SUGAR ACCUMULATION IN THE DEVELOPING GRAPE BERRY

by

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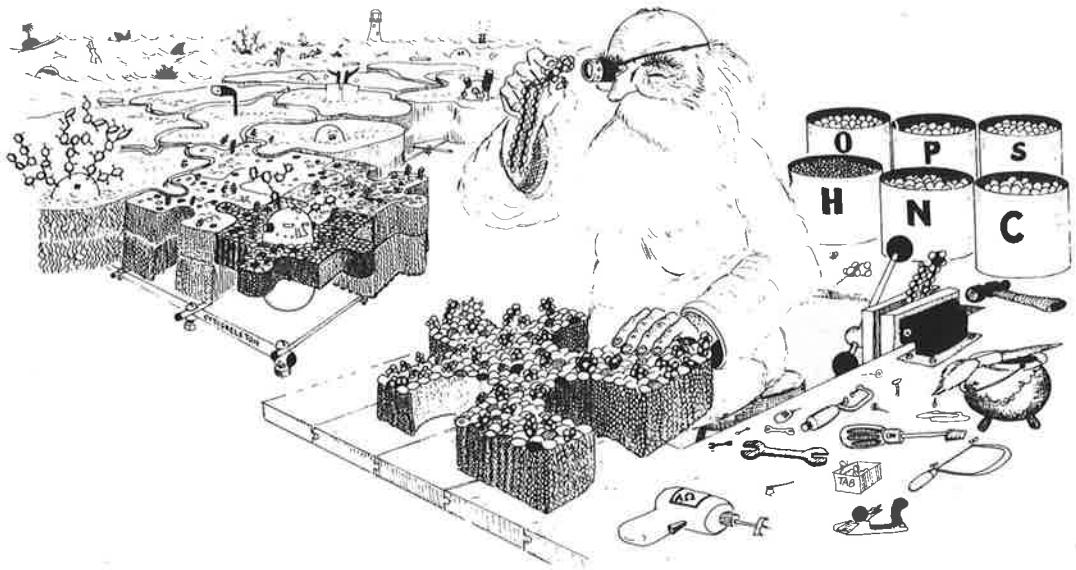
Frontispiece: T.A. Bramley in Finean *et al.* (1978) with permission.

to maarten and alison

from whom we have received much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CHAPTER 14. INTEGRATIVE DISCUSSION

SUMMARY

1. Sugar accumulation was examined in the berry of the grape, Vitis vinifera cv. Muscat Gordo Blanco, at the inception of the ripening stage of berry development.

2. Reviews of the literature are presented pertaining to development of the grape berry, membrane transport of sugars in higher plants, techniques for the isolation of vacuoles from plant material, and the use of isolated protoplasts and vacuoles in uptake studies.

3. The working hypothesis describing the mechanism of accumulation of carbohydrates has been: unloading of sucrose from the phloem into the apoplasm followed by inversion to hexoses and diffusion, movement of hexoses across the plasmalemma, synthesis of sucrose or sucrose phosphate in the cytoplasm with transport to the vacuole where, finally, inversion occurs.

4. Components of the hypothesis were examined stepwise by analysis of sugar compartmentation in vivo, and by in vitro manipulations of excised pericarp tissue, and of protoplasts and vacuoles isolated therefrom.

5. Compartmentation of hexoses in the pericarp of the developing grape berry, proceeding from veraison, was examined by efflux analysis, as too the effect of shading upon this. The method indicated half the hexoses to be diffusible at veraison, and 15 days later 80% (of a larger total) were diffusible. This suggests that the onset of rapid sugar accumulation in the grape berry results primarily from changes in phloem unloading rather than from concentrative accumulation by the cell. Nevertheless the skin of a pre-veraison berry is a glucose accumulator, and, post-veraison, it becomes a fructose and glucose accumulator.

6. Methods were developed for protoplast release from the skin of berries by the action of cellulases, and for subsequent isolation of vacuoles by DEAE-Dextran-induced lysis of these protoplasts. Protoplasts and vacuoles were purified by filtration through coarse Sephadex and by ultracentrifugation in metrizamide. A method is presented for a sequential, enzymatic assay of glucose, fructose and sucrose.

Isolated vacuoles from a grape berry cv. Muscat c. 10^{o} Brix had an isopycnic density, in Percoll and a 600 mOsmol medium at 17^{o}C, of ρ = 1.082 and 1.075 g cm^{-3}, while protoplasts were lighter at ρ = 1.055.

7. The kinetics of radiochemical uptake indicated mediated transport of glucose and fructose in protoplasts, with respective K_Ts (mM) of 5.6 ± 1.4 and 1.7 ± 0.4. Similarly, mediated transport was evident in isolated vacuoles, with K_Ts (mM) of 10.3 ± 1.9 and 2.8 ± 1.3 for glucose and fructose. In each instance there was coincident uptake attributed to passive diffusion across the membrane. The two K_Ts for fructose, into protoplasts and into vacuoles, were not significantly different and could have arisen from the same interaction at the tonoplast. Accumulation from sucrose indicated variable, non-saturating (diffusive) uptake of sucrose by protoplasts and isolated vacuoles superimposed upon a lesser, but saturable, process. Upon incubation of protoplasts in sucrose, rapid initial uptake was observed but not maintained despite hydrolysis of the product, and was interpreted as indicating a potentiated transport system at time zero.

8. The radiochemical products of incubations were analysed by chromatography and electrophoresis. From the incubation of isolated vacuoles in glucose radionuclide, labelled fructose and sucrose were compartmented in addition to glucose. In protoplasts and skin segments the synthesis and compartmentation of sucrose was associated with rapid uptake of ^{14}C from glucose or fructose. Isomerization of glucose to fructose was vectorial and the reaction was unaffected by pool equilibria. These results provide substantial evidence for a group translocator operating in/at the tonoplast. A 1:1 ratio of hexoses in the vacuolar sap would thus arise by virtue of the sucrose translocator at the tonoplast, rather than as a consequence of that ratio in phloem sugar (sucrose).

9. From the incubation of grape protoplasts in ^{14}C-glucose, labelled phosphates of glucose, fructose and sucrose were recovered. Their recovery provides support for the mechanisms proposed. From the incubation of skin segments there was evidence of glucose epimerization to galactose, an uncommon free sugar in higher plants.

Radioactivity was also recovered in compounds partially identified as the galactose-containing sugars melibiose and raffinose. The presence of these metabolites suggests an exaggerated or perturbed in vitro metabolism thought due to high levels of cytoplasmic sugar. This result may also explain the fact that pre-veraison skin in vitro accumulates sugar in the manner of post-veraison skin.

10. It is concluded that group transport of glucose and fructose occurs across the tonoplast of grape pericarp cells, most probably via a multienzyme complex associated at that membrane and including sucrose phosphate synthase and phosphoglucose isomerase. The proposal of such a mechanism for sugar transport is novel for higher plants for which most proposals have been couched in terms of proton/sugar symports. Techniques are available to further examine each element of the postulated accumulatory process.

STATEMENT

I hereby declare that the thesis here presented is my own work, that it contains no material previously published, except where due reference is made in the text, and that no part of it has been submitted for any other degree.

(Spencer C. Brown)

PREFACE

This thesis covers three subjects which warm the soul of many a searcher: the grape, membrane transport, and the developing field of protoplast and vacuole isolation.

I am very grateful to my supervisor, Dr. Bryan Coombe, who displays the many qualities of both teacher, scientist and friend. Moreover, many of the ideas and approaches in this thesis arise from his appreciation of development in the grape berry.

I am also indebted to my alternative supervisor, Dr. Colin Jenner, and to Dr. Gordon Edwards; and to Pauline Phillips who so often assisted and instructed me in the laboratory. My thanks extend to each member of the Department of Plant Physiology. One is fortunate to receive not only aid but friendship from so many. I should especially like to thank Maarten and Alison Ruiter, Daryl Joyce, Priya Aiyar, Jenny Creasey, Allan Robins and each of the postgraduate students.

The assistance of Trevor Hancock (Biometry Section), Brian Palk (Photography), Dick Miles (Electron Microscopy) and Mrs. Fuss (typing) is acknowledged, as too the cheerful attention of the library staff. Financial support was provided by the University of Adelaide Research Grant.

Martine, and little Carmen and Pascal, you have been a great joy and encouragement to me during these years. And I am most grateful to my parents for their unfailing support.

This thesis was undertaken with the dust of India still upon the sandals. Whatever may or may not be achieved with pen and ink, the writer holds affectionate memories of many like Dileep who never learnt to read but learn to till the soil and to tend their beasts.

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SYMBOLS AND MISCELLANEOUS ABBREVIATIONS

Atm.	atmospheres
^{o}Brix	g solute/100 g solution
CAM	Crassulacean acid metabolism
cpm	counts per minute
cv., cvv.	cultivar, (s)
D	diffusion coefficient
Ea	free energy of activation
ER	endoplasmic reticulum
F	Faraday constant
J	flux
k_D	diffusion constant
K_i	inhibitor constant
K_m	Michaelis constant
K_T	affinity constant
LSD	least significant difference
meq.	milliequivalents (m mol x z)
NMR	nuclear magnetic resonance
PMR	protonmotive force
Q_{10}	temperature coefficient, ratio of rates at temperatures differing by 10^{o}C
R	gas constant
R	relative mobility
r	correlation coefficient
RQ	respiratory quotient
S	solute
SD	standard deviation
SEM	scanning electron microscopy
SER	smooth endoplasmic reticulum
T	temperature
z	valence of ion
α	activity coefficient
ϵ	extinction coefficient
η	viscosity
μ	chemical potential
$\bar{\mu}$	electrochemical potential
ρ	density

$\psi_{(m)}$	(membrane) electrical potential
Ω	electrical resistance
ω	mobility coefficient

ABBREVIATIONS (CHEMICALS)

ABA	abscisic acid
ACS	Amersham scintillation cocktail
ADP, ATP	adenosine 5'-diphosphate, 5'-triphosphate
ADPG	adenosine 5'-diphosphate glucose
BSA	bovine serum albumen
CCCP	*m*-chloro-carbonylcyanidephenylhydrazone
CEPA	2-chloroethylphosphoric acid
ConA	concanavalin A
DCCD	N, N'-dicyclohexyl carbodiimide
DEAE-	diethylaminoethyl-
DES	diethylstilbestrol
DHAP	dihydroxyacetone phosphate
Dio-9	antibiotic of unknown structure
DMO	5, 5-dimethyl-2, 4-oxazolidine dione
DMSO	dimethylsulfoxide
DNP	2, 4-dinitrophenol
DTT	dithiothreitol
EDAC	ethyl-3-(3-dimethylamino propyl carbodiimide)
EDTA	ethylenediamine tetraacetic acid
FC	fusicoccin
FDA	fluorescein diacetate
F6P	fructose 6-phosphate
GA	gibberellic acid
GAE	gallic acid equivalents
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
G6P-DH	glucose 6-phosphate dehydrogenase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HK	hexokinase
IAA	indoleacetic acid
MES	2-(N-morpholino) ethanesulfonic acid
NAD(H)	nicotinamide adenine dinucleotide (and reduced form)
NADP(H)	nicotinamide adenine dinucleotide phosphate (and reduced form)
NDP, NTP	nucleoside 5'-diphosphate, 5'-triphosphate
NEM	N-ethylmaleimide
Pi	inorganic phosphate
PCMB	*p*-chloromercuribenzoic acid
PCMBS	*p*-chloromercuribenzene sulfonic acid
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PGA	phosphoglyceric acid
PGI	phosphoglucose isomerase
PM	protoplast medium
PMSF	phenylmethylsulfonyl fluoride
POPOP	1, 4-bis[2-(5-phenyloxazolyl)] benzene
PPase	phosphatase
PPi	inorganic pyrophosphate
PPO	2, 5-diphenyloxazole
RNA	ribonucleic acid
U-^{14}C-sugar	uniformly ^{14}C-labelled sugar
UDP	uridine 5'-diphosphate
UDPG	uridine 5'-diphosphate glucose
VM	vacuole medium
3-O-MG	3-O-methylglucose

CHAPTER 1: DEVELOPMENT OF THE GRAPE BERRY

DEVELOPMENT OF THE GRAPE BERRY

1.1 The Grape Inflorescence

Development of the grape berry proceeds from initiation of Anlagen, in temperate climates, in the summer preceding flowering and fruiting. These primordia develop on the primordial shoot of the latent bud in two ways : highly branched, becoming inflorescences; and sparsely branched, becoming tendrils. (The ontogeny of tendril and floral primordia has been investigated by Srinivasan and Mullins (1976, 1978, 1979, 1980a, b) with the concept that a tendril is a weakly differentiated inflorescence.) In the spring, with additional branching and differentiation of these primordia, the inflorescence develops as a bunch - a pyramidal branched inflorescence with terminal flowers. The period from budburst and initiation of floral primordia to anthesis is about nine weeks; synchrony of development in the primordia (and hence, of anthesis) with that in the shoot has been noted (Coombe, 1973; Pratt and Coombe, 1978).

The flower is syncarpous and biloculate, each locule containing two anatropous ovules. The bunch is a raceme or panicle and the peduncle bears flowers, each on a pedicel.

Anthesis may be conveniently prescribed as 70% of calyptras fallen. Fruit set is completed within two weeks of anthesis. It is "normally" only 5% (Coombe, 1973) in cv. Muscat Gordo Blanco (hereafter referred to as Muscat).

The first stage of fruit growth is characterised by a rapid change of ovary volume, in a sigmoidal manner. Seed development is substantial during this stage, through the sequence: divisions of endosperm nuclei followed by wall formation, cell division of the embryo, and testa hardening by c. 45 days post-anthesis. The testa hardening coincides with a slower growth of ovary volume as the berry enters what is

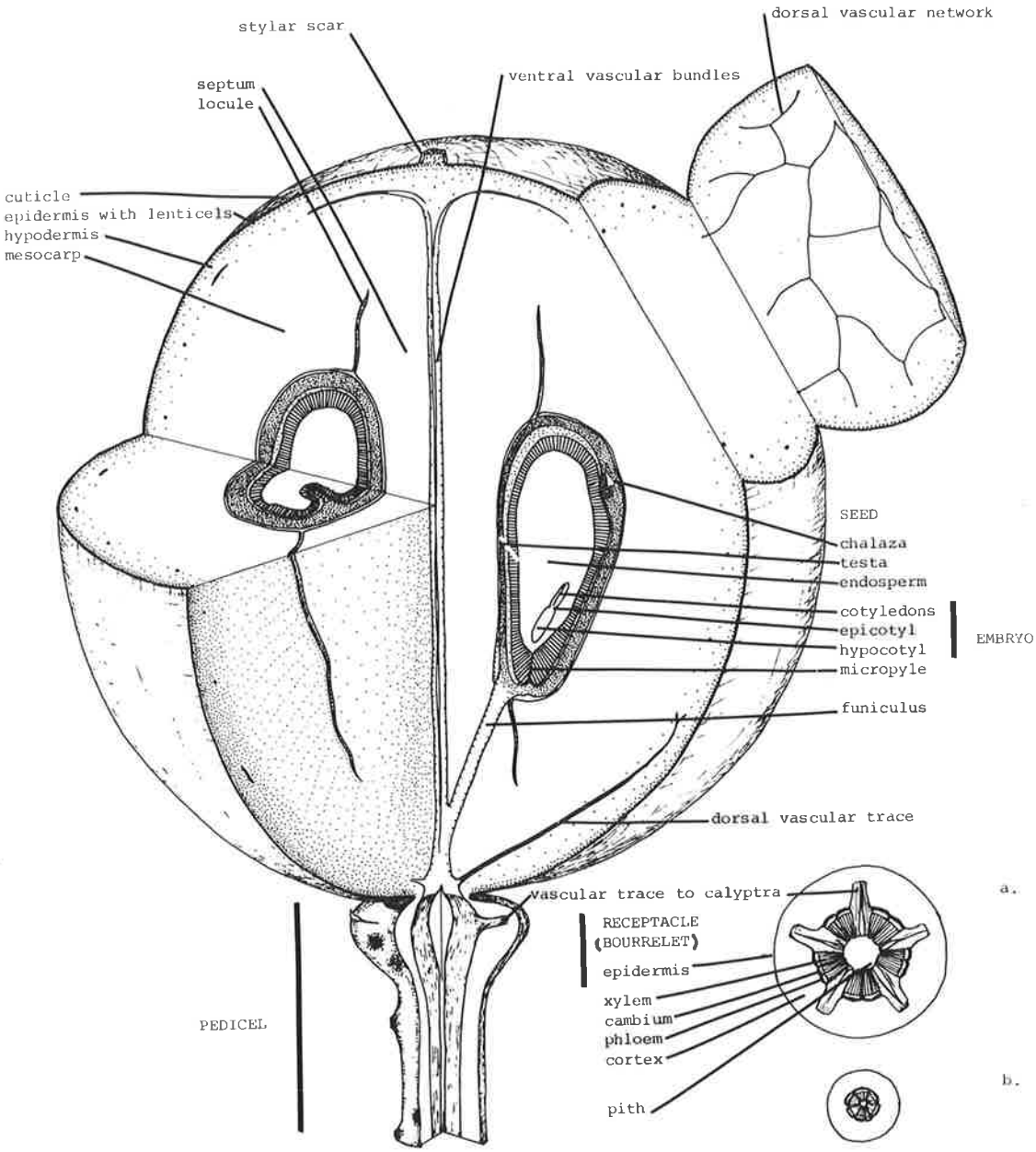


Fig. 1.1 Composite sections of a grape berry, including T.S. of (a) receptacle and (b) pedicel base.
From Coombe (pers. comm.)

