D-GAL})_2$ $(\beta\text{-D-GLU})_1$ 0.5	$(\alpha\text{-D-GAL})_2$ $(\beta\text{-D-GLU})_1$ 0.1	V47 ml (pH7.2)	$(\alpha\text{-D-GAL})_2$ $(\beta\text{-D-GLU})_1$ 0.1	$(\alpha\text{-NAcGLU})_2$ $(\beta\text{-D-GLU})_1$ 0.1	OSI (pH7.2)
$(\beta\text{-D-GLU})_3$	0.05mg/ml.	+++	+++	++++	++++	++++	+++	+++	+++
	0.005mg/ml						++	+	++
$(\beta\text{-D-GAL})_3$	0.05mg/ml						++++	++++	++++
Rabbit serum (PB4)	1/20	++++	++++	+++	+++	+++			
	1/40	++++	++++						
	1/80	+++	++						
	1/160	+	+						
	1/320	-	-						
	1/640	-	-						
Sheep serum	1/8						+++	+++	+++

++++ = 75-100% agglutination +++ = 50-75% ++ = 25-50% + = 25% - = negligible

Hybrid Yariv antigens cannot be used to coat protoplasts with specific glycosides. The presuppositions of model 4 are invalid and therefore the model is invalid.

SECTION 8

8-1 PLANT PHENOLIC AND TERPENOID GLYCOSIDES

8-1-1 Introduction

Plant phenolics are very widely distributed and exist *in vivo* as esters or more usually as glycosides (Ribéreau-Gayon, 1968). The most widely distributed phenolic constituents of plant tissues include phenolic acids, coumarins, flavonols, flavones, chalcones, aurones and anthocyanins. Flavonoid glycosides and other plant glycosides are almost invariably in β-linkage (Harborne, 1967; Harborne *et al*, 1975; Miller, 1973). This makes them attractive possibilities for natural substrates for β-lectins. The probability of this seems to be enhanced by the analogy between the structures of Yariv antigens and the simple phenolic glycosides which inhibit protoplast agglutination (see section 5-2).

The flavonoids have been extensively studied but there has been no unifying role given to explain their abundance. Some have postulated a role in resistance to fungal and bacterial pathogens and cited examples where particular metabolites (phytoalexins) increase in concentration as the tissue is invaded (Wood, 1967; McClure, 1975; Kuć, 1976; Debnam and Smith, 1976; Ebel *et al*, 1976; Sakuma *et al*, 1976; Langcake and Pryce, 1977). Other roles that have been suggested for certain flavonoids include: antioxidants, enzyme regulators, pigmentation for insect attraction, protection against U.V. by absorption, growth regulator activity, synergism with other growth regulators, participants in electron transport processes, morphogenic regulation, toxicity to some insects, antiviral activity (McClure, 1975; Kefeli and Kutacek, 1977). Phloridzin (2'-β-glucoside of phloretic acid) inhibited wheat

coleoptile and tomato roots but promoted apple shoots (Jones, 1976). Rye seedlings produce 2-β-D-glucosyloxy-4-hydroxy-1, 4-benzoazin-3-one which is toxic to *Fusarium nivale* and barley leaves contain an even more complicated phenolic glucoside which is toxic to *Helminthosporium sativum* (Overeem, 1976).

There has been some recent interest in the physiological activities of steryl glycosides and free sterols including growth regulation, auxin synergism, morphogenic regulation and effects on membrane permeability (Greenwald, 1971, 1975; Geuns, 1974, 1977; Kimura *et al*, 1975; Wojciechowski *et al*, 1976; Tietz *et al*, 1977). The diterpene glucoside, fusicoccin, seems to have growth regulator activity on plant cell cultures and protoplasts (Strobel, 1976; Sparapano, 1976; Cimmo and Johnson, 1977; Rubinstein and Conrad, 1977; Rollo *et al*, 1977). Some potatoes have a resistance to the pathogen, *Phytophthora infestans*, which responds to infection by hypersensitive rapid cell death. The hypersensitivity is caused by the rapid accumulation of many terpenoids. It appears that this accumulation is triggered by a terpenoid glucoside in the pathogen (Kuć *et al*, 1976). Oat plants produce a triterpenoid saponin triglycoside which is antifungal and the toxicity is destroyed by removal of the glycosides (Overeem, 1976).

The predominating occurrence of glycosides *in vivo* suggests the importance of the glycosidic linkage. In some instances the activity examined can also be attributed to the aglycone. Popovici and Reznik (1976) found the flavonoid aglycones, kaempferol, quercetin and myricetin, reduced cytoplasmic streaming in oat root hairs at 5×10^{-5}M but stimulated it at

5×10^{-6}M. Certain glycosides of these flavonoids stimulated cyclosis at both concentrations. Plant phenolics are located in vacuoles and cell walls (Kefeli and Kutacek, 1977). Because of this cell surface location for many phenolics it is significant that there are some indications that exogenous flavonoid effects are initiated at cell membranes. Flavonoids reduce blood cell aggregation by some effect on cell surfaces (McClure, 1975) and can inhibit Ca^{++} uptake and membrane-bound Ca^{++}-dependent ATPase (Fewtrell and Gomperts, 1977). The remainder of section 8-1 describes phenolic extracts of plant tissues which interacted with protoplast surfaces in a way which suggests binding to β-lectins. These interactions are the basis for two further models for hybrid protoplast selection. One of these models was tested experimentally.

8-1-2 Materials and Methods

a) Plant tissue extractions. Extraction procedures were chosen to include flavonoid glycosides. Tissues used were: the leaves of *Echeveria gibbiflora* DC., *Ceratonia siliqua* DC. (carob tree), *Hypericum perforatum* L., *Pteridium aquilinum* L. (bracken), and *Pseudotsuga menziersii* (Mirb.) Franco (Douglas fir), flowers of *Zephyranthes citrinus* Herb. and *Hisbiscus* L., *Allium cepa* L. (onion) bulbs, and seeds of *Brassica napus* L. and *Phaseolus vulgaris* L. cv. red kidney. Tissues were ground and homogenised in hot aqueous ethanol (80-95% depending on the moisture content of the tissues) and the extract filtered through muslin and/or Whatman paper (No.1). Chlorophylls, carotenoids, sterols, fats and waxes were removed by petroleum ether and/or chloroform washes (the endpoint being when no further green colouration washed out) (Ribéreau-Gayon, 1968; Markham, 1975).

In the case of the seeds and the Douglas fir leaves the extracts were then mixed with an equal volume of *n*-propanol. Saturation with NaCl caused the *n*-propanol to separate into an upper phase which should contain the flavonoid glycosides (Ribéreau-Gayon,1968). The *n*-propanol fractions were then evaporated, redissolved in ethanol and the NaCl crystals filtered off. All extracts were taken up and/or dialysed in the OSI (pH 7.2) osmoticum (OSVIII for Douglas fir, table 8-3). The pH was readjusted to 7.2 and the extracts were filtered through a 0.45µm membrane filter (Millipore). After periods of storage in the freezer some precipitates formed. These were removed by further filtration and the pH readjusted to 7.2.

b) Preliminary purification of *Ceratonia* extract. A 175g

sample of Ceratonia siliqua leaves were homogenised in hot ethanol (final volume 650 ml) and filtered through muslin and two layers of Whatman No. 1. paper. The volume was reduced to 300ml on a rotary evaporator under reduced pressure at 65°C. Three washes with chloroform left an aqueous phase which was divided into 3 fractions of 100 ml each.

Fraction A was reduced to a brown residue in the rotary evaporator and taken up in 30 ml of OS VIII (pH 7.2)(see table 8-3 later). It was then filtered through a 0.45μm Millipore membrane and readjusted to pH 7.2.

Fraction B was loaded onto a 45 x 100 mm column of Dowex 1X10-400 anion exchange resin and 400 ml of water washed through. The column was then eluted with 400 ml of 0.1 M NaOH and 500 ml collected. This was neutralized with HCl and evaporated to dryness. The residue was dissolved in ethanol and the NaCl crystals filtered off. It was re-evaporated and taken up in 30 ml OS VIII (pH 7.2), membrane- filtered and readjusted to pH 7.2.

Fraction C was mixed with 9g powdered activated carbon and 9g granulated activated carbon. The carbon was collected on filter paper in a Buchner funnel and washed with 400 ml hot methanol, 500 ml hot water and finally with 200 ml 7% crystalline phenol in water. This final eluent was collected and reduced to near dryness on a rotary evaporator (reduced pressure and 65°C). The remaining phenol was removed by 3 diethyl ether washes. The residue was taken up in 30 ml OS VIII (pH 7.2) and then membrane-filtered and readjusted to pH 7.2.

All three extract fractions were stored frozen.