Scale 1:10

often termed the lag phase. Maturation of the seed continues and the embryo attains full size by veraison (anglicised from *véraison* fr.); veraison is the inception of fruit maturation and is marked by rapid increases in berry volume, sugar content, softness and by pigment changes. Parts of the berry are identified in Fig. 1.1.

1.2 Designation of Development

The classical designation of the phases of development in fruits with a double-sigmoid growth curve (Connors, 1914) assigns three stages:

I. the first post-anthesis stage of rapid volume growth;
II. the slow growth or lag phase; and
III. the final rapid volume growth (Fig. 1.2).

For cv. Muscat in temperate Australian conditions these stages are c. 65, 20 and 60 days respectively (Considine, 1979) - stage II being particularly variable, between 8-48 days (Coombe, 1973, 1980). Alternative classifications are discussed in Peynaud and Ribéreau-Gayon, (1971), Pratt, (1971) and Coombe, 1976a. But as Coombe (1976a) clearly indicated, the classification has weaknesses such as the indeterminate nature of the boundaries to the stages. Furthermore, stage II may be a "non-entity", a period best characterised as the slowing of processes

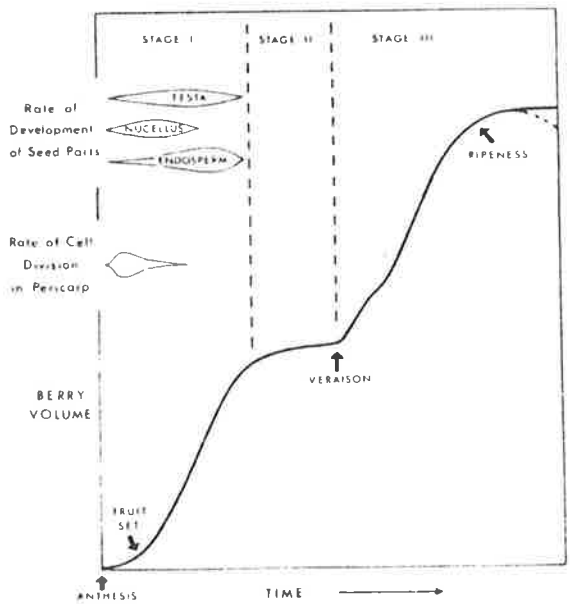


Fig. 1.2 Diagram of the volume-time curve of a grape berry from anthesis to ripeness and of the rate of development of seed parts. The division of growth into stages I, II, and III is indicated. (from Coombe 1973)

of stage I and acceleration of those of stage III. As shall be discussed later, the smooth sigmoidal curves used to portray growth (cumulative curves of diameter, length, volume or weight) may be characteristic of populations whilst individual fruits may, with respect to certain attributes, exhibit discontinuous changes - sharp inceptions rather than gradual accelerations. (However, a sigmoid deceleration may generally be accurate.) To this extent the delineation of stage II and stage III may be tolerated. Following the precedent of Coombe (1980), the terminology here shall be: a first growth cycle (acceleration and deceleration), slow growth or lag phase, and a second growth cycle (acceleration and deceleration).

1.3 Ontogeny of the Pericarp

In an elegant investigation entitled "Biophysics and Histochemistry of Fruit Growth and Development", Considine (1978, 1979; Considine and Knox, 1979a, b) described the ontogeny and properties of the cells of the grape berry from before anthesis through to fruit maturity. Considerable detail of his work is presented here, with emphasis upon the pericarp of berries cv. Muscat Gordo Blanco, the major source of material in experiments of the present thesis.

The grape pistil develops with three phases of pericarp differentiation; ring meristem formation, cell proliferation by anticlinal cell divisions and, just prior to anthesis, periclinal cell division and differentiation.

> At anthesis the pericarp consisted of a uniseriate outer epidermis, a single layer of (small) hypodermal cells, inner and outer mesocarp (with large tanniferous vacuoles), separated by an anastomosing network of vascular bundles and a two-layered endocarp of druse-containing (calcium oxalate) hypodermal cells and inner epidermis.

By anthesis the capital of cells available for fruit development has been established and in particular the hypodermal mother layer is differentiated.

Just after anthesis the hypodermal layer divides periclinally to give rise to the several layers of the skin followed (c. day 10) by proliferative anticlinal cell divisions. The epidermis is conserved with anticlinal divisions. Having assessed berries stereologically at 3-day intervals from floral initiation, Considine established a hypothesis about cell lineage of the dermal system, depicted in Fig. 1.3.

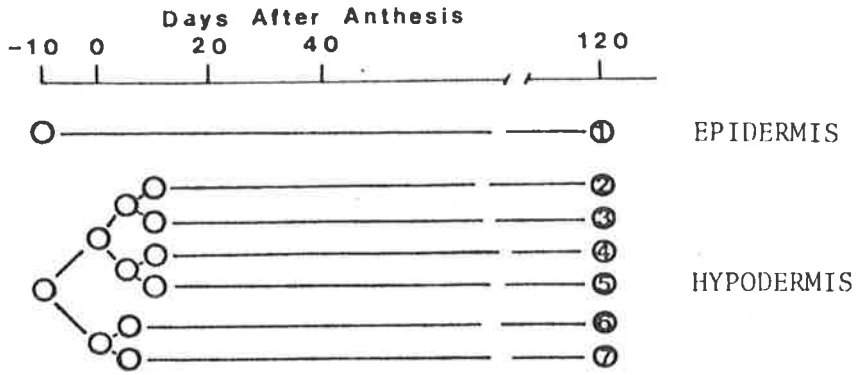


Fig.1.3 A hypothetical cell lineage for the dermal system of fruit of cv. Muscat constructed from frequency of appearance of periclinally oriented new cell layers during early fruit growth.
(from Considine 1979)

Considine considers that the crucial divisions are those which signal the differentiation of new tissue types and these are commonly periclinal. He argues against the concept of a pre-determined number of cell cycles intrinsic to each cell and against the notion that cessation of cell division across the tissue leaves cells at various stages of the cell cycle. He finds indirect evidence, however, for endomitosis in the pericarp, especially in the inner mesocarp, and proposes that such might be target cells for hormone action (as observed in the abscission layer of *Ecballium* flower buds : Wong and Osborne, 1978).

The following attributes (averages) of mature Muscat berries were calculated by Considine: 5.9 g, radius 10.8mm, cuticle 4µm, mean cell number in epidermal layer 1.68×10^6, in hypodermis (layers 2-8, defined as having walls larger than 1µm) 2.2×10^6. The mean cell volume was 8×10^3 $(\mu m)^3$ in the epidermis, 7.5×10^5 $(\mu m)^3$ at layer 7. In Muscat the generalisation holds : cell volume doubles with each layer through the dermal system. The proportion of total volume occupied by cell wall, in the outer 140µm of the berry (the skin), was 12.4%. The cell walls at layer 4 and 5 were thickest (c. 4.2µm).

The collenchymatous cell walls of the dermal system result from thickening which occurred from two weeks after anthesis till the end of the first growth cycle. Histochemical techniques indicated a poly-anionic, lamellate composition in which cellulose microfibrils were embedded in a matrix of hemicelluloses and pectins. In addition to pit fields, an unusual transverse striation of pectic material was observed in the hypodermis.

At veraison a sudden, transitory swelling of the hypodermal cells was observed, suggestive of cell wall hydration consequent upon glycosidase activity. This may have been coincident with the marked increase in fruit elasticity associated with veraison (Coombe, 1974; Coombe and Bishop, 1980). Thereafter the walls thinned and at the end of maturation appeared to be partially degraded. A more substantial dissolution of the cell walls occurs in layers of the outer mesocarp, such that when a berry is peeled the hypodermal and epidermal layers are conveniently removed with neither vascular nor parenchymous tissue. Stretching and partial degradation of the walls of the inner mesocarp parenchyma occurs. The vacuoles of these walls have so expanded that only a very thin cytoplasmic layer remains, appressed against the thin wall, with trapped chloroplasts and nuclei.

Considine remarks upon the occurrence of polyphenols in most pericarp cells at anthesis, though those of the outer epidermis and the mesocarp stained differently. By day 16 there were two cell types between the hypodermal layers and the position of the vascular bundles, one containing polyphenols and the other not, these latter cells occurring in distinct patches and smaller than tanniferous cells. By day 26 there was a reduction in polyphenols in all except the dermal system.

> It seems [that] despite the similar ability of inner and outer mesocarp cells to accumulate sugars during ripening, the cell of these tissues are metabolically differentiated in the first growth phase.

During the second growth cycle there appeared an interspersion of acidic polysaccharide material (by histochemical analysis) amongst the tanniferous precipitates within the vacuole.

1.4 The Cuticle

The cuticle of the pericarp is evident at anthesis and, during the first growth cycle, three layers can be distinguished by fluorescence microscopy. These were termed the inner procutin, the polymerised cutin, and the primary cuticle. The increase in surface and cuticle area of a berry cv. Muscat is c. 400-fold in the first cycle and 1.6-fold in the second. Considine designates a structural function to the cuticle - to withstand the stresses of turgor; he argues against growth of the cuticle by a process of cracking and filling-in.

The cuticle is quite constant in thickness throughout berry development (Radler, 1965). It consists largely of triterpenes (70%) and substantially limits gaseous exchange, including transpiration (Peynaud and Ribéreau-Gayon, 1971), and protects against microbial invasion (Bessis, 1972; Bernard 1977a, b). The developing epidermis of each berry has 25-40 stomata (in cv. Pinot : Bessis, 1972); in older berries there are c. 15-35 suberized lenticels often associated with stomata (Swift et al., 1973; Bernard 1977a, b; Nakagawa et al., 1980).

1.5 Vascular Connections

The vascularization of the ovary consists of four ventral (axial) carpellary bundles with branches to each ovule and an anastomosing network of dorsal bundles lying sub-dermally. The ventral and dorsal systems join at the top of the ovary (Fig. 1.1).

A recent histological investigation has revealed the particular order of the vascular connections between the bunch and leaves, tendrils and shoots (Fournioux and Bessis, 1979). The principal connections are "unilateral", linking structures of the same orthostic. On each side of a shoot are three systems : one linking leaves, bunches and tendrils and two others linking bunches, tendrils and axillary buds. A "bilateral" system links leaves, bunches and tendrils of the same and opposite orthostics. The axillary buds are never directly connected to leaves, only to bunches or tendrils.

1.6 Constituents of the Berry

1.6.a Introduction

The constituents of the grape berry have been reviewed in substantial volumes: e.g. J. Ribéreau-Gayon and Peynaud (1960, 1971), Peynaud and P. Ribéreau-Gayon (1971), Winkler et al. (1974) and an O.I.V. Symposium (McNamara, 1977). It is difficult to abbreviate the extensive research into constituents of the berry, let alone the variation in those constituents which is of fundamental interest to oenologists. The following remarks simply recall elements which, *a priori*, could be considered in a study of mechanisms of sugar accumulation and of changes at veraison.

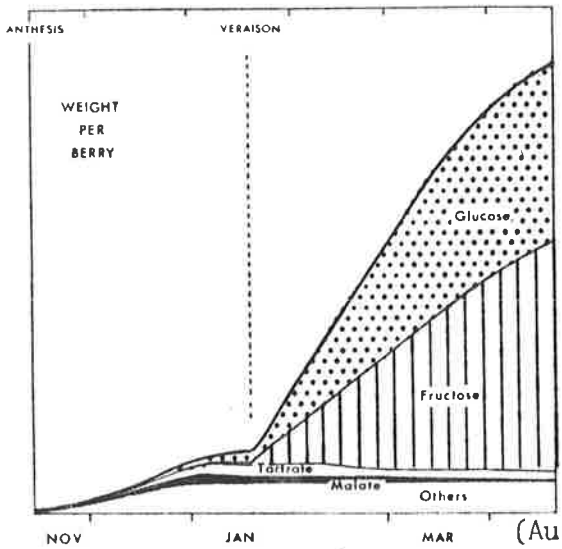
It is unfortunate that many analyses of the berry have not considered the distribution of a constituent between at least the elementary components: the seed, the skin and the fleshy mesophyll. As is evident in Table 1.1 there are major differences.

Table 1.1: Ratio of concentrations of different constituents in the skin relative to the flesh of ripe grape berries cv. Sauvignon, deduced from Peynaud and Ribéreau-Gayon, 1971. (by FWt).

Constituent	Ratio $\frac{\text{conc. in skin}}{\text{conc. in flesh}}$
reducing sugars	0.46
$[H^+]$	0.14
free acids	7.6
titratable acids	33
tartaric acid	11
malic acid	16
citric acid	38
soluble phenols	essentially, in skin only

1.6.b Water and carbohydrates

The major constituent of berries is water, which shall be considered in chapter 2. The next major constituents, in the ripe berry, are the carbohydrates (Fig. 1.4).



(Australian)

Fig. 1.4 Typical amounts of glucose, fructose, tartrate, malate and "other" components of dry weight, on a per-berry basis. (from Coombe 1975)

Glucose accounts for 85% of the sugar in grapes in the first growth cycle, but after veraison the ratio glucose:fructose approaches unity (Kliewer, 1967; Harris et al., 1968). In ripe and over-ripe grapes fructose may be in excess of glucose. Though sucrose is the main sugar translocated in the grapevine (Swanson and Elshishiny, 1958) it is usually less than 1% F.Wt. in the berry (Kliewer, 1966; Downton and Hawker, 1973; Rapp et al., 1977). Pentoses (mainly arabinose) may be present in small amounts, as also are stachyose, verbascose, manninotriose, raffinose, melibiose, maltose and galactose (Kliewer, 1966; Winkler et al., 1974). Traces of starch are found in most cells of the pericarp at anthesis but at maturity only in the epidermis (Downton and Hawker, 1973; Swift et al., 1973).