They were membrane-filtered and readjusted to pH 7.2 after 4 days

and after 5 weeks storage. The procedure for *Ceratonia* B should partially purify polyhydroxy aromatic acids and glycosides (Jermyn, pers. comm.). The procedure for *Ceratonia* C should give a reasonably pure preparation of flavonoid glycosides (Mabry *et al*, 1970).

Ceratonia C was further fractionated by loading onto a 45 x 150 mm column of Amberlite IR-120(8% cross linked; 20-50 mesh) cation exchange resin. This was washed with 500 ml water. *Ceratonia* C-a was collected by elution with 115ml 20% 2-propanol. *Ceratonia* C-b was then collected by elution with 90% ethanol. These fractions were reduced to near dryness with a rotary evaporator (reduced pressure, 80°C) and then taken up in the original volume (5 ml) of OS VIII (pH 7.2). Both were membrane-filtered and readjusted to pH 7.2. Ribéreau-Gayon (1968) indicated that flavonoid glycosides were more likely to be in *Ceratonia* C-a than C-b. However Markham (1975) claimed that under these conditions fractionation would not be on the basis of ionic exchange but rather on the basis of adsorption and partition.

c) *Chemicals*. Malvidin -3-β-D-glucoside, malvidin-3, 5-β-D-diglucoside, quercetin, quercetin-3-α-L-rhamnoside and catechin were gifts from Dr. T.C. Somers (The Australian Wine Research Institute). Rutin (quercetin-3-β-D-rutinoside), naringin (naringenin-7-β-D-rutinoside), hesperetin, hesperidin (hesperetin-7-β-D-rutinoside), 6-bromo-2-naphthyl-β-D-galactoside, 8-hydroxyquinoline-β-D-glucoside, deoxycorticosterone glucoside, digitoxin, phloridzin and esculin hydrate were purchased from Sigma Chemical Co. Hyperoside (quercetin-3-β-D-galactoside) and p-β-D-glucosyloxyphenylazo-4-hydroxybenzene were gifts from Dr.

M.A. Jermyn (C.S.I.R.O. Division of Protein Chemistry).

Carmine was from B.D.H. Chemicals.

8-1-3 The Blocking of β-Lectin Effects on Protoplasts

Protoplasts were preincubated at room temperature with an equal volume of one of the extracts. The extract was described as inhibitory if the protoplasts would not agglutinate in $(\beta\text{-D-GLU})_3$ or $(\beta\text{-D-GAL})_3$ or serum while the extract was still present ($\frac{1}{4}$ the original concentration). The extract was described as having 'blocking' activity if the protoplasts were still unable to be agglutinated after having been washed free of extract (by at least 2 centrifugation and resuspension cycles and sometimes also by collection on Lymphoprep). In each case controls were used consisting of untreated protoplasts.

The Phaseolus and Brassica seed extracts were tested only once with Nicotiana tabacum cv. White Burley leaf protoplasts. They were not inhibitory to agglutination in 0.05 or 0.005 mg/ml $(\beta\text{-D-GAL})_3$ or 0.05 mg/ml $(\beta\text{-D-GLU})_3$ or 1/14 rabbit serum (TB4). The Zephyranthes and Hibiscus extracts were tested only once with Vicia faba leaf protoplasts and were not inhibitory to agglutination in 0.05 mg/ml $(\beta\text{-D-GLU})_3$ or 1/10 sheep serum. In each of these experiments the protoplast suspensions were 80% FDA-fluorescing both before and after incubation with the plant extracts. This was also true for all the other extracts except that of Pteridium aquilinum which destroyed the integrity of the plasmalemma.

The Hypericum extract was completely inhibitory and blocked agglutination in all experiments over a 3 month period. The experiments included 4 independent preparations of N.tabacum protoplasts, 3 independent preparations of V.faba protoplasts, and 1 preparation of Avena sativa leaf protoplasts. The Pseudotsuga

extract blocked both *Petunia hybrida* leaf and *Daucus carota* culture protoplasts. The *Echeveria* and *Allium* extracts both showed blocking activity when first examined but thereafter were inactive (6 separate occasions for *Echeveria* and 2 for *Allium*). Conversely the *Ceratonia* extract showed no initial activity but developed both inhibitory and blocking activity after storage. The blocking activity of the original unfractionated *Ceratonia* extract was observed with 7 independent protoplast preparations including *N.tabacum*, *V.faba*, *P.hybrida* and *A. sativa* leaf protoplasts (figs 8-1 and 8-2).

The various fractions of the *Ceratonia* extract were tested for activity and the results are presented in table 8-1. The blocking activity was retained in fraction C which Mabry *et al* (1970) claimed should be enriched in flavonoid glycosides. The activity was further retained by C-a. Ribéreau-Gayon (1968) suggested that C-a and C-b should represent glycosides and aglycones respectively on the assumption that cation exchange is the fractionating principle. However Markham (1975) claims that size partitioning may be occurring under these conditions. The active molecules may be larger and move more slowly through the column. The size of the molecules may also be an explanation for the variation of activity of some of the extracts during storage. Variation was also observed in *Ceratonia* C in table 8-1. Activity may reside only with small oligomers of the appropriate phenolic glycosides. Monomers may be inactive and polymers which are too large may precipitate and thus be inactive. The degree of polymerization of such compounds is known to vary with storage.

The liklihood that this explanation is valid is enhanced

Fig. 8-1 Oat leaf protoplasts agglutinating with 0.05mg/ml $(\beta\text{-CELL})_3$ in OSI (pH 7.2).

Fig. 8-2 Ceratonia-extract blocked oat leaf protoplasts failing to agglutinate with 0.05 mg/ml $(\beta\text{-CELL})_3$ in OSI (pH 7.2).

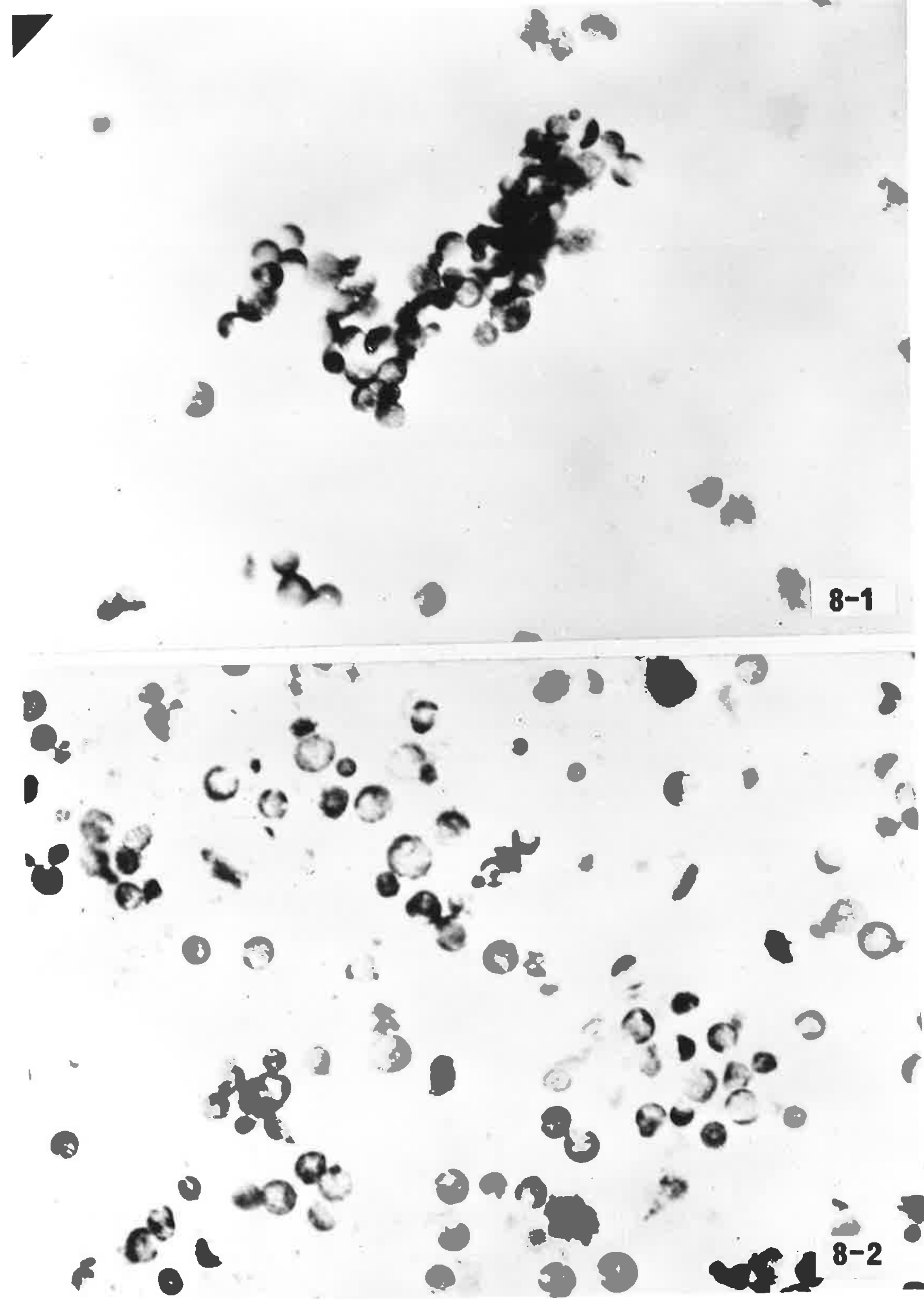

8-1
8-2

TABLE 8-1 Blocking activity of *Ceratonia* extract fractions

EXTRACT	PROTOPLASTS		
	Petunia leaf	*Daucus carota* suspension	
	3/6/77	21/6/77	19/7/77
Ceratonia A	+	n.a.	n.a.
Ceratonia B	-	n.a.	n.a.
Ceratonia C	+	+	-
Ceratonia C-a	n.a.	n.a.	-
Ceratonia C-b	n.a.	n.a.	+