Current leaf photosynthesis is the main source of sugar for maturation of the berry; remobilization from stem reserves probably only occurs under abnormal conditions such as defoliation (Matsui et al., 1979). Ruffner and Hawker (1977) determined the activities of the enzymes of gluconeogenesis and moreover calculated that the maximal observed consumption of malic acid in a berry could only account for 5% of sugar accumulation (assuming no fresh malate was formed after veraison).

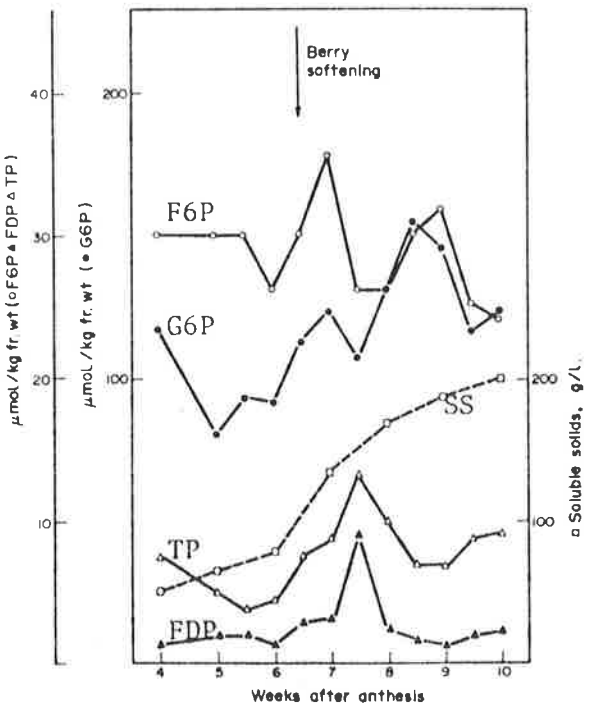


Fig. 1.5 Contents of fructose-6-phosphate (○), fructosediphosphate (▲), triose phosphates (△), glucose-6-phosphate (●) (larger scale) and soluble solids (□) of developing Sultana berries.
(from Ruffner & Hawker 1977)

Fig. 1.5 indicates the level of several phosphorylated carbohydrates from the latter part of the first growth cycle. Rapid rates of sugar accumulation in the berry are associated with peaks of triose phosphates, fructose diphosphate, fructose-6-phosphate and increasing levels of glucose-6-phosphate. The G6P/F6P ratio goes from 3 to c. 5.

Pectins and pentosans comprise about 0.5% F.Wt. of the berry. Other polysaccharides have been mentioned.

1.6.c Organic acids

The acidity of berry juice at veraison is c. 300-500 meq. ℓ^{-1} and at ripeness 100 meq. ℓ^{-1}. Most of the osmotic potential of cells in a berry before veraison is attributable to organic acids. At veraison L-malic and D-tartaric comprise 90% of the total acidity and the concentration of each declines thereafter to different degrees depending, amongst other factors, upon the climate. Malate accumulation has a temperature optimum of about 20°C (Lakso and Kliewer, 1974). Malate may attain 150 mM in the berry and a high level is maintained during the lag phase even if this is lengthened by auxin application (Hale, 1968). After veraison malate decline tends to be initially rapid. Tartaric acid per berry tends to remain constant.

Ribéreau-Gayon demonstrated that the main biosynthetic pathway of malic acid was by β-carboxylation of pyruvic acid via PEP and oxaloacetate by PEP-carboxylase with oxidation of NADH. Degradation occurs via NADP-dependent malic enzyme and respiration of the resultant pyruvate (Peynaud and Ribéreau-Gayon, 1971). In green grapes glucose can be converted to malate, just as the reverse may occur in riper grapes, by gluconeogenesis through PEP-carboxykinase (Ruffner and Kliewer, 1975; Ruffner et al., 1975).

Tartaric acid is believed to be an oxidation product of glucose (Loewus, 1971; Ruffner and Rast, 1974; Saito and Kasai, 1978). It is synthesised in young leaves and berries (Ribéreau-Gayon, 1966).

The free acids tend to convert to salts (mono- and di-basic) during ripening. As potassium is the major counter-ion, potassium bitartrate crystals may form. A steady increase in pH of the juice, from c. 3.0 to 3.8, results from this net decrease in acid, and also from the conversion to salts and from the dilution of acids by water due to berry growth.

1.6.d Minerals

During the second growth cycle there is an increase per berry in cations such as K^+, Na^+, Ca^{2+} and Mg^{2+} and in anions such as P_i.

Studies on the developing legume fruit (Pate and Hocking, 1978; Pate et al., 1978) indicated that 97% of dry matter entered the fruit via the phloem (including most of the C, N, S, P, K^+, Mg^{2+}, Zn^{2+} in various forms) while 73% of the water entered by the xylem (including much of the Ca^{2+} and Na^{2+}).

Studying the effect of salinity upon compositional changes during berry ripening, Downton and Loveys (1978) found chloride advanced veraison but the sequence of events was unaffected, namely, ABA and reducing sugars increased, softening occurred, proline, arginine, K^+ and Cl^- increased and titratable acidity declined.

Of the total K^+ in a Shiraz berry, 45% may be in the skin (Somers, 1975). Hrazdina and Moskowitz (1981) have determined the potassium content of skin vacuoles in ripe berries cv. Dechara (red) to be 2.73 M (tartrate 1 M; osmotic potential 1.2 Osmolal)! This value, if correct, must be affected by potassium depositions, notably the potassium bitartrate salt.

Boulton (1980a, b, c) noted that discrepancy between titratable acidity and total acidity (the pool of acid anions) and showed that monovalent metal cations, particularly potassium, could account in a stoichiometric sense for this. Subsequently he postulated the existence of a plasmalemma-bound proton-translocating K^+-ATPase in the berry.

Hale (1977) examined the relationship between K^+ and malate in the skin and pulp of Sultana berries of differing K^+ concentrations due to varied K^+-nutrition and to rootstock effects. K^+ constituted 70% of mineral cation content in ripe grapes. In a two-month period from the lag phase potassium concentration doubled in the skin and pulp while malate concentration declined to one-tenth of its initial level. Hale found no significant correlation between K^+ and malate during the acid accumulating stage of berry development, but between treatments on the same day during ripening there were significant direct correlations.

Similarly, berries removed from the vine at this stage lost malate during seven days in inverse proportion to their potassium content. Citing efflux studies with tomato pericarp, Hale speculated upon a causal relationship:

(i) that tonoplast permeability to malate determines the acid's catabolism and

(ii) that potassium content and tonoplast permeability are inversely related.

But this is contrary to the cited work in which Vickery and Bruinsma (1973) indicated that during ripening, and despite the climacteric and its associated ethylene release, permeability of neither the plasmalemma nor the tonoplast changed. "Increasing activities of ions in the cytoplasm, rather than increasing membrane permeabilities, may explain ... that the efflux of solutes (not specifically malate) from fruit tissues increases during ripening."

1.6.e Nitrogen (N)

Ammonium cations account for 50% of N in immature grapes and for 25% of N in ripe grapes (Peynaud and Ribéreau-Gayon, 1971). The predominant free amino acids of grape are proline and arginine (Nassar and Kliewer, 1966) and each increases during maturation. Proline in centrifuged juice from ripe berries may be 6 mM (Coombe and Monk, 1979) or up to 40 mM (Downton and Loveys, 1978). If this were concentrated in the restricted cytoplasmic volume it would establish an osmotic potential of 2500 kPa, similar to that in the vacuole due to sugars.

1.6.f Adenylates

Ruffner and Hawker (1977) found an increase in the ATP/ADP ratio from one week prior to veraison of berries cv. Sultana. This persisted except for one sampling just after veraison and at maturity.

Ben-arie and Faust (1980) reported high ATP levels in green strawberries, decreasing with ripening. They proposed that a peculiar Mg^{2+}-inhibited ATPase activity in the same fruit was associated with sugar uptake.

1.6.g Phenolics

The demonstration of phenolics in many cells of the developing pericarp has already been mentioned (Considine). These constituents have been intensively studied by Ribéreau-Gayon (1964). It appears that

shikimic acid is synthesized in leaves of the vine, then is translocated to the berries where synthesis of phenols occurs.

Phenols constitute c. 0.1-0.6% F.Wt. of grapes. Per berry, phenols increase during 30 days beginning one week after the large increase in reducing sugars; however, the concentration decreases (Singleton, 1966; Pirie and Mullins, 1980). Most phenolics are in the skin with some in the seeds and conducting tissue (Hawker et al., 1972). Juice contains principally non-flavonoid, chlorogenic acid-like phenols, especially the tartaric acid esters of caffeic acid (caftaric acid) and *p*-coumaric acid (coutaric acid) (Ribéreau-Gayon, 1964; Singleton et al., 1978). Skin contains some of these esters but especially the flavan phenolics such as *d*-catechin and *epi*-catechin and various dimeric and polymeric flavonoids (Lea et al., 1979). Somers (1976) reported no qualitative change in composition of total anthocyanins during ripening (cv. Shiraz). The pigments of white grapes are leuco-anthocyanins.

Pirie and Mullins (1976, 1977) proposed that, in the ripening berry, sugars and phytochrome in the skin regulate the production of anthocyanins, and polyphenols in general. The one week lag between sugar and phenol increases in the skin does not support this. Nor could Wisseman and Lee (1980) find any correlation between total phenolics and polyphenoloxidase activity.

Phenols readily condense to tannins (Swain, 1965) and artefactual products may often arise during extraction procedures by such condensation (Somers, 1976).

1.6.h Enzymes

Certain enzymes have already been mentioned in the context of their substrates.

The marked softening of the berry at veraison is attributed to modification of the cell wall properties by enzymes such as polygalacturonases, pectinesterases and, especially in the skin, pectin methylesterases (Peynaud and Ribéreau-Gayon, 1971). The activity of the latter was unaffected by growth of vines in a saline medium, though rate of berry softening was affected. Thus cell wall modification does not associate directly with any single enzyme activity, despite a general increase at veraison (Datunashvili et al., 1977).

Hawker (1969a, c) has shown in cv. Sultana the following changes in enzyme activity per berry : invertase reaches a maximum at veraison; hexokinase activity parallels berry F.Wt.; and glucose-6-phosphate dehydrogenase decreases from a peak late in the first cycle. Sucrose phosphate synthase and sucrose phosphate phosphatase showed similar trends to reducing sugar concentration. After a marked decrease in sucrose synthase prior to veraison (almost the inverse of the invertase trend) sucrose synthase activity increased in a manner which paralleled the reducing sugar curve. (Meynhardt et al., 1974 found similar activities in an extract from Barlinka berries.)

In these Sultana berries the maximal rate of sugar accumulation was 1.1 μmoles hexose $(g\ F.Wt.)^{-1}\ (h)^{-1}$. *In vitro* activities of these enzymes were sufficient to maintain such accumulation if it involved sucrose breakdown by sucrose synthase or invertase, hexose phosphorylation, synthesis of sucrose phosphate, and its hydrolysis, as in the mechanism proposed for sugar uptake in sugarcane (Glasziou and Gayler, 1972a, b).

Figs 1.6 and 1.7 are reproduced from Hawker's paper. Enzyme activity per berry is indicated on different scales : invertase and sucrose phosphate phosphatase activities are c. 100- and 10-fold greater, respectively, than the activities of the other sucrose enzymes.

Downton and Hawker (1973; Hawker and Downton, 1974) attributed the very low starch content of berries to low activities of ADPG-starch glucosyltransferase and ADPG pyrophosphorylase, while phosphorylase, amylase and UDPG pyrophosphorylase activities increased 6- to 7-fold from early in growth to just after veraison. Phosphofructokinase remained low from four weeks after anthesis (cv. Cabernet Sauvignon : Ruffner et al., 1976). Berries also contain α-glucosidase (Peruffo et al., 1978).

1.7 Studies of Gaseous Exchange

Recent investigation of photosynthesis and respiration in grape berries have shown changes with time that differ somewhat from the early work of Geisler and Radler (1963). Refixation of O_2 and CO_2 inside berries may be considerable and this should be taken into con-

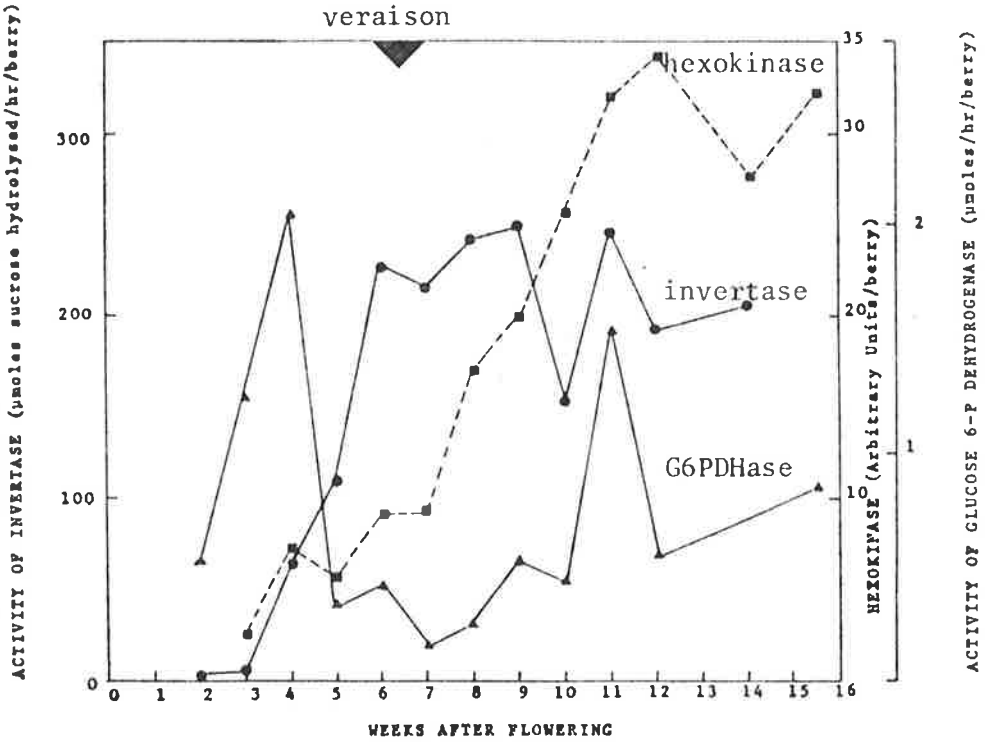


Fig.1.6 ACTIVITIES PER BERRY OF INVERTASE (●), HEXOKINASE (■) AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE (▲) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.
Note the different scales on the ordinate axes.
(from Hawker 1969a)