+ = blocking activity in the presence of 0.05mg/ml $(\beta\text{-CELL})_3$ or $(\beta\text{-LAC})_3$

- = no blocking activity

n.a. = not ascertained

by the observation that simple phenolic glycosides and terpenoid glycosides are unable to block the β-lectins of plant protoplasts. The compounds tested and their structures and the concentrations used (usually the maximum possible) are shown in fig. 8-3.
It may also be that flavonoids need to be glycosylated on the B ring (i.e. forming 2'-, 3'-, 4'-, or 5'- glycosides) before they can block β-lectins. Certainly such glycosides would more resemble Yariv antigens. The simple flavonoids known to be present in Allium cepa bulbs include: cyanidin-3-β-D-glucoside, cyanidin-3-β-D-diglucoside, peonidin-3-β-D-glucoside, quercetin-3, 4'-diglucoside, quercetin-7, 4'-diglucoside, quercetin-4'-glucoside and peonidin-3-β-L-arabinoside (Harbone, 1967; Timberlake and Bridle, 1975; Harborne and Williams, 1975). The flavonoids reported for Hypericum perforatum leaf include quercetin-3-β-D-galactoside and quercetin-3-β-D-glucoside (Falco and de Vries, 1964; Harborne, 1967; Harborne and Williams, 1975). Quercetin-3'-β-D-glucoside has been found in Pseudotsuga menziersii (Harborne and Williams, 1975).

FIG. 8-3 Chemical structures with no blocking activity

Malvidin-3-β-D-glucoside (1mM)

Malvidin-3,5-β-D-diglucoside (1mM)

Hesperidin (65μM)
(Hesperetin-7-β-rutinoside)

also Hesperetin (130μM)

Naringin (1.7mM)
(Naringenin-7-β-rutinoside)

Rutin (200μM)
(Quercetin-3-β-rutinoside)

Hyperoside (1mM)
(Quercetin-3-β-D-galactoside)

Quercetrin (1mM)
(Quercetin-3-α-L-rhamnoside)

also Quercetin (1mM)

Catechin (1mM)

FIG. 8-3 continued.

Carmine (1mM)

Phloridzin (2.25mM)
(phloretin-2'-β-D-glucoside)

Esculin hydrate (5.05mM)
(6,7-dihydrocoumarin-6-β-D-glucoside

8-Hydroxyquinoline-β-D-glucoside
(325μM)

6-Bromo-2-naphthyl-β-D-galactoside
(200μM)

p-β-D-Glucosyloxyphenylazo-4-hydroxybenzene
(1.65mM)

Deoxycorticosterone-β-D-glucoside
(400μM)

Digitoxin (50μM)
(Digitoxigenin-3-β-D-digitoxose)

8-2 MODEL 5 AND 5a FOR HYBRID SELECTION USING β-LECTIN BLOCKERS

The model in fig 8-4 employs the ability of certain substances to block β-lectins and in so doing the parental protoplasts are coated differentially. Since the blocking effect prevents the non-antibody mediated agglutination by serum, it is proposed that antisera specific for the blocking molecules can be used to select hybrid protoplasts as shown. In a sense model 5 is analogous to model 1 (fig 4-1) but with the added advantage of complete generality. Once 2 suitable blockers and 2 corresponding specific antisera are found then any two parents, irrespective of genotype, can be used.

The feasibility of model 5 may be established by testing model 5a (fig 8-5). Both model 5 and 5a presume that flavonoid glycosides are blockers. Even if this is not the case the availability of an extract with unidentified blocking activity should allow model 5a to be evaluated. In model 5a one parent is blocked and eliminated by its inability to agglutinate with $(\beta\text{-D-GLU})_3$. The other parent and the fusion hybrids should agglutinate because at least half of their β-lectin sites are available for binding to the $(\beta\text{-D-GLU})_3$ molecules. It may also be possible to employ nutritional traits to select against the other parent in culture.