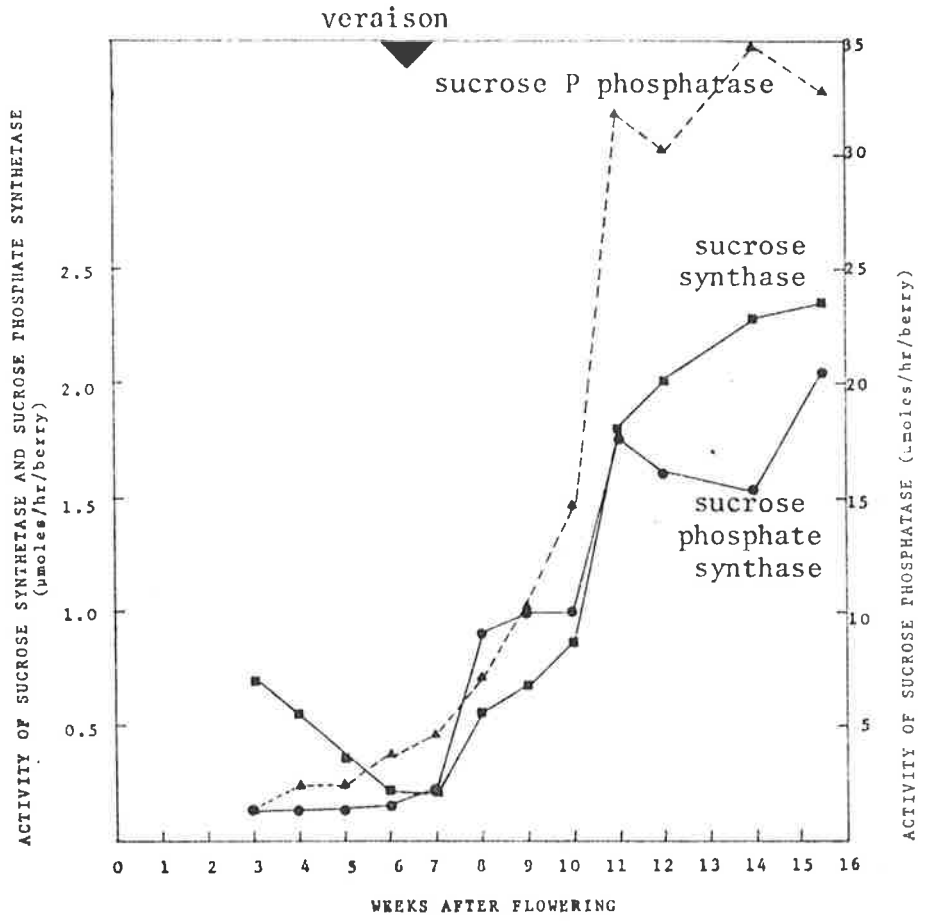


Fig.1.7 ACTIVITIES PER BERRY OF SUCROSE PHOSPHATASE (▲), SUCROSE SYNTHETASE (■) AND SUCROSE PHOSPHATE SYNTHETASE (●) EXTRACTED FROM DEVELOPING SULTANA GRAPE BERRIES.
Note the different scales on the ordinate axes.
(from Hawker 1969a)

sideration; likewise experiments with $^{14}CO_2$ fixation at different stages of berry development (e.g. Matsui et al., 1980) should take account of an effective variation in specific activities due to this. Kriedemann (1968) demonstrated that dark CO_2 fixation was relatively high and may exceed the previous rate of photosynthesis. Both decline from veraison. Fermentation may also occur at high temperatures (above 35°).

The following is the situation in several cultivars (Koch and Alleweldt, 1978).

- Per berry, respiration (both as O_2 uptake and CO_2 release) and photosynthesis (as CO_2 release) are maximal at the beginning of the lag period and decline thereafter except for a small respiratory rise at veraison, especially at high temperatures.
- Per g F.Wt., respiration and photosynthesis are highest at the middle of the first growth cycle (no earlier stage was assessed). Photosynthesis is always less than respiration (in CO_2 units). Per unit FWt., photosynthesis in the berry is about one-tenth that in the leaf (Ribéreau-Gayon, 1966).
- The respiratory quotient (RQ) of the berry in the first growth cycle is about unity, and rises at veraison to c. 1.3, consistent with a change from oxidation of carbohydrate to oxidation of malic acid (Harris et al., 1971).
- The volume of extractable gas per berry is at a peak during the lag phase (40 ml kg^{-1} berry) and rapidly declines thereafter (Alleweldt and Koch, 1977).

Other reports differ as to the timing of peaks (cf. cv. Delaware : Niimi and Torikata, 1979) but there is agreement on the change in RQ and the trend to very low photosynthesis and respiration during maturation.

Kriedemann (1968) states, without reference, that chlorophyll concentration in the grape berry declines at and after veraison. However, in berries cv. Delaware, Matsui et al. (1980) showed a 90% decline of chlorophyll per cm^2 of skin occurred during the 20 days preceding the rapid sugar accumulation of veraison. The same workers reported a surprising 30-fold increase in light-dependent incorporation by berries of $^{14}CO_2$ into glucose and fructose for several weeks following veraison.

That this was not due to altered CO_2 diffusion or specific activity effects was indicated by a constant unchanged dark fixation as also the considerable shift in labelling pattern. By what process might this ^{14}C incorporation to sugars occur if it is not photosynthetic yet is photodependent? Gluconeogenesis is a possible pathway, with the novel condition that it cease in the dark - such as by unavailability of substrate (e.g. malate) or of $^{14}CO_2$.

1.8 Malate, its Enzymes and Gluconeogenesis

PEP-carboxylase activity was high throughout berry development (Ruffner and Kliewer, 1975) except for a low value at veraison coincident with cessation of malic acid synthesis. The unique enzyme for gluconeogenesis, PEP-carboxykinase, showed consistently high activity from a peak four weeks post-anthesis (Ruffner and Kliewer, 1975). Essentially a change from glycolysis to gluconeogenesis occurs about veraison. Ruffner and Hawker (1977) concluded that phosphofructokinase and pyruvate kinase were the two major sites of control in the reaction sequences between sugars and acids in the grape. Moreover, Ruffner et al. (1976), noting the activation of sucrose phosphate synthase in *Vicia faba* (Turner and Turner, 1975), suggested that malate and citrate may not only mediate glycolysis (via phosphofructokinase) but also direct the incoming sugars towards accumulation by stimulating the synthase.

Gluconeogenetic activity of berries cv. Pinot noir was 5-fold greater at veraison than at maturity (Ruffner et al., 1975).

Malic enzyme, pyruvate decarboxylase, and malate dehydrogenase show no clear trend following veraison (Hawker, 1969b). Any increase in these enzymes was toward maturity. Malate decline is temperature-dependent (Kliewer, 1973) but neither the temperature-dependency of these enzymes nor that of PEP-carboxykinase (Ruffner and Kliewer, 1975) accounts for this. Therefore the decrease in malic acid is attributed to changed compartmentation thought to be temperature-dependent in the manner so characteristic of malate decline.

Steffan and Rapp (1979) envisaged two pools of malate in two tissue types : a metabolized (respired) pool in the outer pericarp and a virtually non-metabolized pool in the inner pericarp, with exchange

between the two after veraison. This proposition was based upon the contrasting metabolism of malate applied to berries by the pedicel (and metabolized) or injected into the flesh (less metabolized). As mentioned, Hale (1977) proposed two intracellular pools of malate, and Lakso (in Ruffner and Kliewer, 1975) estimated the level of malic acid in the cytoplasmic pool of grape berries at 20°C to be 8.5 times greater than at 40°C.

One may further speculate upon the unusual light-dependent $^{14}CO_2$ fixation after veraison reported by Matsui et al. (1980). Across a permselective membrane malic acid - as a weak acid - will tend to accumulate in the more alkaline of two compartments by virtue of anionic trapping. Therefore the notion of a "leaky membrane" is inconsistent with the presumed vacuolar compartmentation. As gluconeogenesis is predicted to be, in part, a compartmented metabolism (Ap Rees et al., 1974), might not the activity of key enzyme(s) be dependent upon malate distribution between the cytoplasmic matrix and a subcellular entity other than the vacuole? That distribution might be determined by pH and indeed be light-dependent. An analogy is found in the light-sequestration of ABA by chloroplasts (Heilman et al., 1980).

1.9 Hormones

Though the significance of hormones in fruit physiology has long been evident (Nitsch, 1965) little progress has been made in clarifying their specific mechanisms of action (McGlasson et al., 1978). But techniques are available for more fundamental questions to be investigated than in the past, such as concerning cell physiology, molecular interactions and cell differentiation.

A perspective of hormone interactions in the grape berry is summarized in the following abstract from Coombe, 1973:

> Grape flowers and berries react in diverse ways to growth substances, depending on the time of application. Pre-anthesis treatment with auxins causes premature growth of the ovule, while gibberellins inhibit seed growth. Gibberellins applied at or after anthesis promote pericarp growth mainly through cell enlargement. Seeds prevent this response. Auxins and ethylene applied during the slow-growth phase delay the onset of ripening, but ABA hastens it.
>
> It is hypothesized that: (a) The hormonal control of the first rapid growth of the pericarp resides in interactions between auxins, cytokinins, gibberellins and abscisins and the relative importance of each changes as development progresses from cell division to cell enlargement. (b) The seed is the chief source of these hormones. (c) The second rapid growth phase is associated with ABA and sugar accumulation, initially in the skin.
>
> For a period after anthesis the ovary appears to exert no control over the flow of organic nutrients to it. As a result, its abscission and early development are dependant on the overall supply of nutrients in the vine. Later, the berry develops an ability to attract nutrients and different limitations to accumulation then operate.

Further material was reviewed by Düring et al. (1978) and Düring and Alleweldt (1980).

High cytokinin activity in the berry coincides with periods of rapid cell division before and after anthesis (Niimi et al., 1977). Furthermore the level of cytokinins (and gibberellins) are higher in leaves of vines carrying fruit (Hoad et al., 1977). The formation and development of vascular tissue is much influenced by auxin and cytokinins (Nitsch, 1970) and there is a strong positive correlation between the number of berries on a bunch (manipulated on uniform bunches) and phloem area per cross-section of the peduncle (Singh and Sharma, 1972). That phloem area is not a unique determinant of berry growth was shown by data of the same workers in which gibberellic acid treatment of bunches at full bloom affected bunch weight (F.Wt. and D.Wt.) independently of phloem area of the peduncle.

Skene (1971) demonstrated increased mobilization of reserves from grapevine internodes into a perfusing solution when cytokinins, gibberellins and auxins were included. This was attributable to activation of cambium.

During the lag phase endogenous ethylene is very low, while ABA first declines then rapidly increases. The matter of which hormone, if any, triggers the process of veraison is discussed in Paragraph 2.

1.10 Interactions of Other Factors

As many constituents have been overlooked in this review, so too have many factors of the whole plant and the environment such as temperature (Kliewer 1973; Klenert et al., 1978), light (Kliewer and Schultz, 1964; Kliewer et al., 1967; Kliewer and Lider, 1968), vine vigour and leaf area (Kliewer and Antcliff, 1970). An interesting series of correlations published by Schneider and Staudt (1980) are simply represented in Fig. 2.8. Must density may be taken as an indication of sugar content at maturity.

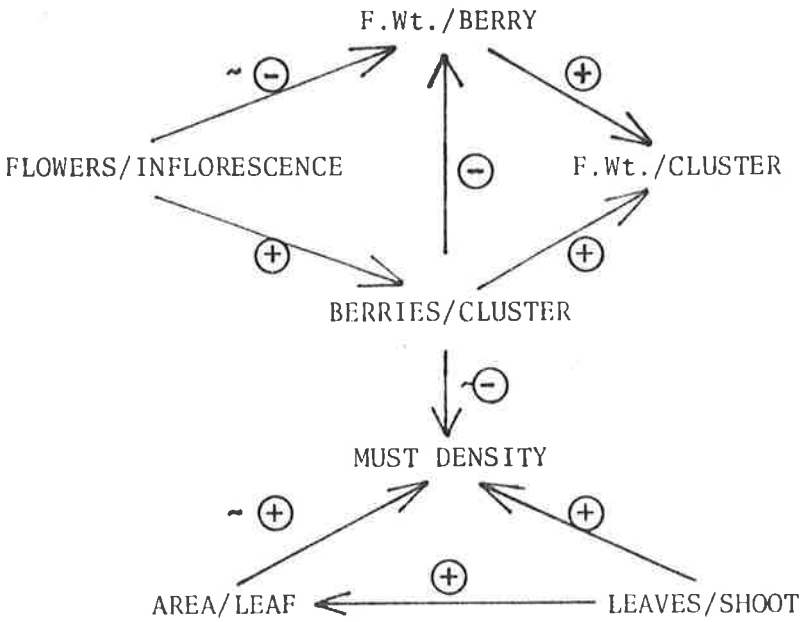


Fig. 1.8: Representation of positive and negative correlations observed in ripe berries, constructed from Schneider and Staudt (1980). (~ indicates a weak correlation.)

CHAPTER 2: THE PROCESS OF VERAISON

THE PROCESS OF VERAISON

2.1 What Regulates Ripening?

Since the notable contribution of Nitsch (1965) there have been many reviews of fruit development, including the volumes of Hulme (1970, 1971) and the briefer contributions of Sacher (1973), Coombe (1975) and Fellenberg (1978).

That membranes are altered during ripening is a reasonable assertion. But the misleading extrapolation has often been made that ripening proceeds by increased membrane permeability (Sacher, 1973) with the inference of progressive disorganization in the cell. This is finally embraced in the notion of a "leaky membrane". On the one hand this is contrary to much evidence (e.g. Vickery and Bruinsma, 1973). Furthermore, it is irreconcilable with notions of charge separation, pH gradients and cotransport mechanisms which depend upon permselective membranes discriminating molecular species much smaller than, for instance, the accumulating fruit solutes. Rhodes (in Hulme, 1970) criticises the notion of ripening as a consequence of disorganization. Taking observations such as the increased synthesis of enzymic protein during the climacteric of fruits, he proposes an analysis based upon "reorganization and redevelopment" in the cell.

Ho (1980) investigated carbon translocation in the tomato with the hypothesis that sucrose concentration in the fruit determines rate of sucrose translocation to or from the fruit. This sucrose concentration is regulated by invertase which is therefore controlling the phloem unloading process. During 24 h the rate of import of translocate to a fruit was predetermined by the developmental stage of the fruit and unaltered by the rate of current carbon fixation in the source leaf (1979). Upon injecting either U-^{14}C-sucrose or U-^{14}C-hexoses into tomatoes at 5°C, the larger export of label (as ^{14}C-sucrose in each case) derived from hexose injection (Walker and Ho, 1978). From such work Ho postulated that sucrose unloaded by the

phloem must first by hydrolysed, then compartmented as hexoses, before being susceptible to resynthesis and re-export as sucrose.

In a tomato truss the tip fruits tend to import less assimilates even when the supply is non-limiting and competitive factors have been removed. Ho sought a parameter to describe the relative "sink strength" of tomatoes on a truss, and found that a lower ratio C/N correlated with lower import of assimilate and the ratio K^+/Ca^{2+} declined. Interpreting K^+ as an indicator of phloem import, and Ca^{2+} of xylem import, he described sink strength of tomato fruits as the capacity of the fruits to receive the phloem mobile assimilates through the selective phloem unloading process.

Others have suggested that ripening is regulated in various tissues: by shifts in the redox potential (Frenkel, 1976) due to phytochrome activity (Jen et al., 1977); by break-down of organizational resistance in the cell leading to decontrol (not uncoupling) of respiration with protein and sucrose synthesis consequent upon a surge of energy substrates (Solomos and Laties, 1973); to a decline in bound ions, especially Ca^{2+} (Suwwan and Poovaiah, 1978); by polygalacturonase causing release of bound enzymes and subsequent changes in compartmentation (Ng + Tigchelaar, 1977; Tigchelar et al., 1978a, b).

Chalmers and Rowan (1971; Woodrow and Rowan, 1979) proposed that in climacteric fruits activation of phosphofructokinase by increased cytoplasmic inorganic phosphate leads to a respiratory rise and subsequent events of ripening. Indeed the climacteric of tomato pericarp slices is preceded by increased permeability of the tonoplast and plasmalemma to ^{32}P. Moreover Salminen and Young (1974) attributed the 5-fold increase in phosphofructokinase activity to removal of a negative cooperativity rather than *de novo* synthesis upon ripening.

In Golden Queen peach Chalmers has distinguished an acceleration of F.Wt. growth from a later D.Wt. increment coincident with ethylene production. Extensive investigations have led to a model in which ethylene establishes "sink strength" (ability to accumulate solute) : the final D.Wt. growth phase is stimulated by the seed, activating autocatalytic ethylene production in the pericarp and maintaining accumulation therein (Chalmers and van den Ende, 1975a, b, 1977; Jerie and Chalmers 1976a, b; Dann and Chalmers, 1978).

Ethylene is a stimulus to ripening in many climacteric fruits (McGlasson et al., 1978). But with regard to the initial stimulus for the events of veraison in the grape berry, ethylene does not appear to be a primary signal (Hale et al., 1970; Alleweldt and Koch, 1977). Its slight activity depends upon some prior "conditioning" of the berry during the lag phase.

ABA levels decline during early lag period and increase just before veraison, then more rapidly following veraison (Hale and Coombe, 1974); in particular, accumulation of ABA and sugars was coincident, each occurring first in the skin and later in the flesh, such that Coombe (1973, 1976b) demonstrated a seven-week correlation of ABA with sugar, there being $10^{5.6}$ moles of sugar per mole of ABA.

Similar observations have been reported (Düring, 1973; Downton and Loveys, 1978; Scienza et al., 1978) with evidence that ABA is transported from leaves to the berries about veraison (Düring et al., 1978). ABA applied to bunches late in the first growth cycle stimulated reducing sugar accumulation in the second cycle; however, bunches maintained in the dark did not contain more sugar, whilst anthocyanin content did increase in both treatments (Matsui et al., 1980). It has been proposed that ABA "inhibits phloem loading or at least raises the retention of assimilates ... " (Düring and Alleweldt, 1980), and that ABA, as an antagonist of FC, might decrease the PMF and depolarize the phloem membrane, thereby favouring unloading at that site by reversal of the proton-sugar transport system purported to produce phloem loading (Tanner, 1980b).

McGlasson et al. (1978) cite further correlations between increasing ABA levels and the progress of ripening in other fruits (e.g. in tomato : McGlasson and Adata, 1976; McGlasson and Franklin, 1979).

2.2 ^{14}C Studies

Kliewer (1964; Kliewer and Schultz, 1964) fed $^{14}CO_2$ to the leaves of vines and assayed the label in berries for four days. Green berries incorporated ^{14}C predominantly into organic acids. The ratio of labelled glucose:fructose increased markedly with temperature. In nearly-ripe fruit most label was incorporated into sugars and the hexose pattern was scarcely influenced by temperature.

Ribéreau-Gayon (1966) noted the appearance of ^{14}C in stachyose (especially in leaves and rarely in green berries), raffinose (in leaves and green berries, but not in ripe berries) and a compound thought to be melibiose (in green berries). In nearly-ripe berries label from $^{14}CO_2$ and ^{14}C-sugars did not appear in organic acids till six days after feeding.

Hardy (1967, 1968) administered labelled sugars (glucose, fructose, uniformly - and asymmetrically-labelled sucrose) via the pedicel of excised berries in both growth cycles (cv. Sultana) to find that sucrose was rapidly converted to hexoses; and fructose mostly remained as such whereas from glucose he recovered equal amounts of labelled glucose and fructose. Small amounts of sucrose were synthesized by the berries in both growth stages. From ^{14}C-sucrose Hardy recovered more labelled fructose than glucose at 7.5 h but by 24 h each hexose was similarly labelled. Hardy concluded that upon inversion of sucrose during the first growth cycle glucose was penetrating more rapidly than fructose to metabolic sites in the berry: in 24 h fructose had similarly penetrated and been metabolized, while a portion of each had been sequestered to a non-metabolizable pool. He did not contrast this with observations in the berry post-veraison where labelled fructose persisted at both 7.5 and 24 h. Given that berries at this stage accumulated both hexoses, it appears that fructose was compartmented without loss to metabolism. Re-interpretation of his data is difficult as it was reported as percentile.

Hardy (1968) contrasted his own results with those of Kliewer, both having used cv. Sultana in the first growth cycle: (i) Of the hexoses supplied via the cut pedicel, glucose was metabolized faster than fructose. (ii) When $^{14}CO_2$ was supplied via leaves both hexoses were labelled and fructose was the more metabolized in the berry. Hardy concluded that "entry of the pedicel-fed sugars into the berry may be different from the route taken by sugar translocated from the leaf, ... by the transpiration stream as opposed to phloem transport." Yet subsequent autoradiography of ^{3}H-glucose movement (Kriedemann, 1969) indicated greater activity in the phloem than the xylem of the pedicel.

2.3 Does the Route of Solute Transfer Change?

The apoplasmic transport of solutes in plant tissues was reviewed by Läuchli (1976); he points out that little is known concerning the hydraulic and solute conductivity of the cell walls, nor the manner in which pectins and their hydrolysis affects this.

There is evidence against assimilate movement by the apoplasmic route in the immature berry. As cell-wall invertase activity has been commonly reported (review : Eschrich, 1980), sucrose movement by an apoplasmic route should yield free hexoses. Indeed, sucrose applied to the cut pedicel is very rapidly hydrolysed (Hardy, 1967). Secondly, from the very low levels of labelling into free hexoses during the first growth cycle, it appears that the hexose moieties of sucrose are directly incorporated into metabolism, (Saito and Kaisai, 1978) by sucrose synthase for example. Thirdly, glucose is metabolized from inverted sucrose faster than fructose; one would expect to recover fructose in the tissue (including in the extra-cellular space). This is not the case.

Conversely during the second growth cycle hydrolysis of sucrose by invertase is evident in the high levels of free hexoses, especially in the diffusible volume (Coombe and Matile, 1980).

This suggests that sucrose transfer in the berry changes from symplasmic to apoplasmic during the lag phase. The atypical result of Coombe and Matile (1980) with pre-veraison berries - fructose levels higher than glucose in both the diffusible and compartmented spaces - would thus arise from transfer in the apoplasm though the tissue still shows some characteristics of the earlier cycle, namely preferential metabolism of glucose and synthesis of malate.

2.4 Specific Studies of Veraison

In a series of papers Coombe has commenced a detailed study of veraison (Coombe, 1980; Coombe and Bishop, 1980; Coombe and Phillips, 1980; Coombe and Matile, 1980).

Coombe (1980) examined berry development in cv. Muscat at six times of flowering involving quite different climate and crop competit-

ion. Berries from late flowers had a prolonged lag phase. But the inceptions of rapid solute accumulation and of the second growth (volume) cycle were coincident within treatments; the rates of increase in °Brix were uniform between treatments, despite large differences in berry volume.

> It would seem that, for this cultivar and these growing conditions, the accumulation of sugar proceeded after triggering at a rate influenced by concentration itself, and was able to adjust to the large differences in the volume increment of the solvent, water.

And subsequently (Coombe and Phillips, 1980):

> ... sugar input is controlled by a mechanism which takes a signal from vacuolar (sugar) concentration.

A different hypothesis, that water increments are adjustments to previous sugar uptake (Coombe, 1960), was dismissed on two accounts.

(i) Confining the volume of a berry with perspex enclosures also inhibited sugar accumulation, while release was followed by both accumulation and growth (Coombe, 1973).

(ii) Rapid sugar accumulation and water uptake have often been measured as asynchronous.

As noted earlier, some physiological changes occurring at veraison are dramatic events, sudden changes involving less than one day, rather than gradual processes. If an asynchronous population is sampled such an event becomes smoothed to a gradual acceleration. On the contrary such detail may emerge if one can sample a synchronised population; or subsequently synchronise the data mathematically about a precise event; or use non-destructive methods to sequentially assay individual berries.

This matter is clear in a paper by Coombe and Bishop (1980) where, using statistical techniques to synchronise data, most berries were shown to increase markedly in deformability on average 6 days before a marked diameter increment. "The increase in deformation beyond a specific value appears to be a useful objective measure of veraison." This paper notes that softening - a decrease in the modulus of <u>elasticity</u> of the tissue - may arise from cell wall changes and the relative elastic and turgor properties of the skin compared with those of the flesh. Growth is a <u>plastic</u> change "due, for instance, to the breaking of cross-

links in the cross-linked polymers of the cell wall" which affects cell wall viscosity.

In situ sampling was used (Coombe and Phillips, 1980) to demonstrate the coincidence of marked increases in berry deformability and sugar concentration in the hypodermal juice. Rapid malate decline followed one day later, and diameter increased markedly six days later.

Coombe and Matile (1980) studied sugar uptake by skin segments of berries cv. Pinot noir just before veraison: this tissue was chosen because of indications that sugar accumulation by grape pericarp may proceed in the skin (Coombe, 1976b) and because the tissue was tractable.

- The concentration of glucose in the skin was double that in the flesh while malate was 4-fold in the flesh.
- Apparent free space($\bar{c}$ glucose) was c. 0.28 ml g^{-1} F.Wt.
- c. 80% of the sugars (hexoses) present in the skin were diffusible (by 30 min, into a complex isotonic medium).
- The concentrations of fructose, both "free" and "compartmented", were double that of glucose in green skin but not different in ripening berries.
- Glucose was taken up from the medium more rapidly than fructose during 20 h, and malate concentration increased during such incubations.
- In particular, sugars were accumulated linearly for 20 h at rates higher than those found in whole berries even during the period of massive sugar accumulation. Though the incubations were at glucose concentrations similar to endogenous levels in the diffusible volume " ... it seems that the experimental procedure of peeling the skin and incubating skin segments in medium plus glucose in some way removes an inhibition of a sugar transport process."
- Glucose uptake was evidently concentrative, active and associated with alkalization of the incubation solution. Effects of GA, ABA, DNP, FC were consistent with this interpretation.

2.5 Water and Osmotic Relationships

In a paper entitled "Fundamental Water Relations Parameters" Zimmermann and Steudle (1980) make many points relevant to water and solute flux in tissue, with innovative techniques to obtain necessary data.

In principle, turgor pressure driven extension growth can be regulated at three different levels i.e. the levels of (i) water uptake; (ii) solute modification or transport; and (iii) mechanical wall extensibility. These authors present data which indicate that:

- the water exchange rates of individual cells of higher plants are rapid, with $t_{\frac{1}{2}}$ of seconds or minutes, such that no significant long-term differences in chemical potential of water would be built up in tissues. Moreover, if an osmotic increment is established in a cell (e.g. starch hydrolysis), rapid water adjustment is expected and any solute flux occurs later.
- the pathway of least resistance for water flux would often be symplastic rather than apoplastic.
- the cell wall elastic modulus as measured is usually volume dependent, increasing (i.e. less elastic) with cell size. Presumably plasticity shows a similar relationship with size.
- the membrane is an electro-chemical entity in which transmembrane charge affects physical thickness and conversely thickness changes (i.e. compression) affect the transmembrane charge.
- pressure-dependent active solute transport processes (e.g. K^+ flux in *Valonia*) may sense either stretching or direct compression of the membrane.

In fruit one would expect very high turgor to be generated when rapid solute accumulation occurs with relatively little growth. This will not be the case if the fruit tissue is somewhat discontinuous with the transpiration stream and thereby extracellular fluid may attain an unusually high osmotic potential. Cram alluded to the osmotic role of the extracellular space (in Spanswick et al., 1980) :

> Turgor, for instance, could be adjusted *in vivo* via changes in the extracellular osmotic pressure very much faster than via the slow changes in intracellular osmotic pressure observed *in vitro*.

Moreover, if there existed in the pedicel a zone in which the xylem was discontinuous and which acted as a substantial resistance to solute diffusion (stopping efflux), the extracellular space of the berry could represent an osmometer of some sort, capable of generating a pressure (albeit weakly) against the cuticle.

A corollary is that the apoplasm may approach the osmotic potential of phloem sap. (In mature sugarcane the concentrations of sugar in the apoplasm and the symplasm are similar; in grape, the final sugar content of the whole berry is marginally above that of sucrose in the phloem.)

Coombe's (1973) experiments with perspex enclosures on berries and the protraction of the lag phase by water stress (Hardie and Considine, 1976) suggest that water relations and hence turgor are linked with sugar accumulation.

Hence the following propositions might be considered:

(i) During the second growth cycle the extracellular space is substantially discontinuous with the transpiration stream of the vine. (Hardy noted that ripening berries take up water via the pedicel at a much slower rate than green berries.)

(ii) This arises as the first growth cycle decelerates.

(iii) In the lag phase water movement and sugar transfer are substantially symplasmic, by which fructose is the more rapidly metabolized hexose moiety (as Kliewer determined in intact berries).

(iv) The extracellular solution will increase in osmotic potential by diffusion of solute, notably glucose, from the cytoplasm.

(v) Characteristics of cells adjacent to the vascular tissue will be imposed upon sugar transfer in the entire berry.

(vi) Changes in the plasmalemma of the phloem and/or the cell wall properties will result in apoplasmic sucrose transfer, sucrose hydrolysis, and further loss of turgor.

(vii) A pressure-sensitive sugar transport mechanism tends to stabilize turgor by sugar uptake at the tonoplast, which affects sugar flux at the plasmalemma.

(viii) Extracellular sugar levels will be high from phloem unloading. But if phloem is c. 20% sucrose (in osmotic terms equivalent

to a 10% hexose solution) the extracellular fluid can attain a maximum of 10% hexoses before phloem loses turgidity (ignoring other solutes). Thus sugar accumulation to 20% hexoses in grapes indicates a concentrative, active process. Conversely, it is untenable on osmotic grounds that 20% hexose accumulation result alone from hydrolysis of unloaded sucrose and diffusion through the tissue.

2.6 Summary

The following observations upon veraison in the grape berry are important to this thesis:

Glucose and fructose content, and fresh weight, increase first slowly then very rapidly with a typical accumulation of 500 µmoles hexoses per berry over 10 days (Coombe and Hale, 1973; Ruffner and Hawker, 1977). Net accumulation of fructose is marginally greater than of glucose.

In a similar manner ABA concentration, having declined from late stage 1, again increases at about the same time as sugars, first slowly then rapidly in correlation with sugars during seven weeks (Coombe and Hale, 1973; Coombe, 1976b). While ethylene and auxins may affect the timing of veraison, there are not correlations from either exogenous treatments nor endogenous levels to clearly implicate them at veraison.

Similarly a slow softening of the berry alters to a sudden increase in deformability which steadily increases thereafter for one week (Coombe and Bishop, 1980). Berry diameter increments follow the same pattern; but the increase in deformability usually, though not always, precedes the increase in diameter, the median difference being 6 days (cvv. Muscat and Doradillo : Coombe and Bishop, 1980). Some previous measurements of sugar accumulation and volume growth (water uptake) show different timing (e.g. Coombe, 1973 : water increments without sugar increment during the lag phase; Coombe, 1980 : coincidence of the events), but still one concludes there not to be a nexus between the two. At some time during these changes a transient "hydration" of the cell walls is observed (Considine, 1979).

Malate accumulation ceases as rapid sugar accumulation is initiated whereupon malate declines (Hale, 1977).

From about one week after sugar accumulation commences, phenolics rapidly accumulate in the skin during 30 days (Pirie and Mullins, 1980). There is loss of chlorophyll from the skin, and in red and black cultivars, accumulation of anthocyanins.

There is a decline in the volume of extractable gas; and an increase in activity of certain enzymes having sucrose as substrates.

Some pertinent questions are:

Which of these events are directly related?

Which, if any, can be considered a primary event?

How is the primary event initiated and controlled?

In which manner are the cells of the skin and flesh different with respect to these processes?

How does sugar move from the phloem to the vacuole?

Which is the concentrative step of sugar accumulation?

What is the relationship between sugar import and water import?