MODEL 5

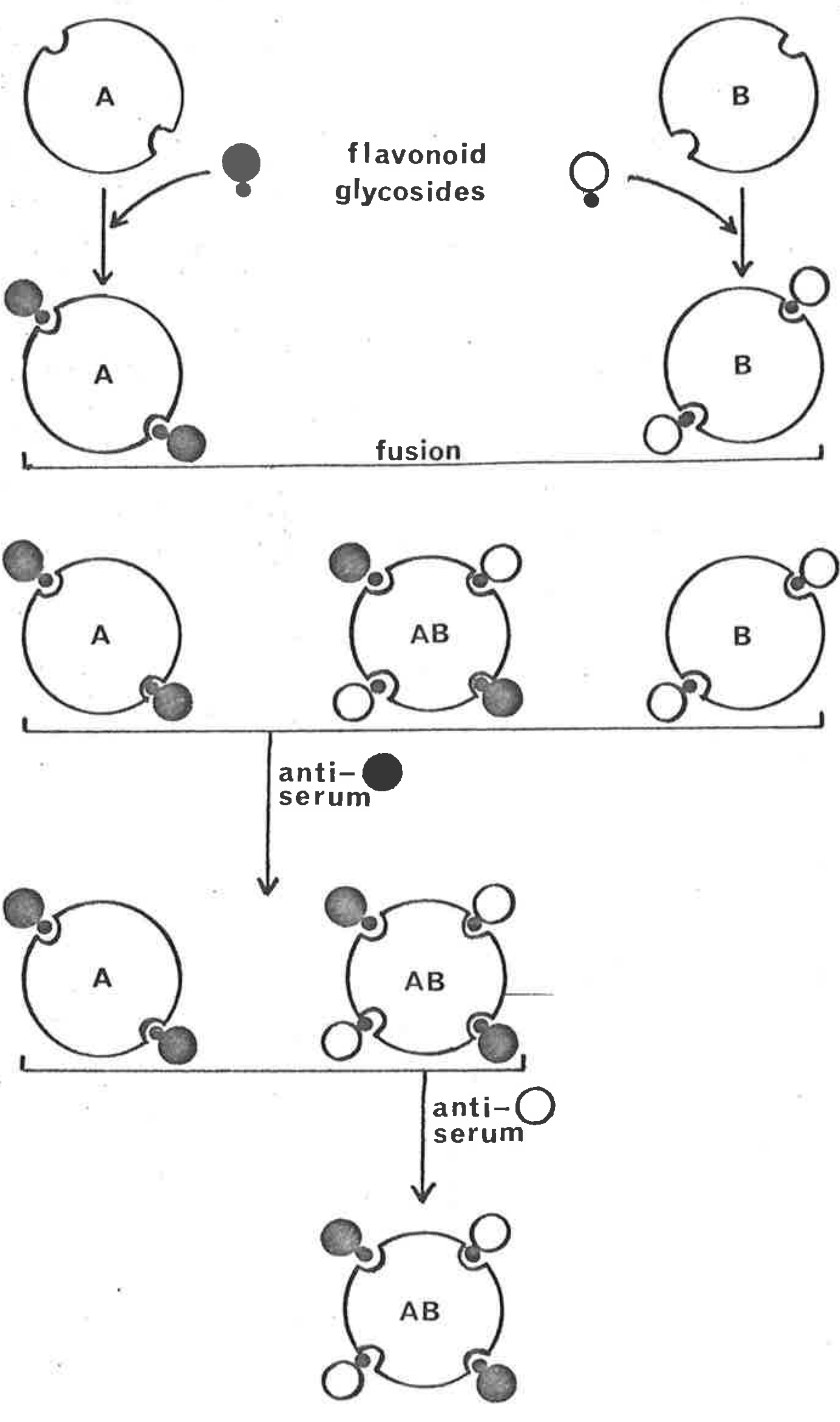


Fig 8-4

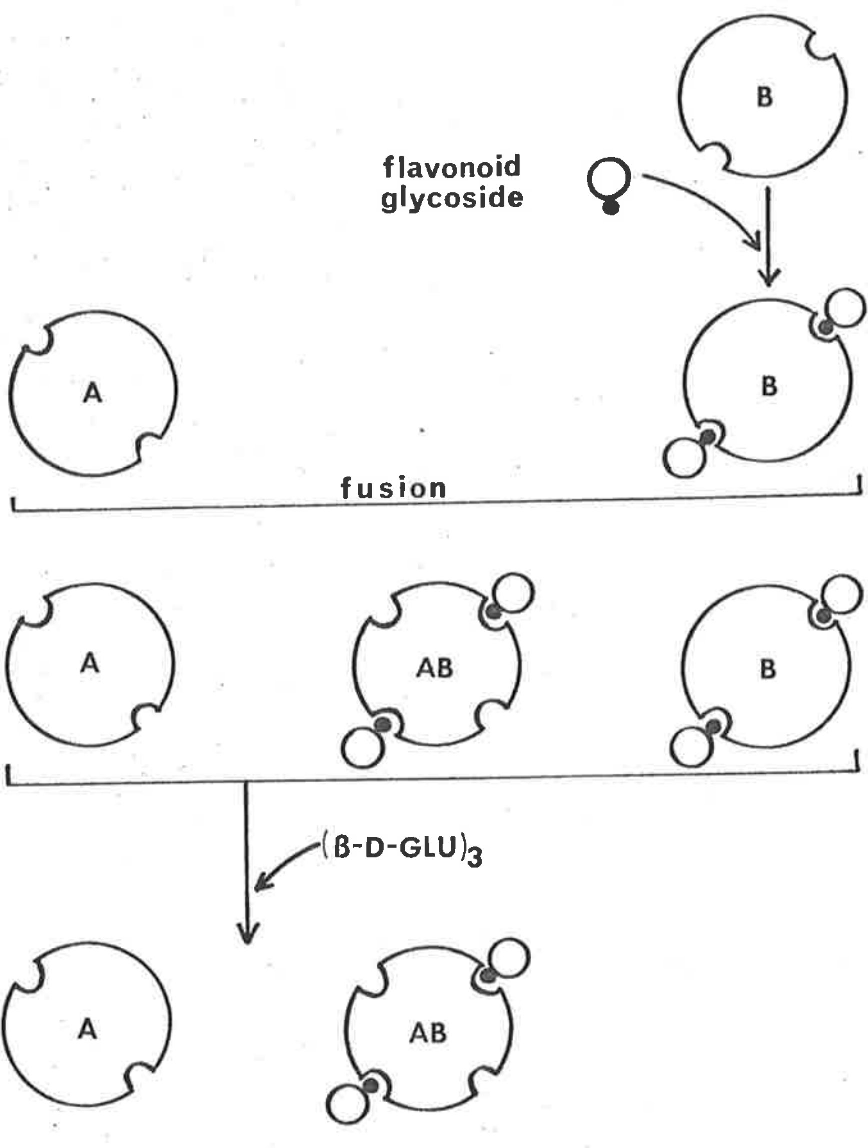


Fig 8-5

8-2-1 Differential Abilities to Use Different Carbon Sources.

Certain carbon sources can be used by some species in culture but not by others. Tobacco cultures and protoplasts are unable to grow on galactose (Sievert and Hildebrandt, 1965; Thorpe and Meyer, 1972; Thorpe, 1974; Thorpe and Laishley, 1974; Uchimiya and Murashige, 1976). However carrot cultures can grow almost as well on galactose as on sucrose (Verma and Dougall, 1977). Galactose utilization requires only a few extra enzymes such as galactose phosphokinase, uridine diphosphate galactose pyrophosphorylase, and phosphogalactose uridyl transferase. Thus this ability may be a characteristic inherited by fusion hybrids of tobacco and carrot at least prior to genetic loss.

Table 8-3 Osmotica and media composition (mg/l)

	KCMG3	V47	OS VII	OS VIII
KH_2PO_4	.	68	.	27.2
$NaH_2PO_4.H_2O$	150	.	109	.
$CaCl_2.2H_2O$	900	1062	882	150
$CaH_4(PO_4)_2.H_2O$	50	.	.	.
KNO_3	2500	1465	.	100
NH_4NO_3	250	280	.	.
$(NH_4)_2SO_4$	134	.	.	.
$MgSO_4.7H_2O$	250	1095	.	250
KI	0.75	0.25	.	0.16
$MnSO_4.H_2O$	10	5.2	.	.
H_3BO_3	3	3.1	.	.
$Na_2MoO_4.2H_2O$	0.25	0.1	.	.
$CuSO_4.5H_2O$	0.025	0.015	.	0.025
$CoCl_2.6H_2O$	0.025	0.012	.	.
$ZnSO_4.7H_2O$	2	1.4	.	.
Na_2-EDTA	.	37.5	.	.
$FeSO_4.7H_2O$	.	28.8	.	.
Fe sequestrene 330 (Ciba-Geigy)	28	.	.	.
$Fe_2(SO_4)_3.6H_2O$	.	.	.	2.5
casein hydrolysate	250	.	.	.
glucose	.	.	126,000	.

	KCMG3	V47	OS VII	OS VIII
yeast extract	100	.	.	.
myo-inositol	100	100	.	.
citric acid	100	.	.	.
malic acid	100	.	.	.
xylose	250	.	.	.
ribose	250	.	.	.
thiamine	10	4	.	.
nicotinic acid	1	3.9	.	.
pyridoxine.HCl	1	0.7	.	.
folic acid	0.5	0.4	.	.
pantothenic acid	1	.	.	.
biotin	0.01	0.04	.	.
ascorbic acid	2	.	.	.
riboflavin	0.2	.	.	.
glutamine	50	.	.	.
glycine	5	1.4	.	.
mannitol	*	*	.	90,000
sucrose	*	17,100	.	.
NAA	1	1.1	.	.
BA	0.2	0.45	.	.
MES 2-(morpholino) ethane sulfonate	.	.	585	585

* KCMG3m1 and KCMG3m3 have 20 g/l sucrose
KCMG3m2 and KCMG3m4 have 20 g/l galactose in place of sucrose
KCMG3m1 and KCMG3m2 have 0.45 M mannitol (82 g/l)
KCMG3m3 and KCMG3m4 have no mannitol
V47(mannitol) has 0.5 M mannitol (90 g/l)

8-2-2 Materials and Methods

Vicia faba and *Nicotiana tabacum* cv. White Burley plants were grown for 36 and 60 days respectively in a growth cabinet with 10,000 lux (cool white fluorescent light) for 16 h per day at 28°C. Fully expanded leaves were stripped of lower epidermis, preplasmolysed for 4 h in OS VIII (*V. faba*) or for 1 h in OS VII(*N. tabacum*) (see table 8-3), and incubated in 2% Cellulysin + 0.5% Macerozyme R 10 in the same osmoticum (pH 5.9) for 9 h in the dark at 27°C. *Daucus carota* suspension cultures were initiated from root slices and grown through 8 or 11 subcultures in SH medium (Schenk and Hildebrandt, 1972) before incubation for 9 h at 27°C in 2% Cellulysin + 0.5% Driselase + 0.5% Sigma pectinase + 0.5% Rhozyme HP150 + 0.5% Macerozyme R10 in OSVII or OS VIII (pH 5.9). Blocking extracts were made and used as described in sections 8-1-2 and 8-1-3. The media used in attempts to culture the protoplasts are listed in Table 8-3.

In order to induce fusion, protoplasts were suspended in 25 drops of PEG solution (Table 8-4) giving a cell density of about 5 x 10^6/ml. After 10 min 3 drops of high Ca^{++}/high pH solution (Table 8-4) were added every 20 seconds for 3 min. Then 0.5, 1.0, 2.0, 2.0, 3.0 and 4.0 ml of KCMG3 ml were added successively at 5 min intervals.

Table 8-4 Fusion solutions

	PEG solution	high Ca^{++}/high pH solution
mannitol		0.4M
$CaCl_2.2H_2O$	10.5 mM	0.05M
PEG 6000 (Koch-Light)	15% (w/v)	
$KH_2PO4.H_2O$	0.7 mM	
sucrose	4% (w/v)	
pH	5.5	10.5

8-2-3 Testing of Model 5a.

In a preliminary experiment a sample of *V.faba* leaf protoplasts were mixed with an equal volume of *Ceratonia siliqua* extract (unfractionated) for 30 min. They were then washed twice in OS VIII (pH 7.2). Control samples of *D.carota* culture and *V.faba* leaf protoplasts were incubated and washed with OS VIII (pH 7.2). The results of agglutination studies are shown in table 8-5.