CHAPTER 3: METABOLISM OF SUCROSE, GLUCOSE AND FRUCTOSE

METABOLISM OF SUCROSE, GLUCOSE AND FRUCTOSE

1. Introduction

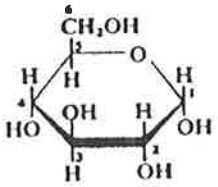
The biochemistry and metabolism of sucrose, glucose and fructose is briefly considered in this chapter.

The standard texts are the Carbohydrates (Pigman and Horton, 1972), Methods in Carbohydrate Chemistry (Whistler and BeMiller, 1972-76) and Biochemistry of Carbohydrates (Whelan, 1975); and in enzymology, Barman (1969), Boyer (1971), Bergmeyer (1974), Walsh (1979) and volumes of the 2 series Advances in Enzymology and Methods of Enzymology. The regulation of carbohydrate metabolism has been reviewed by Turner and Turner (1975), Ap Rees (1977), Latzko and Kelly (1978), Gibbs and Latzko (1979); and carbohydrate storage by Jenner (1980) and Willenbrink (in press). The regulation of synthesis and activity of relevant enzymes was reviewed by Smith (1977).

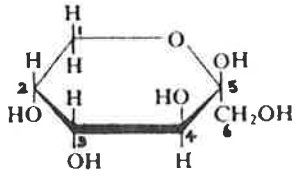
2. Structures

The Haworth projections of several sugars considered in this dissertation are presented in Fig. 3.1. Naturally most of these sugars have several isomeric forms in aqueous solution: the α- and β-anomers, the cyclic (e.g. pyranose, furanose) and trace acyclic conformations.

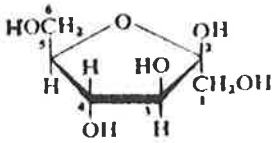
A series of sugars with structural similarity to glucose is shown. These have been used to test for specificity in mediated transport (Maretzki and Thom, 1972a). 3-O-methyl-D-glucose and 2-deoxy-D-glucose, though commonly termed non-metabolizable, are in fact substrates for some enzymes. For instance, 2-deoxy-D-glucose can be metabolized to 2-deoxy-glucose phosphate, UDP-2-deoxyglucose and 2-deoxy-sucrose which can in turn be hydrolysed by invertase (Pavlinova et al., 1978; Ehwald and Zippel, 1980).



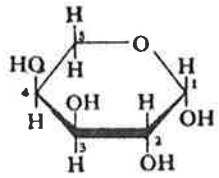
α-D-Glucopyranose



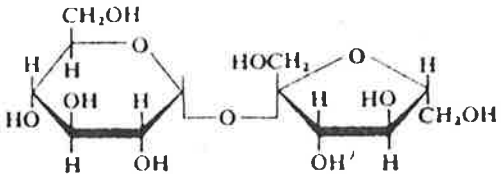
β-D-Fructopyranose



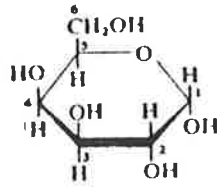
β-D-Fructofuranose



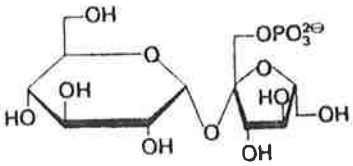
β-L-Arabinopyranose



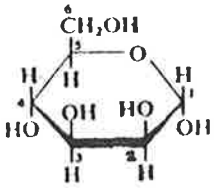
α-Glucose-1→2-β-D-Fructofuranose



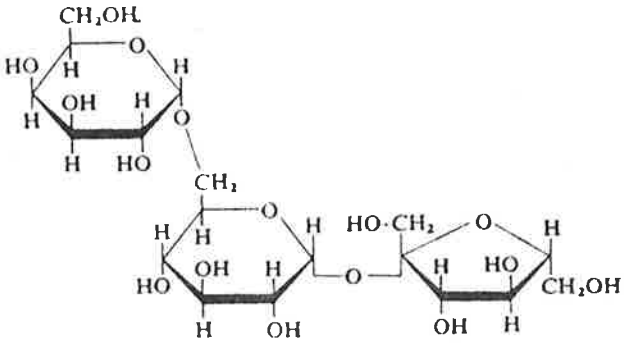
α-D-Galactopyranose



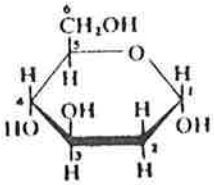
sucrose phosphate



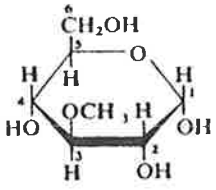
α-D-Mannopyranose



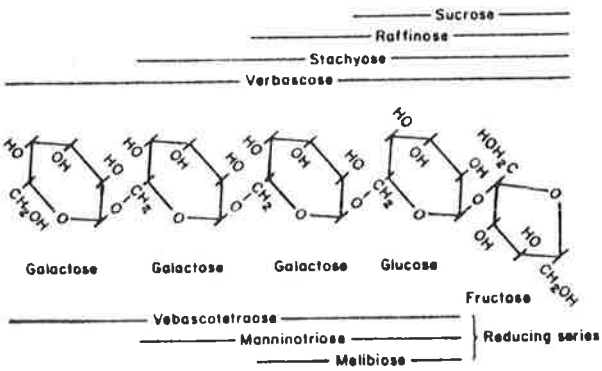
Raffinose



2-Deoxy-α-D-glucopyranose



3-O-Methyl-α-D-glucopyranose



The raffinose family of oligosaccharides. From Zimmermann and Brown (1971).

Fig.3.1 Haworth projections of several sugars referred to in the text. The structure for sucrose phosphate is that postulated by Walsh (1979).

Mannose can be phosphorylated and thereby used to sequester inorganic phosphate (Herold and Lewis, 1977).

A galactosyl moiety is produced by the action of an UDP-glucose 4'-epimerase (EC 5.1.3.2) of alkaline optimal pH and requiring NAD^+ as cofactor (Fan and Feingold, 1969; Davies 1974). Beck (1975) reviewed the metabolism of galactose and synthesis of the raffinose series.

The structure of sucrose phosphate has been proposed from the following two observations:

(i) Fructose-6-phosphate is a more reactive precursor than fructose and is incorporated without exchange of phosphate (Hassid, 1970).

(ii) The stereochemistry of the glucosyl unit during catalysis is consistent with this structure (Walsh, 1979).

The raffinose family of oligosaccharides is presented as it shall be argued that these sugars reflect the level of phosphorylated hexoses in the cytoplasm, especially in a perturbed cell. It should be noted that the fructose moiety is cleaved by β-fructofuranosidases to expose an α-glucosyl moiety of the reducing series which may then be cleaved by α-glucosidases (Flowers and Sharon, 1979).

3. Metabolism

Ap Rees (1977) observed that the division of translocated sucrose between storage, polysaccharide synthesis and respiration is determined by the relative activities of sucrose synthase and the two invertases (Table 3.1).

3a Starch biosynthesis and degradation

Starch biosynthesis from sucrose requires production of, first, UDPG by sucrose synthase, then glucose-1-phosphate by a pyrophosphorylase. Hexoses may also be phosphorylated to glucose-1-phosphate, the common precursor to the formation of ADPG by ADP-glucose pyrophosphorylase (Table 3.1). Starch is formed from ADPG by starch synthase (EC 2.4.1.b) (Preiss, 1978).

UDP-glucose pyrophosphorylase (Table 3.1) catalyses interconversion of glucose-1-phosphate and UDPG. This nucleoside is a precursor for polysaccharide synthesis, including epimerizations, in the cytoplasm. While a glucose moiety may be incorporated *in vitro* from UDPG to an α-(1→4)-glucan, ADPG is probably the major precursor *in vivo* (Preiss and Levi, 1979).

Starch degradation in chloroplasts is sufficient to result in continuous interchange of saccharide between sucrose and starch. This occurs via the oxidative pentose phosphate pathway and glycolysis to 3-phosphoglycerate and triose phosphates which are transported to the cytoplasm (Stitt and Ap Rees, 1980: this transport is discussed in chapter 5).

3b Sucrose synthesis

Sucrose formation proceeds by sucrose-phosphate synthase, and subsequently, sucrose phosphate phosphatase or unspecific phosphatases (Table 3.1) (Pontis, 1977).

Herold and Walker (1979) pointed out that photosynthesizing chloroplasts are dependent upon cytoplasmic sucrose formation in order that released inorganic phosphate be available at the envelope for triose-phosphate export into the cytoplasm. To this extent sucrose phosphate synthase and fructose-1, 6-diphosphatase (EC 3.1.3.11) are regulatory (Latzko and Kelly, 1978).

Simple sugars may be involved in many other pathways: for example, in the synthesis of the benzene ring via shikimic acid, of malic and tartaric acids, of glycosides and cell wall polysaccharides (Beck and Wieczorek, 1977), and of storage products (mannans, fructans, glucans: Darbyshire and Henry, 1978).

Phosphorylation and interconversions of glucose, fructose and sucrose occur through hexokinases, phosphoglucose isomerase and phosphoglucomutase (Table 3.1).

4. Pathways of Carbon Metabolism

It is pertinent to list the various metabolic pathways to which these sugars are indirectly either substrates or products. Chapter 2 assessed the relative contribution of these pathways to the sugar accumulating in a fruit.

Major degradation of carbohydrate occurs by *glycolysis* in which glucose- or fructose-6-phosphate is oxidised to pyruvate with production of NADH and NADPH. Under anaerobic conditions ethanol may be a product. The initial non-equilibrium reaction with phosphofructokinase is regulatory.

Gluconeogenesis may be envisaged as a reversal of glycolysis with formation of hexoses and their phosphates via phosphoenolpyruvate from intermediates of the tricarboxylic acid cycle (Ap Rees et al., 1974).

In the oxidative pentose phosphate pathway hexose phosphates are oxidised to pentose phosphate and subsequently to glyceraldehyde-3-phosphate: the initial step is essentially irreversible, yielding 6-phospho-glucono-δ-lactone from catalysis with glucose-6-phosphate dehydrogenase (EC 1.1.1.49). NADPH is produced.

Essentially the reverse of this process occurs in the reductive pentose phosphate pathway (Calvin cycle) during photosynthesis with fixation of CO_2 through to fructose-1, 6-diphosphate.

Where the oxidative pentose phosphate pathway is coupled to the glycolate pathway as in photorespiration there is a loss of hexose carbon via oxidation to CO_2.

4a Regulation

Turner and Turner have proposed the following regulation. Glycolysis is controlled at the steps catalysed by phosphofructokinase and pyruvate kinase: the former enzyme is activated by inorganic phosphate and Mg^{2+} and inhibited by ATP and citrate. The pentose phosphate pathway is essentially regulated through the activity of glucose-6-phosphate dehydrogenase, which is competitively inhibited by NADPH and ATP. Gluconeogenesis, as yet poorly understood, may be controlled by the activity of fructose-1, 6-diphosphatase. Ap Rees (1974) concluded there

was no evidence for organisation of each pathway into membrane systems and proposed the existence of multienzyme complexes in the cytoplasm. However, compartmentation is important in gluconeogenesis (Ap Rees et al., 1974) where intermediates do not enter oxidative pathways.

4b Examples of integration

The integration of these pathways involves compartmentation, energy charge, pH of microenvironments, activation and inhibition of enzymes - to mention only a few mechanisms. Two examples are given.

The three hexose monophosphates (glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate) may be regarded as forming a common pool. Two of the enzymes competing for this pool are ADPG pyrophosphorylase and phosphofructokinase. These two enzymes are affected in opposite ways by a number of metabolites such as inorganic phosphate and 3-phosphoglycerate.

Fructose-6-phosphate is a common substrate for the enzyme pair sucrose-phosphate synthase and phosphofructokinase. Citrate activates the former and inhibits the latter. Sucrose formation as well as glycolysis may be regulated by citrate, a compound of the tricarboxylic acid cycle.

4c Ambiquitous enzymes

Some enzymes are "ambiquitous" (Wilson, 1978), partitioning reversibly between kinetically distinct soluble and membrane-bound forms. Brain hexokinase is the classical example, being solubilized off the mitochondrion by glucose-6-phosphate or ATP.

Kursanov et al. (1969) identified in sugar beet vascular tissue three forms of hexokinase with different catalytic properties: two were membrane-bound and one soluble. The hexoses were competitive toward the bound form but without interaction toward the soluble form. These workers proposed that the kinetics of hexose transport across the vascular membrane could be explained by the kinetics of two of these hexokinases.

Several hexose transport studies have proposed two pools of hexoses, one metabolic and one transport pool (liable to storage or to export).

This phenomenon could arise from compartmentation, or from localization of necessary enzymes. If an hexokinase were actually ambiquitous the relative pool size and the relative rate of hexose metabolism (or transport) might be regulated by reversible solubilization of the enzyme.

Table 3.1: Characteristics of certain enzymes of sugar metabolism.

SUCROSE SYNTHASE

EC 2.4.1.13 UDP-glucose: D-fructose 2-glucosyltransferase

$$NDP\text{-glucose}^{2-} + \text{D-fructose} \rightleftharpoons NDP^{3-} + \text{sucrose} + H^{+}$$

N = A, U, T, C, G but the K_m for UDPG is one tenth of the other nucleosides.

Pontis 1977 : review Salerno et al. 1979 : assay

pH optima synthesis 7.5-8.5
cleavage 6.0-6.5

divalent cations may inhibit sucrose cleavage and stimulate synthesis (Tsai, 1974).

contains essential sulfhydryl groups

the only sugar nucleotide transglucosylation reaction to be readily reversible and exhibiting broad specificity for the nucleoside base, unlike other enzymes of sugar nucleotide metabolism.

SUCROSE PHOSPHATE SYNTHASE

EC 2.4.1.4 UDP-glucose: D-fructose-6-phosphate 2-glucosyltransferase

$$\text{UDP-glucose}^{2-} + \text{fructose-6-phosphate}^{2-} \longrightarrow UDP^{3-} + \text{sucrose phosphate}^{2-} + H^{+}$$

Salerno and Pontis, 1976, 1977, 1978a, 1978b : assay and review
Salerno et al., 1979 : assay Pontis, 1977 : review

synthesis opt. pH 6.5
practically irreversible

substrate specific; activated by divalent cations
inhibited by anions such as citrate and phosphate
sucrose inhibits sucrose phosphate synthesis
contains essential sulfhydryl groups

SUCROSE PHOSPHATE PHOSPHATASE

EC 3.1.3.00 sucrose-6-phosphate phosphohydrolase

$$\text{sucrose-6-phosphate} \longrightarrow \text{sucrose} + P_i$$

Pontis, 1977 : review Hawker and Hatch, 1975 : assay

opt. pH 6.7

absolute requirement for Mg^{2+} (c.f. unspecific phosphatases)
inhibited in presence of EDTA and sucrose: Ki (sucrose) 10 mM

▶

Table 3.1 continued

HEXOKINASE

EC 2.7.1.1 ATP: D-hexose-6-phosphotransferase

$$\text{ATP} + \text{D-hexose} \rightleftharpoons \text{ADP} + \text{D-hexose-6-phosphate}$$

specific for ATP
Bergmeyer 1974 : assay Barman 1969 : data

opt. pH 7.2-7.6
physiologically irreversible

$$\frac{[\text{ADP}]\,[\text{D-glucose-6-phosphate}]}{[\text{ATP}]\,[\text{D-glucose}]} = 3.86 \times 10^2 \quad (\text{no } Mg^{2+}, \text{ pH6, } 30°)$$

Baker's Yeast hexokinase at pH7.4, phosphate buffer, 30° :

substrate	relative rate	K_m (mM)
D-glucose	1.0	0.1
D-fructose	1.8	0.7
2-deoxyglucose	1.0	0.3

glucose-6-phosphate inhibits K_i 9.1 mM at pH8.0

EC 2.7.1.2 glucokinase Turner et al. 1977
glucose K_m = 0.070 mM
relative rate of phosphorylation at 5 mM : glucose/fructose = 100/11
glucose-6-phosphate does not inhibit except at high concentrations (Barman, 1969)

EC 2.7.1.4 fructokinase Barman 1969
fructose k_m - 1 mM
fructose-6-phosphate does not inhibit

PHOSPHOFRUCTOKINASE

EC 2.7.1.11 ATP : D-fructose-6-phosphate 1-phosphotransferase

$$\text{NTP} + \text{fructose-6-phosphate} \rightleftharpoons \text{fructose-1, 6-diphosphate} + \text{NDP}$$

N = A, U, T, C, G

Turner and Turner 1975 : review
Hobson 1976, Ruffner et al. 1976 : assays

opt. pH 8.0

plant PFK: P_i stimulates ; citrate, ATP, ADP inhibit

▶

Table 3.1 continued

PHOSPHOGLUCOSE ISOMERASE

EC 5.3.1.9 D-glucose-6-phosphate ketol-isomerase

glucose-6-phosphate$^-$ $\rightleftharpoons$ fructose-6-phosphate$^-$

opt. pH 7-8
specific for glucose-6-phosphate (K_m 0.7 mM) and fructose-6-phosphate

Yeast isomerase, tris buffer, pH8, 30° :

$$\frac{[\text{D-fructose-6-phosphate}]}{[\text{D-glucose-6-phosphate}]} = 0.3$$

PHOSPHOGLUCOMUTASE

EC 2.7.5.1 α-D-glucose-1, 6-bisphosphate : α-D-glucose-1-phosphate phosphotransferase

D-glucose-1-phosphate + D-glucose-1, 6-diphosphate $\rightleftharpoons$
D-glucose-6-phosphate + D-glucose-1, 6-diphosphate

opt. pH 7.4

Rabbit muscle PGM, veronal buffer, pH 6.2-7.5, 30° :

$$\frac{[\text{D-glucose-6-phosphate}]}{[\text{D-glucose-1-phosphate}]} = 17.2$$

UDPG PYROPHOSPHORYLASE

EC 2.7.7.9 glucose-1-phosphate uridylyltransferase

UDP-glucose + PP_i $\rightleftharpoons$ UTP + α-glucose-1-phosphate

opt. pH 7.8 requires Mg^{2+} specific for UDPG

ADPG PYROPHOSPHORYLASE

EC 2.7.7.27 glucose-1-phosphate adenylyltransferase

ADP-glucose + PP_i $\rightleftharpoons$ ATP + glucose-1-phosphate

Preiss 1978 : review McCracken et al. 1980 : assay

opt. pH alkaline activation is highly pH dependent
P_i inhibits, 3 phosphoglycerate activates

▶

Table 3.1 continued

INVERTASE

EC 3.2.1.26 β-D-Fructofuranoside fructohydrolase

sucrose + H_2O ——→ D-glucose + D-fructose

Ap Rees 1974 : review

acid invertase opt. pH 4-5.3
alkaline or neutral invertases opt. pH 7.0-7.8

Bergmeyer 1974 : assay

K_m (sucrose) 9.1 mM

heavy metal ions reversibly inhibit, as also ammonium molybdate (Prado et al., 1979)

carrot root invertases (Ricardo 1974):
alkaline - raffinose not hydrolysed
fructose inhibits (K_i 40 mM)
acidic - raffinose hydrolysed
fructose inhibition K_i 300 mM

PHOSPHATASES

orthophosphoric-monoester phosphohydrolase
EC 3.1.3.1 alkaline optimum
EC 3.1.3.2 acid optimum

orthophosphate monoester + H_2O → alcohol + P_i

Bergmeyer 1974 : assay

inorganic pyrophosphate is hydrolysed, not phosphodiesters
vanadate inhibits ATPase, acid and alkaline phosphatase (Van Etten et al., 1974; Simons, 1979; Cocucci et al., 1980)
P_i inhibits alkaline phosphatase competitively
NaF inhibits acid phosphatase (Torriani, 1960; Vyskrebentseva and Semenov 1976)

EC 3.6.1.3 adenosine triphosphatases
Hodges 1976, Bakker-Grunwald : reviews
refer chapter 7 : criteria for ATPases

CHAPTER 4: TRANSPORT IN HIGHER PLANTS : CONCEPTS AND TERMINOLOGY

TRANSPORT IN HIGHER PLANTS : CONCEPTS AND TERMINOLOGY

4.1 Introduction

The broad topic of transport in higher plants has been the subject of many reviews, texts and conferences during the past 20 years. Some of these are listed in Table 4.1. The purpose of this chapter is to define in an elementary manner many concepts which are used in Chapters 5, 6 and 7.

In vascular land plants long distance transport of water is essentially a transpiration stream through primary and secondary xylem. The plant may be represented as a series of resistances to the net flux occurring between the high water potential in the soil and the low water potential of the atmosphere. Inorganic ion transport occurs principally by the same route.

Long distance transport of assimilates occurs predominantly as phloem translocation by processes yet to be elucidated, though a pressure-flow mechanism no doubt contributes (Zimmermann and Milburn, 1975).

Hormones and external agents such as viruses and herbicides variously move through either elements of the vascular system.

Gross phenomena of transpiration and translocation are an integration of multiple events some of which are quite localized and are subject to control. Such examples are guard cells affecting transpiration, or the mass transfer of solutes stimulated by localized events such as onset of fruit ripening, nectary maturity, or parasitic invasion.

Though short distance transport or transfer involves many processes (diffusion, mass flow, cyclosis, active transport) and structures (plasmodesmata, transfer cells, salt glands, nectaries), of particular relevance to this study is membrane transport.

Table 4.1: Selected texts and reviews of membrane transport, with annotation.

Reference	Title / notes
Azzone et al. 1978	"The proton and calcium pumps." / Proc. ; incl. measurement of ΔpH.
Baker 1978	"Transport phenomena in plants." / Introductory student text.
Bowling 1976	"Uptake of ions by plant roots."
Bronner and Kleinzeller 1970- .	"Current topics in membranes and transport." / vol.X: H^+/K^+ in solute transport in microorganisms (Eddy); vol.XII : identifying carrier proteins (Tanner).
Bunow 1978	"Chemical reactions and membranes : a macroscopic basis for facilitated transport."
Carafoli and Semenza 1979	"Membrane biochemistry. A laboratory mannual on transport and bioenergetics."
Clarkson 1974	"Ion transport and cell structure in plants." / a basic text.
Colowick and Kaplan 1978- .	"Biological oxidations", "Bioenergetics." / vols. LII-LVI, Meth. of Enzymol.
Fillingham 1980	"The proton-translocating pumps of oxidative phosphorylation.:
Finean et al. 1978	"Membranes and their cellular functions." / Introductory text.
Giebisch et al. 1978- .	"Membrane transport in biology."
	Vol. 1: "Concepts and models." Vol. 2: "Transport across single biological membranes." / incl. triose phosphate transport in chloroplasts. Vol. 3: "Transport across multi-membrane systems." / incl. turgor and transport; MacRobbie on plant vacuoles. Vol. 4a, b: "Transport organs."
Heinz 1978	"Mechanics and energetics of biological transport." / as thermodynamics of irreversible processes.
Kaback et al. 1975	"Molecular aspects of membrane phenomena." / Proc.
Kedem 1961	Original treatment of transport by principles of irreversible thermodynamics.
Lorenzen 1980	"Phloem loading and related processes." / Proc. ; H^+-sugar transport in plants (Tanner); Phloem loading (Giaquinta).
Lüttge and Higinbotham 1979	"Transport in plants." / the contemporary text.
Maddy 1976	"Biochemical analysis of membranes."
Maretzki and Thom 1978	"Transport of organic and inorganic substances by plant cells in culture."
Markham et al. 1974	"The electron microscopy and composition of biological membranes and envelopes." / freeze-etching methods.
Marré and Ciferri 1977	"Regulation of cell membrane activities in plants." / Proc. ; auxin and FC effects; pH stat (Raven and Smith); H^+-sugar cotransport in Chlorella.
Marré 1979	"Fusicoccin : a tool in plant physiology."
Minchin and Troughton 1980	"Quantitative interpretation of phloem translocation data."
Neame and Richards 1972	"Elementary kinetics of membrane carrier transport."
Pate 1980	"Transport and partitioning of nitrogenous solutes."
Pirson and Zimmermann 1975- .	"Encyclopedia of plant physiology."
	Vols. I-III / Transport in phloem (I), cells (IIA), tissues and organs (IIB); Intracellular interactions and transport processes (III).
Pitman 1977	"Ion transport into the xylem."
Raven 1977	"Regulation of solute transport at the cell level."
Semenza and Carafoli 1977	"Biochemistry of membrane transport." / incl. ionophores; reconstituted systems; ATPases; bacterial PEP:sugar phosphotransferase.
Spanswick et al. 1980	"Plant membrane transport : current conceptual issues." / Smith and Raven on chemiosmosis; Zimmermann on water relations.
Wardlaw and Passioura 1976	"Transport and transfer processes in plants." / Proc. ; incl. transfer cells, sucrose pathway in wheat endosperm (Jenner), sorbitol in pear leaf slices (Bieleski).
Zimmermann and Dainty 1974	"Membrane transport in plants."

4.2 Membrane Structure

The plant cell membrane is a flexible intracellular structure composed primarily of lipids and proteins, 3-5 nm thick, which serves both as a barrier to separate aqueous compartments with different solute composition and also as a structural base to which certain enzymes, receptors and transport systems are bound. Bound water represents an equivalent of one third of the membrane dry weight. Sterols are important components of certain membranes.

The present widely accepted model for membrane structure (the Fluid Mosaic model of Singer and Nicolson, 1972) consists of a lipid bilayer into which proteins penetrate to varying degrees (Capaldi, 1974). The lipids are predominantly amphiphilic. This model requires modification to accommodate: (a) specific association of part of the lipid with protein molecules, (b) possible asymmetry of lipid distribution in the bilayer, and (c) localized domains of lipid in the membrane. Most biological membranes carry, and are subject to, both fixed and mobile charges which affect their properties (Zimmermann and Steudle, 1980).

Considering a membrane from one side, the term cis applies to the proximal and trans to the distal face or position.

4.3 Membrane Flux

The study of permeant flux across membranes has produced a number of characteristic groupings and consequent classifications. The definitions which follow are not universally accepted - and synonyms are given with caution. While these predict kinetic phenomena, and have a theoretical base, there are few membrane transport systems in which the actual molecular mechanism has been elucidated. Despite this, the definitions reflect mechanisms rather than the kinetics.

Flux is defined as the amount of substance which, per unit time, passes a unit of area placed at right angles to the direction of the transport flow (Ussing, 1978). It has the dimensions :

$$J \equiv m^{-2}\ s^{-1}.$$

A membrane may be semi-permeable, transmitting the solvent only ; nonselective, transmitting solvent and solutes of all kinds ; and perm-

selective, transmitting water and solutes to a different extent. Where an osmotic or hydrostatic pressure acts upon one side of a biological membrane, bulk flow (rather than diffusion) may occur as solvent moves through the membrane without loss of the cohesive forces typical of a fluid.

An electrical transmembrane potential, the Donnan potential, results from unequal activities of diffusible ions across a membrane due to impermeable charged sites (macromolecules) in one compartment. Vieth (1979) demonstrated that Donnan charges alone could produce a sucrose accumulation ratio of 1.5 in reconstituted, collagen-containing, membrane vesicles.

The existence of an activity gradient of solute molecules (the permeant) across a permselective membrane tends to cause a net movement of permeant molecules towards the point of lower activity. In fact fluxes will occur in both directions. In the simplest case flux of uncharged molecules in the direction of the gradient can be described by the law of simple diffusion in single compartment (Fick's Law):

$$J = -D \cdot \frac{dS}{dx} \qquad \text{Eq. 4.1}$$

where dS/dx is the activity gradient of S and D, the diffusion coefficient, is characteristic for a solute in given conditions and may be replaced by the constant ωRT where ω is a mobility coefficient. This is an osmotic phenomenon.

An electrical gradient across the membrane will influence flux of charged permeants in a manner described simply by the Nernst-Planck equation:

$$J = -\omega S \left(\frac{RT}{S} \cdot \frac{dS}{dx} + zF \cdot \frac{d\Psi}{dx} \right) \qquad \text{Eq. 4.2}$$

where z is the number of electrical charges on the permeant and F the Faraday constant. The bracketed terms represent the electrochemical gradient.

Where the permeant flux can be described by either of these general equations the process is defined as non-mediated diffusion (syn. free diffusion, passive diffusion).

In an asymmetrical system about a membrane, a net flux of permeant may result from an activity gradient (an osmotic process) or from an electrical gradient (an electrophoretic process).

4.4 Mediated Processes

Many transport processes appear to be mediated: the flux may be more rapid than that which is predicted by Fick's and the Nernst-Planck equations, or may attain a flux equilibrium state different from the predicted value. Flux may be saturable, stereo-specific, inducible, subject to competitive and non-competitive inhibition, to trans effects, and exhibit a higher activation energy than expected of physical processes.

On the assumption that a specific interaction of permeant with mediator limits the kinetics of such transport, unidirectional flux across the membrane is described by an equation of the Michaelis-Menten type:

$$J_s^{ct} = \frac{J_{max}^{ct} \cdot [S^c]}{K_T + [S^c]} \qquad \text{Eq. 4.3}$$

where J_s^{ct} is the flux of permeant from cis to trans, J_{max}^{ct} is its maximum, K_T is the affinity of the interaction, and $[S^c]$ is the concentration of the permeant at the cis position. For linear systems the principle of superposition is applicable, so the total flux of a membrane in which both mediated and non-mediated transport occur may be described simply as the sum of the two processes.

Where the driving force of the transport process is the electrical or chemical potential of the permeant species itself the process is termed mediated diffusion (syn. facilitated diffusion): a spontaneous transport process which is more rapid for a particular species than for other species of similar molecular properties.

Particularly relevant to studies of isotope flux are two diffusion phenomena. Exchange diffusion is the rapid exchange of permeant molecules across a membrane by mediated diffusion. If a membrane separates two compartments having equal activities of a permeant but with a tracer in only one compartment, a rapid unidirectional flux of the tracer is observed though no net permeant flux occurs. Counter transport (syn. counter

flow, competitive exchange diffusion) occurs where a single mediating mechanism exists for two permeants A and B. At equilibrium for A the fluxes in each direction cancel out. If B is now added to the cis compartment, competition by B reduces one flux of A without initially affecting the opposite flux ... a net flux of A into the cis compartment is observed.

4.5 Active Transport

Active transport occurs when transport of particles is energetically coupled to chemical reactions without the particles themselves participating in the chemical reaction (Kedem, 1961). It is therefore capable of working against an electrochemical gradient of the permeant. The nonconjugate driving force may itself be a chemical or electrical gradient. This definition does not preclude from active transport an energetic process moving a permeant down its electrochemical gradient: possibly under these conditions such biological mechanisms revert to mediated diffusion.

Kedem's definition does preclude the sequential process wherein a permeant moves independently of any chemical reaction but is subsequently modified or sequestered in the trans compartment, termed a trapping mechanism. A hypothetical system would be sucrose diffusion through a permselective membrane into an inner compartment containing invertase whereupon hydrolysis of sucrose maintains disequilibrium by substrate conversion. Flux of carbon atoms will be concentrative though sucrose moves by diffusion.