Table 8-5 Mixed protoplast agglutination

Protoplasts	(β-CELL)$_3$ 0.05 mg/ml			OS VIII (pH 7.2)
	V.faba (green)		*D.carota* (non-green)	
untreated *V.faba*	++++		•	-
Ceratonia-blocked V.faba	-		•	-
D.carota	•		++++	-
D.carota + untreated *V.faba*	++++	MIXED	++++	-
D.carota + blocked *V.faba*	-		++++	-

++++ = 75-100% agglutination

\- = negligible agglutination

• = none present

All protoplasts, blocked and unblocked, were FDA-viable. The results are illustrated in figs 8-6, 8-7, 8-8, 8-9 and 8-10. This experiment confirmed that the blocking factors, once attached to a protoplast surface, are not free to diffuse to another protoplast. Therefore blocked and unblocked protoplasts retain their original status even when mixed. The agglutinated carrot protoplasts could be separated from the blocked *V.faba* protoplasts (fig 8-8) by sedimentation and gentle washing.

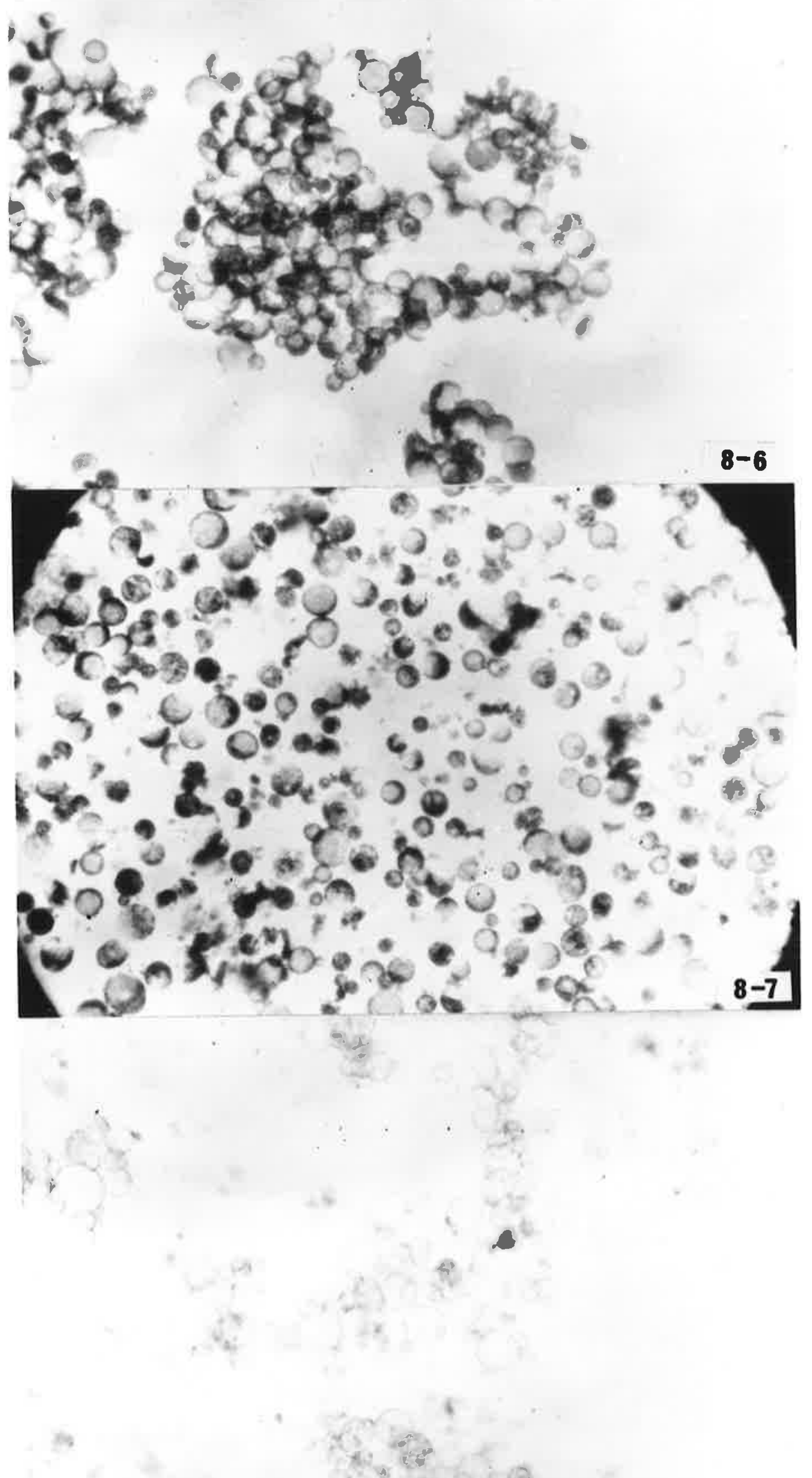
Fig 8-6 *Vicia faba* leaf protoplasts agglutinating with 0.05 mg/ml (β-CELL)$_3$ in OS VIII (pH 7.2).

Fig 8-7 *Ceratonia* - extract blocked *V.faba* protoplasts failing to agglutinate with 0.05 mg/ml (β-CELL)$_3$ in OS VIII (pH 7.2).

Fig 8-8 *Daucus carota* culture protoplasts agglutinating with 0.05 mg/ml (β-CELL)$_3$ in OS VIII (pH 7.2).

Fig 8-9 Mixed agglutination of *D.carota* protoplasts (light) and *V.faba* protoplasts (dark) with 0.05 mg/ml (β-CELL)$_3$ in OS VIII (pH 7.2).

Fig 8-10 Mixed suspension of *D.carota* and *Ceratonia*-blocked *V.faba* protoplasts with 0.05 mg/ml (β-CELL)$_3$ in OS VIII (pH 7.2). Only the carrot protoplasts agglutinated.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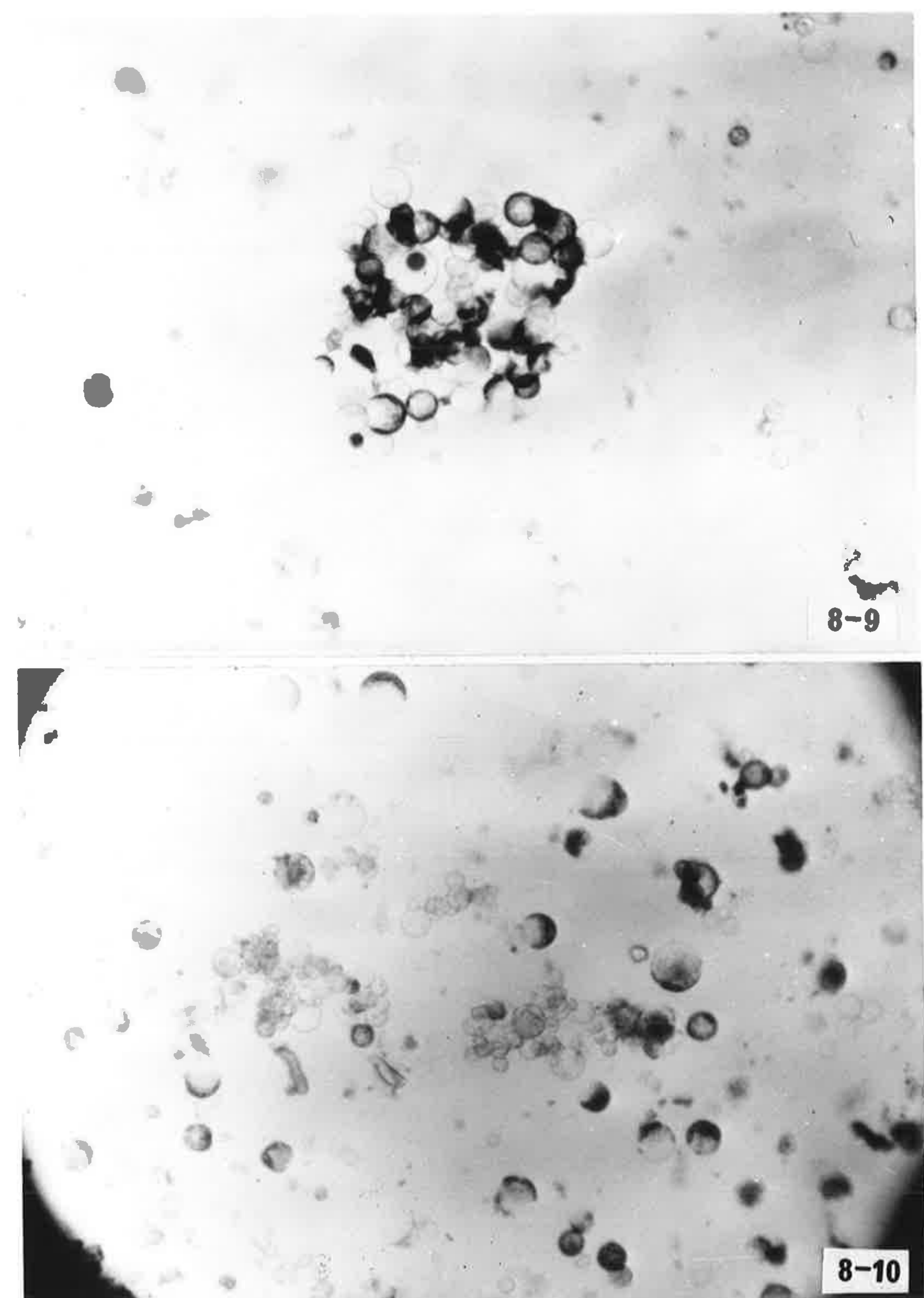
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Following this experiment tobacco leaf and carrot culture protoplasts were chosen as parents for a fusion experiment because of their differential ability to utilize galactose as a carbon source (see 8-2-1). Protoplasts were isolated and the suspensions in KCMG3ml were divided and treated as outlined in fig 8-11 using aseptic techniques. Some fusion hybrids were quite unambiguously observable as mixtures of tobacco-chloroplast-containing cytoplasm and carrot-non-green cytoplasm (fig 8-12). The critical selective branch of the experiment was where the carrot protoplasts had been blocked and the fusion mixture was agglutinated with $(\beta\text{-CELL})_3$. In this selective experiment it was quite clear that the population surviving the selection had a very greatly reduced proportion of carrot protoplasts. The blocking effect of Ceratonia C on carrot protoplasts in this experiment is illustrated in fig 8-13 and 8-14. It was not possible with certainty to observe fusion hybrids within the agglutinated mass of cells. The presupposition of model 5a that fusion hybrids will also agglutinate remains unverified.

The cultures from this experiment were in parafilm-sealed Petri-dishes and were kept at 21°C in a moistened plastic container to maintain high humidity. The tobacco protoplasts formed walls rapidly but were prone to forming subprotoplasts (fig 8-15). Nevertheless rapid cytoplasmic streaming (cyclosis) was observed in many healthy tobacco protoplasts and chloroplasts redistributed throughout the cytoplasm and faded in colour over 7-10 days (fig 8-16). Walls and cyclosis were observed in cultures B, C, G, I and J after 7 days. Culture I was particularly noteworthy in this respect because it represented the experiment selecting

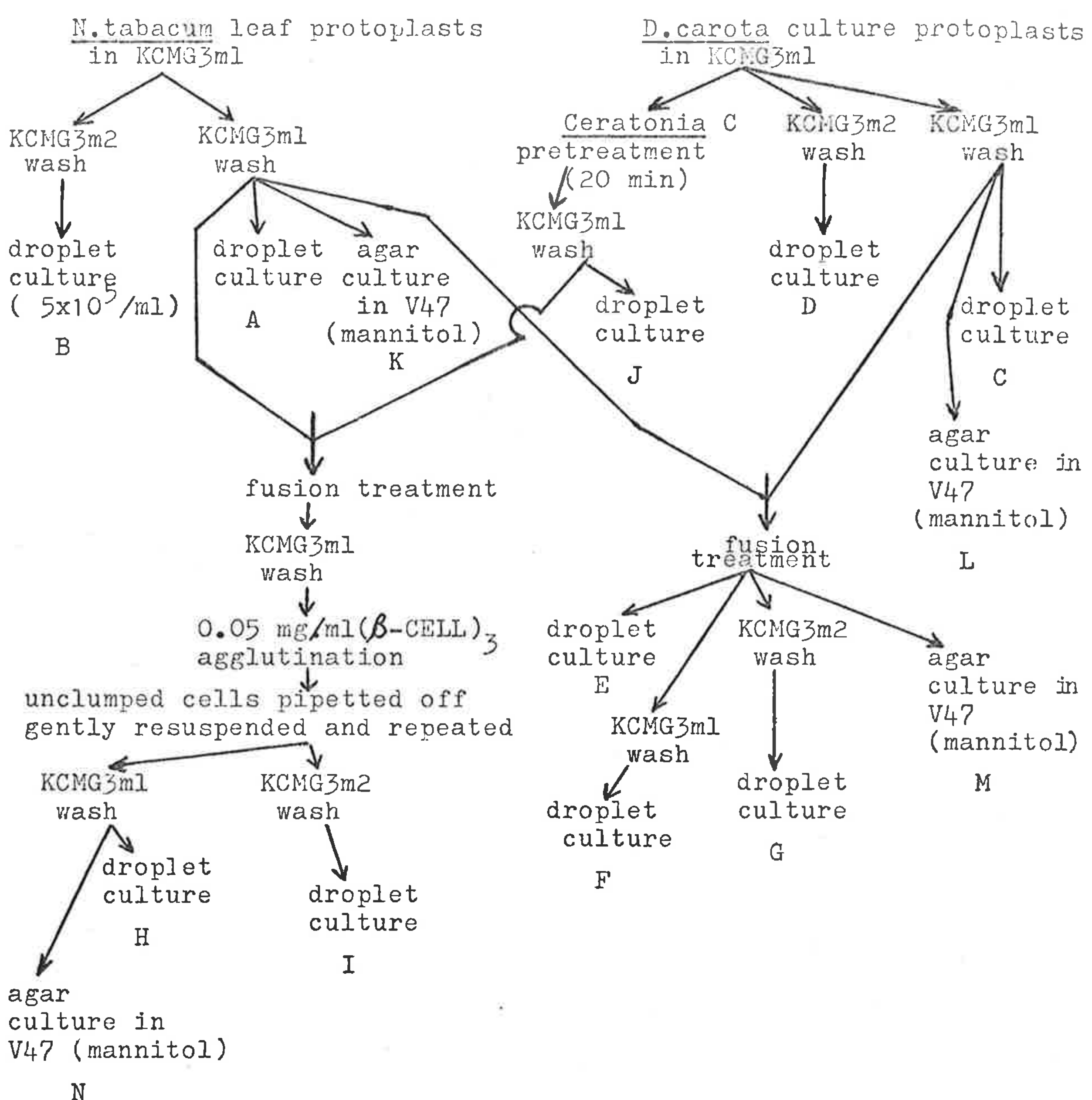
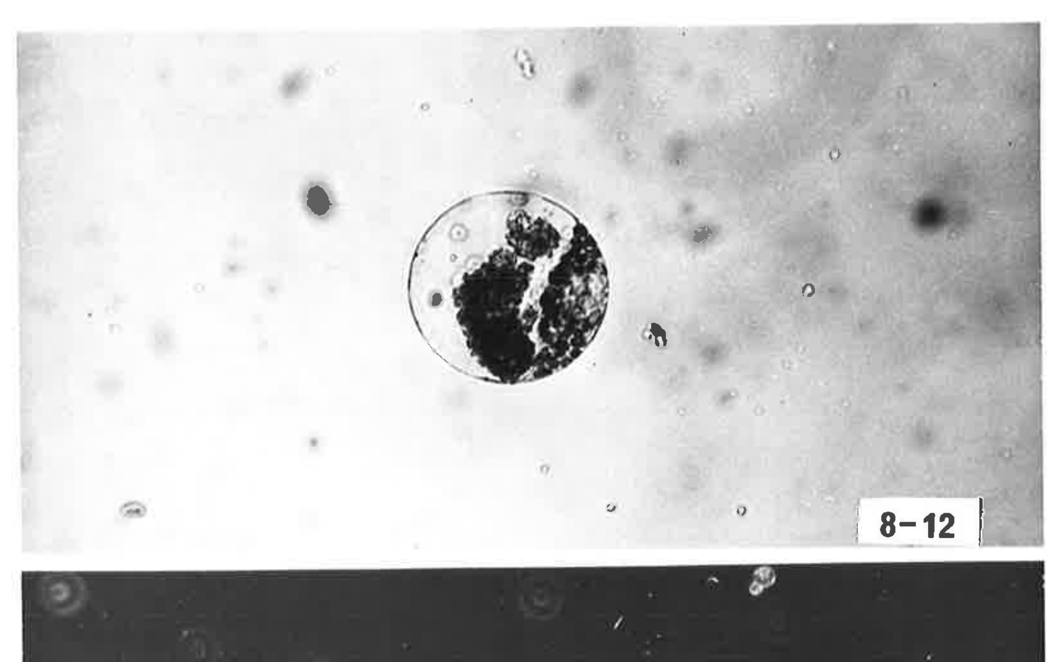


Fig 8-11 Tobacco/carrot protoplast fusion experiment.

Fig 8-12 A fusion hybrid between tobacco and carrot having resumed its spherical shape.

Fig 8-13 Untreated carrot protoplasts agglutinating in 0.05 mg/ml $(\beta\text{-LAC})_3$.

Fig 8-14 Ceratonia C blocked carrot protoplasts failing to agglutinate in 0.05 mg/ml $(\beta\text{-LAC})_3$.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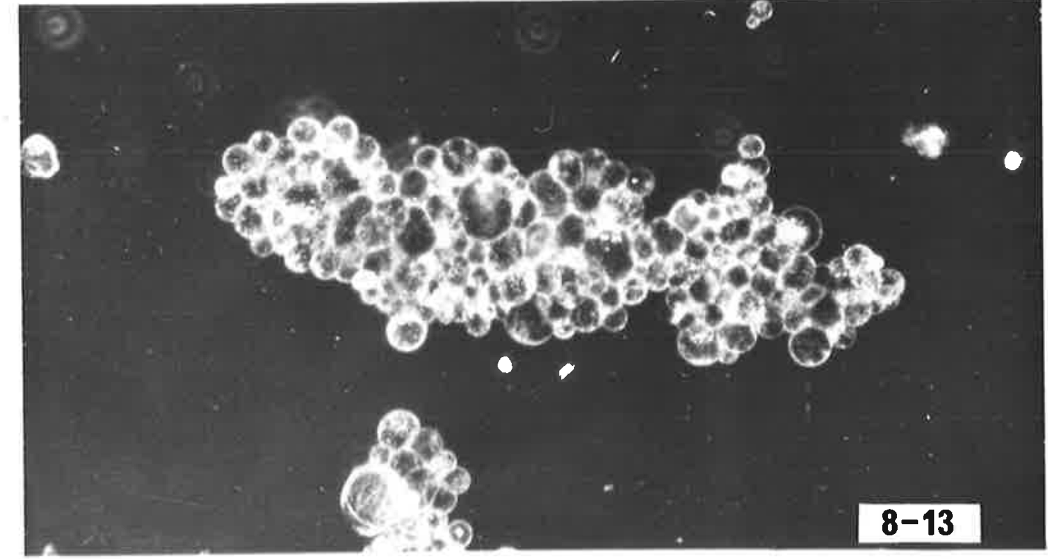
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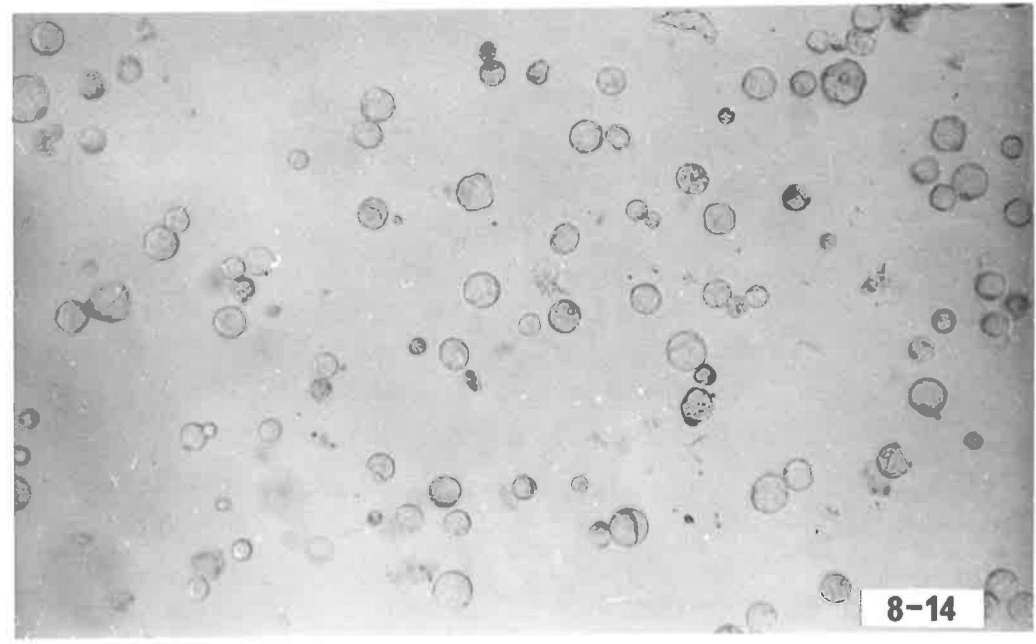
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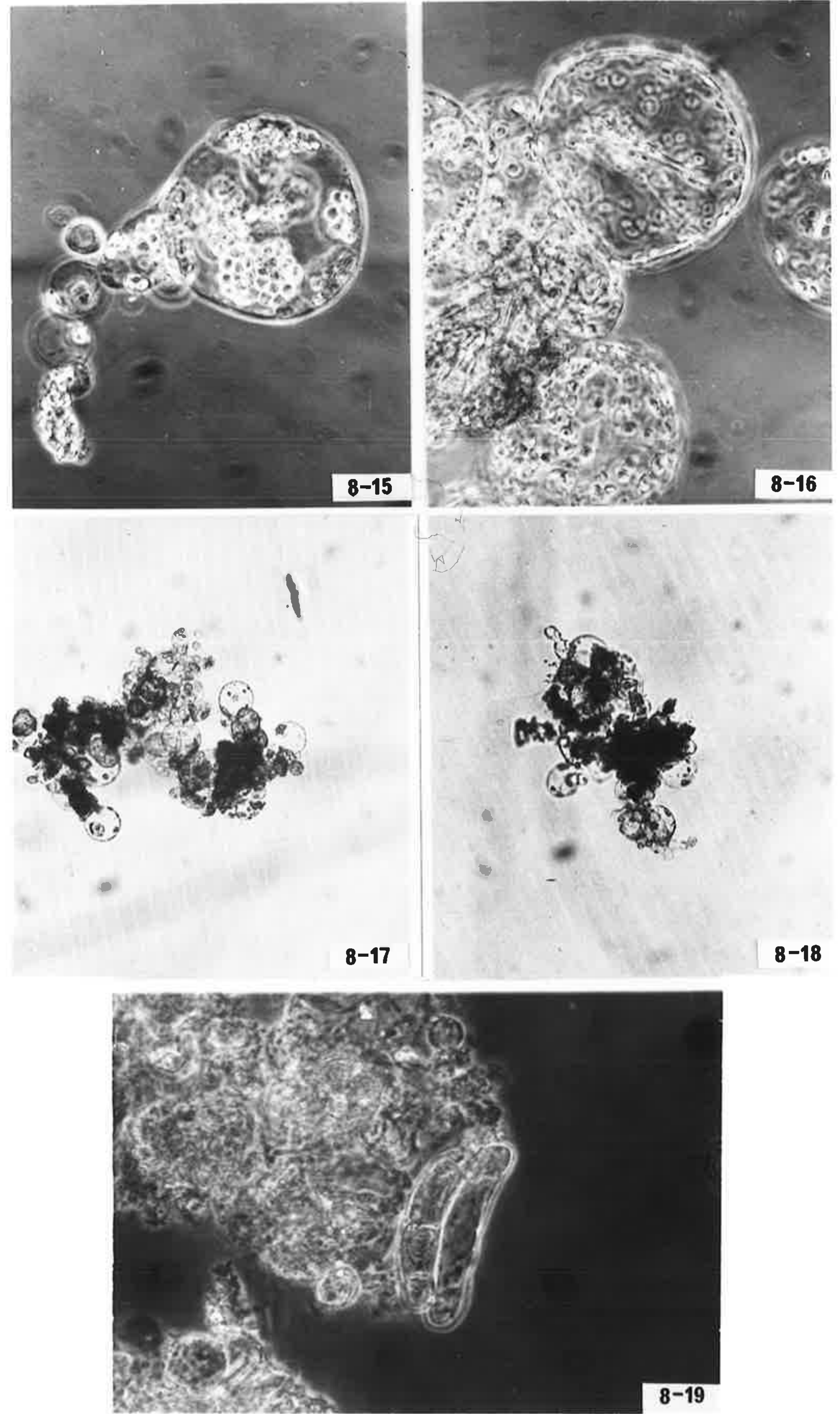
Fig 8-15 *N.tabacum* protoplast (culture A) forming cytoplasmic subprotoplasts after 6 days.

Fig 8-16 *N.tabacum* protoplasts (culture A) after 6 days. These showed rapid cyclosis along cytoplasmic strands.

Fig 8-17 Protoplasts of the tobacco/carrot fusion mixture 14 days after removal of carrot protoplasts being cultured in galactose medium (culture I). The clump is not due to colony formation but due to $(\beta\text{-LAC})_3$ agglutination. Many cells have collapsed but some are still healthy.

Fig 8-18 as Fig 8-17.

Fig 8-19 Regenerated and dividing carrot cells from protoplasts (culture J).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against both parents—against carrot by agglutination and against tobacco by culturing in galactose. However the visual marker system was invalid within 10 days because of the degeneration of chloroplasts. Many of the cells in culture I had lysed by the 14th day but others were still intact (fig 8-17 and 8-18). In the carrot cultures there were some unambiguous walls and divisions (fig 8-19) and also some extrusion of extracellular amorphous material. This material may represent cell wall molecules.

Attempts to induce further divisions included spreading drops of each of the cultures onto the surfaces of KCMG3m3 and KCMG3m4 agar-solidified media and also mixing samples with melted V47(no mannitol)(0.3% agar). None of the cultures was successful to the point of forming colonies.

8-3 CONCLUSIONS FOR MODELS 5 AND 5a

The experiments of section 8-2-3 did establish that the blocking of one parent prior to fusion and the agglutination of the fusion mixture can result in a dramatic elimination of one of the parents. It has not been possible in these experiments to verify whether fusion hybrids agglutinate along with the unblocked parent. Differential stains may help to verify this presupposition of model 5a (Constabel *et al*, 1975c). In the absence of colony formation in the control cultures it was also not possible to assess the potential usefulness of galactose utilization as a selective trait.

The agglutinating principle of model 5 is different to that of model 5a. It will require independent assessment using specific antisera. However before such experiments can be conducted a well-defined stable blocking system is required. Once pure blocking molecules are available highly specific antisera to the aglycone moieties can be produced. For example the phenolic molecules can be conjugated to human serum albumin prior to injection into rabbits enabling small changes on the phenolic to be recognised (Weiler and Zenk, 1976).

There are a number of ways that the actual cell sorting procedure could be modified to avoid the problem of non-agglutinating protoplasts sedimenting with the agglutinated protoplasts. This problem was not extensive but did reduce the selective efficiency. Each of the specific antibody preparations could be immobilized onto a column of Sepharose 2B or 6MB beads (Kinzel *et al*, 1976, 1977). The fusion mixture would be passed through one column. The protoplasts retained would be washed and then

released by an excess of blocker or by digesting a gelatin bridge between the antibodies and beads (Thomas and Phillips, 1973). These protoplasts would then be passed through the second column. Retained protoplasts should be hybrids. Alternatively antibodies could be conjugated to nylon fibres in meshes (Edelman and Rutishauser, 1974). Incubation of the fibres in the protoplast fusion mixture would allow those protoplasts to bind which carried suitable blocking molecules on their surfaces. Cells which bind to nylon fibres in this way can usually be released by plucking the taut fibre.

SECTION 9 GENERAL CONCLUSIONS

Exogenous antisera and lectins did not display sufficient specificity to enable the sorting of protoplasts of different species. Also the endogenous membrane-bound β-lectins of different species responded similarly to various multi-liganded antigens. However the experiments of section 8-2 demonstrated that certain plant phenolic molecules (possibly flavonoid glycosides) can be stably coated onto protoplast surfaces prior to fusion. On the basis of such artificially applied surface differences it was possible to greatly enrich the fusion mixture for one parental type (and presumably for the hybrids also).

With further investigation, including positive identification of the blockers and examination of their antigenicity, it should be possible to extend this principle to model 5a. For this model to operate 2 stable and antigenically- distinct blockers and 2 corresponding specific antisera are required. With these 4 components it should be possible to select fusion hybrids of any 2 plant protoplast populations irrespective of the genotypes of those protoplasts.

There has been an increased interest in recent years in cell sorting techniques. Most of the work has generated from a need to fractionate natural mixtures of animal cells into the component subpopulations. A brief examination of these cell sorting techniques suggests some possible alternative approaches to hybrid protoplast selection:

(i) Sorting according to differences in cell volume using velocity sedimentation. A gradient of protein or Ficoll is

usually used without centrifugation. Cells separate into layers after 3-18 h (Dicke, 1973; Denman and Pelton, 1973; Miller, 1973). Protoplasts often vary considerably in size even in the one preparation and therefore velocity sedimentation is unlikely to be useful for hybrid selection.

(ii) Sorting according to differences in cell density using equilibrium density-gradient centrifugation (Dicke, 1973; Harwood, 1974; Munthe-Kaas and Seglen, 1974). This technique has already been investigated as a means of separating hybrid protoplasts from parental protoplasts. The hybrids had an intermediate density (Harms, Lörz and Potrykus, 1976).

(iii) Sorting according to differences in surface characteristics using affinity chromatography. Plasma cells producing antibodies to a given antigen have been purified by affinity to polyacrylamide beads conjugated with the antigen (Truffa-Bachi and Wofsy, 1970; Henry et al, 1972). Schlossman and Hudson (1973) purified mouse lymphocytes by affinity to Sephadex beads conjugated with rabbit antiserum to mouse antibodies. Cells which were bound were released by digesting the beads with dextranase. Thomas and Phillips (1973) similarly purified human lymphocytes but a gelatin bridge was placed between the antibodies and beads. This gelatin could be easily digested with collagenase to release the cells. Human tissue culture cells with an affinity for lentil lectin were separated using a column of large Sepharose 2 B beads conjugated with the lectin (Kinzel et al, 1977). Polyacrylamide has been described as the preferable matrix for cell chromatography because cells do not adhere non-specifically to polyacrylamide beads (Lowe and Dean, 1974). Model 5a for hybrid protoplast selection may be modified so as to employ 2 polyacrylamide matrices, one conjugated with antibodies to the first

blocker and the other with antibodies to the second blocker. Gelatin bridges could be digested to release protoplasts.

(iv) Sorting according to differences in cell surface charge using cell electrophoresis. This technique uses an electrophoretic chamber or capilliary which is optically suitable for following cell movements microscopically (Bangham et al, 1958). The cells can also be electrophoresed through density gradients so that there are two major fractionating principles, surface charge and cell density (Griffith et al, 1975). Plant protoplasts can be electrophoresed without apparent loss of viability and different species show different electrophoretic mobilities (Grout and Coutts, 1974; Pilet and Senn, 1974). Fusion hybrids may have an electrophoretic mobility intermediate between the parents.

(v) Sorting according to differences in applied fluorescence. Herzenberg et al (1976) developed apparatus which created a stream of microdroplets from a suspension of cells such that each droplet contained one cell. The fluorescence of each droplet was detected and the value of this fluorescence used to determine the direction of electrostatic deflection so that the droplet falls into one of several collecting vessels. As little as 5% difference in fluorescence was sufficient to divide two types of cells. It may be possible to apply this type of technology to protoplasts. One parent suspension could be labelled with fluorescent molecules either in the cytoplasm (Hess, 1973) or on the plasmalemma (Ruesink, 1975) prior to fusion with the unlabelled parent. Presumably the fusion hybrids would have an intermediate fluorescence and could be collected accordingly.

A variation on this approach would be to use the fluorescence detection signal to trigger a cell killing device. Protoplasts passing through the detection aperture could be destroyed by an electronic laser flash if its fluorescence was above or below given values. Laser flash units are readily available with the required characteristics to kill cells and the required cycle times to hit successive droplets as they fall.

Mention has already been made of the potential usefulness of being able to culture individual isolated protoplasts (section 2-1-3). Indeed there has already been limited success in this regard (Kao and Michayluk, 1975; Abo El-Nil and Hildebrandt, 1976). If culturing techniques can improve to the point where this is generally possible, then individual fusion hybrids could be cultured without selective techniques. This approach requires that hybrids be visually distinctive at least immediately following fusion and this can be achieved by using differences in chlorophyll or anthocyanin content (Gosch _et al_, 1975b) or non-toxic cytoplasmic stains (Kameya, 1975a). In addition the available micromanipulation apparatus and techniques will need to be adopted for use with protoplasts (Diacumakos, 1973).

Another alternative approach may be to so modify the fusion techniques that only fusion hybrids occur. For example A protoplasts could be bound to surfaces or fibres at a density at which they will not be in contact. B protoplasts may then be pretreated so that their surface charge is opposite to that of the A protoplasts. Incubation of B protoplasts with immobilized A protoplasts may result in A/B adhering pairs. The free B protoplasts would be rinsed away from the immobilized A/B pairs which

could then be fused and cultured.

Cell sorting techniques, as examined and discussed in this thesis, may be valuable tools for those concerned with selection of somatic hybrid protoplasts. Such methods offer a form of hybrid enrichment which is rapid, more or less general, and independent of the genetic loss which may occur from one or both genomes. The application of cell sorting procedures does not preclude the subsequent use of nutritional pressure to select for the function (and thus presence) of certain desirable genes.

SECTION 10 LITERATURE CITED

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