In primary active transport (syn. chemi-osmotic transport) movement of the permeant is directly coupled to the energetic process (but is nonconjugate). (Where the coupled process affects the association or dissociation of the carrier/permeant complex it is termed scalar coupling; where the translocation complex is directly coupled to the energetic process it is termed vectorial coupling.) Systems exist in which a gradient so generated or maintained is in turn the driving force for transport of a second permeant: this is termed secondary active transport (syn. osmo-osmotic transport).

Where a single permeant is transported the term uniport may be used. In the case of cotransport the movement of one permeant is dependent upon

simultaneous movement of a different permeant in either the same direction (symport) or in the opposite direction (antiport).

The experimental criteria for active transport are presented in Table 4.2.

Chemiosmosis, which conceptually is the reverse of primary active transport, is a process by which the free energy of an electrochemical gradient performs work in driving the flow of a chemical reaction.

An ion transport which is driven by metabolism and which generates an electropotential is said to be electrogenic. Evidently many electrogenic pumps (and hence transport processes) are energized by ATP. It is unresolved as to which other sources function to energize transport processes in particular plant membranes. For instance, cytochromes and oxido-reductases of the plasmalemma and tonoplast have been reported by some workers.

Specifically, a difference of proton activity across a membrane represents a proton motive force. In proposing this term in his chemiosmotic theory, Mitchell was describing the primary form of conserved redox energy (across the inner mitochondrial membrane) which might be transduced to the chemical energy of ATP. But the term has been generalised to describe the force, however generated or dissipated. The electrochemical gradient of protons ($\Delta\overline{\mu}_{H}{}^{+}$) consists of the charge difference across the membrane due to the protons ($\Delta\Psi$) and the chemical potential difference ($\Delta\mu_{H^+} \equiv \Delta pH$). The relative contribution of each component in energized biological membranes varies according to the movement of other ions and the compartments' buffering capacities.

In group translocation covalent modification of the permeant occurs during the transport event. Group translocators consist in part of enzymes which are often found in the soluble phase. By contrast, most carriers are proteins showing lesser selectivity than their related enzymes (higher affinity for analogues and isomers) but specificity for particular ions. In this thesis "group transport" is used synonymously with group translocation.

As Bunow (1978) stressed, the vectorial process is merely a consequence of the topology of an asymmetrical membrane system functioning between media of different constitutions. Clearly such systems will be

Table 4.2: Active transport

There is no adequate single criterion for active transport. Several of the following are required to be tested (Heinz, 1979; Lüttge and Higinbotham, 1979):

1. The flux of a permeant at the membrane does not conform to the equations 4.1 and 4.2. Moreover the two equations predict (respectively) a distribution at flux equilibrium as follows: - for uncharged permeants, equal activities ($\alpha_s^c = \alpha_s^t$) on either side of the membrane; - for charged permeants,

$$\Psi_M = \frac{RT}{zF} \cdot \ell n \frac{\alpha_s^c}{\alpha_s^t} \qquad \text{Eq. 4.4}$$

where Ψ_M is the membrane electrical potential.

2. For a charged permeant : considering the mobilities of anions and cations, Ψ_M exceeds the maximum value predicted from diffusion.

3. For a charged permeant : withholding an ion induces depolarization of the membrane.

4. For a charged permeant : metabolic inhibitors rapidly and reversibly depolarise the cell.

These are negative criteria. The following positive criteria are particularly relevant to transport of uncharged solutes.

5. Flux has a high Q^{10} value.

6. Flux exhibits various properties of carrier/permeant interaction e.g. enzyme kinetics (saturation, competitive inhibition, analogue effects).

7. Stoichiometric coupling of transported permeants is evident.

8. The following ratio (Eq. 4.5) - the maximum initial net flow of the permeant over the Michaelis constant of this flow, divided by the corresponding ratio of the initial net flow in the opposite direction - is unity for passive processes.

$$\text{Haldane Ratio} = \frac{J_{max}^{ct}}{K_T^c} \cdot \frac{K_T^t}{J_{max}^{tc}} \qquad \text{Eq. 4.5}$$

From this one can also calculate the maximum static head ratio α^t/α^c at flux equilibrium, when $J^{ct} = J^{tc}$.

□

sensitive to many factors: substrate activity, cofactors, competitors, pH, ions, hormones, phytochrome, lipid domain, temperature and pressure. Certain sugar transport proteins have SH-groups essential to their activity.

4.6 Other Transport Processes

Ionophores (of synthetic or microbial origin) enhance the ion permeabilities of membranes, and are generally highly specific for an ion. Flux is maintained by an electrochemical gradient and is obviously influenced by the presence and nature of other anions and cations.

In some excitable membranes (e.g. nerve axon) a phenomenon, visualized as specific ion gates, has been demonstrated to allow momentary transfer of ions (Na^+) at extremely rapid rates.

Membrane vesicles may transfer both small molecules and macromolecules. Exocytosis is the fusion of membrane vesicles with the plasma membrane and external release (secretion) of the contents. In apocrine secretion free droplets of material at the cytoplasmic face of the plasmamembrane become surrounded by part of that membrane thus forming a vesicle which then pinches off to the exterior. The topological converse of exocytosis is endocytosis, transfering into the cell particulate or soluble material ... respectively, phagocytosis and pinocytosis.

The sequestration of cytoplasmic material by fusion of several membrane segments is observed, such as in the formation of autophagic vacuoles (literally, "self-digestion").

Generally these membrane processes each require metabolic energy: poisoned cells do not exhibit the phenomena.

Several biochemical tools for the study of membrane processes should be mentioned. The fungal toxin, fusicoccin, a diterpene glucoside affecting many physiological phenomena, apparently stimulates proton pump ATPase linked with K^+ uptake, with consequential hyperpolarization of the transmembrane potential difference (Marré, 1978). Jung and Lüttge (1980) suggest that it acts directly upon a chloride pump. Conversely, diethyl stilbestrol (DES) will inhibit proton extrusion by a membrane K^+, Mg^{2+} -ATPase (Colombo et al., 1978; Delrot and Bonnemain, 1979). Various

polyene antibiotics, e.g. nystatin, act as protonophores (Palacios and Serrano, 1978) and so will reduce a proton gradient: a proton-sugar cotransport of *Chlorella* is thereby reduced to a mere facilitated diffusion system (Komor, et al., 1978).

Specific ionophores have been mentioned. Some may not simply perturb, but may also damage, the membrane (e.g. CCCP : Willenbrink, 1980). DMSO selectively affects the permeability of the plasmalemma (Delmer, 1979).

As sugar transport proteins have often been shown to possess sulfhydryl groups essential to their function, the inclusion of reductants and SH-groups is usually required in incubation media. Sulfhydryl-specific reagents can be used to deactivate these proteins, thereby giving evidence about their location. Non-penetrating parachloromercuribenzenesulfonic acid (PCMBS) will only affect proteins on the exposed membrane, whereas N-ethylmaleimide (NEM) will penetrate (Delrot, et al., 1980).

Finally, non-metabolized analogues such as 3-0-MG and 2-deoxyglucose are useful in distinguishing transport from subsequent non-vectorial metabolism which, by sequestering a transported species, affects the transport process.

CHAPTER 5: TRANSPORT PROCESSES IN HIGHER PLANTS : SPECIFIC EXAMPLES

TRANSPORT PROCESSES IN HIGHER PLANTS : SPECIFIC EXAMPLES

5.1 Cotransport of Sugars

Membrane transport of sugars in higher plants has been covered in recent reviews (Table 4.1). The purpose of this section is to elaborate upon the preceding chapter by presenting some of the better characterised sugar transport systems.

The depolarization of a membrane attributed to cotransport of sugar with an ion has been demonstrated in large-celled microorganisms (e.g. proton-glucose symport in *Neurospora* : Slayman and Slayman, 1974), algae (e.g. in *Chlorella* : Komor and Tanner, 1974, 1976) and in higher plants (e.g. glucose uptake in *Lemna gibba* : Lüttge, 1977 ; sucrose transport in the nyctinastic plant *Samanea* : Racusen and Galston, 1977). With the availability of microelectrodes (Findlay and Hope, 1976) and of weak, lipophilic ions (Skulachev, 1970; Rottenberg, 1979) membrane potentials are increasingly being assessed. Similarly pH gradients can be measured by the distribution of weak acids and bases (Walker and Smith, 1975; Azzone et al., 1978) and by ^{31}P NMR (Roberts et al., 1980).

Komor, Tanner and coworkers have described a proton-sugar symport, thought to be phloem loading, in cotyledons of *Ricinus communis*. Uptake is electrogenic such that a transient efflux of K^+ occurs, as charge compensation, until there is no net H^+ flux due to an activated proton pump (Komor, 1977; Komor et al., 1977; Cho and Komor, 1980; Komor et al., 1980; Martin and Komor 1980; Tanner, 1980).

Hutchings (1978a, b) monitored changes of pH and K^+ in the apoplast of *Ricinus* cotyledons during sucrose loading and postulated the same mechanisms as Komor.

Giaquinta (1977) incorporated such a symport into his model for phloem loading, accommodating the following properties : the alkalinity (pH8-8.5), high K^+ and ATP concentrations of the phloem content, ATPase activity of the plasmalemma, an acid apoplasm in leaves next to the veins, and the cessation of loading when a non-permeant buffer was used to reduce apoplasm $[H^+]$.

However, Malek and Baker (1977, 1978), studying sucrose loading into leaf petioles of *Ricinus*, proposed that protons were transported to the apoplasm by an ATPase/proton-K^+ antiport (primary active transport). The resultant PMF energized sucrose loading as a proton-sucrose symport (secondary active transport).

Table 5.1 presents (in two parts) the published proposals for proton-sucrose symports and for proton-hexose symports. In Table 5.2 kinetic parameters for mediated uptake of various sugars are listed. Some of these proposals have been thoroughly investigated, whilst most remain tentative. Sucrose uptake may be without prior hydrolysis (e.g. Kriedemann and Beevers, 1967; Chin and Weston, 1975; Dick and Ap Rees 1975) or with hydrolysis (see Table 5 of Geiger, 1975).

5.2 Transport Metabolites

Lüttge and Higinbotham (1979) review the so-called transport metabolites and the intracellular shuttles, of which one is the dihydroxyacetone phosphate (DHAP) - phosphoglyceric acid (PGA) shuttle across the chloroplast envelope. Triose phosphate (DHAP) transport at the inner chloroplastic membrane is coupled with orthophosphate exchange (1:1 stoichiometry). Heldt et al. (1977; Herold et al., 1980) postulate a key role for orthophosphate in regulation of assimilated carbon flow, the feedback control of photosynthesis, and starch formation. The chloroplast envelope is not permeable to sucrose, but facilitated diffusion of glucose (and other hexoses) is observed (Schäfer et al., 1977).

5.3 Group Transport of Sugars

The bacterial phosphoenolpyruvate-linked phosphotransferase system which catalyses group transport of various glycosyl groups has been well documented (Semenza and Carafoli, 1977). Though group transport systems probably occur in plants, none has been characterised. Nor has any redox-driven transport system been demonstrated in plants. It has been proposed that sucrose inversion can be vectorial. Similarly, the presence of trehalase in plants despite very low levels of trehalose has led to a proposal of a trehalose-mediated glucose transport (Glasziou and Gayler, 1969;

Table 5.1a: Published proposals for proton-sucrose symports in higher plants

Reference	Plant tissue	Remarks
Delrot and Bonn 1979; Delrot 1980	*Vicia faba* minor veins	at less than 20 mM
Doll et al. 1979; Willenbrink and Doll 1979	red beet root vacuoles	———
Giaquinta 1977, 1979	sugar beet minor veins	———
Gumber et al. 1980	lily pollen	K_T 1.8 mM sucrose; E_a-11 Kcal mol^{-1}; pH opt 5; D-glucose, D-galactose inhibit
Heyser 1980	maize leaf slices	———
Humphreys 1978	maize scutellum	K_T 20 mM sucrose
Hutchings 1978a, b	*Ricinus* cotyledons	———
Komor et al. 1977	*Ricinus* cotyledons	K_T uptake 25 mM; K_T export 35 mM
Lichtner and Spanswick 1978, 1979	soybean cotyledons	———
Malek and Baker 1977, 1978	*Ricinus* petiolar phloem	———
Racusen and Galston 1977	*Samanea* pulvini	———
van Bel and van Erven 1979	tomato internodes	———

Table 5.1b: Published proposals for proton-hexose symports in higher plants

Reference	Plant tissue	Remarks
Colombo et al. 1978	maize roots	glucose
Coombe and Matile 1980	grape pericarp	glucose
Ehwald and Jahn 1976	wheat roots	hexoses
Guy et al. 1978, 1980	*P. sativum* mesophyll protoplasts	glucose
Jones et al. 1975	*Impatiens balsamina* root cells	glucose
Novacky et al. 1978; Ullrich et al. 1978	*Lemna minor* leaves	glucose, fructose

Table 5.2: K_T for mediated uptake of various sugars

Reference	Plant tissue	Values in mM
Bieleski 1977	Rosaceae leaf slices	glucose : I 1 , II 60 sorbitol : I 3 , II 100 these not competitive
Bowen 1972	Immature sugarcane parenchyma	glucose : 6.7 ; fructose 8.4 ; competitive
Cameron-Mills and Duffus 1979a, b	Immature barley embryos	sucrose : 36
Carlier 1975	Pelargonium leaf discs	glucose or 3-O-MG : I .19 II .51 III 2.9 IV 140
Cataldo 1974	tobacco leaf discs isolated mesophyll cells isolated vascular bundles	sucrose : 32 glucose : 17 30 16 16 58
Grant and Beevers 1964	maize, carrot root tissue	glucose 11 ; fructose 15 ; galactose 5
Hampson et al. 1978	cotton hypocotyl segments	glucose 1 ; fructose 8 ; sucrose 8
Linask and Laties 1973	aged potato tuber slices	glucose or 3-O-MG : I 0.027-0.40 II 0.52 -0.53 III 3-7.7 IV 24-54 Data from 3 years; always 4 phases to isotherm.
Maretzki and Thom 1972	cultured sugarcane cells	glucose : system I 0,02 (preincubate w/o glucose) II 1.40 (preincubate with glucose)
Schäfer et al. 1977	spinach chloroplast	glucose : E_a 17 kcal mol^{-1}
Sokolova et al. 1979	cowparsnip leaf petiole phloem tissue cowparsnip cells with xylem	sucrose : 7.6 :14.3
Sovonick et al. 1974	sugar beet discs	sucrose : 88
Turkina and Sokolova 1972	sugar beet leaf vascular bundle	sucrose :12.5
Vickery and Mercer 1964	bean leaf	sucrose : 10
Willenbrink and Doll 1979	red beet storage root	sucrose : 22

and criticism in 1972a, b). Bieleski (1980) proposed that in nectaries of *Rosaceae* sorbitol (the translocated sugar) is converted to hexoses during phloem unloading.

Certainly the high phosphatase activity of nectary glands (Ziegler, 1956) and transfer cell membrane suggests a role for phosphorylation of sugars in export, but whether this is an integral part of the transport step is unclear (Lüttge and Schnepf, 1976). Shiroya (1978) used neutron activation and autoradiography to assay the sucrose phosphate produced by different sunflower leaves, and found a strong correlation between the proportion of sucrose phosphate in the leaf and the rate of translocation from it.

5.4 Sugar Accumulation in Sugarcane

Maretzki and Thom (1972) found that upon addition of ^{14}C-glucose to sugarcane cells in suspension half the incorporated label was phosphorylated within five seconds. The two pentoses, L-arabinose and L-ribose, did not compete for uptake : the authors deduced that the carbon-6 must be available for phosphorylation. Galactose and 3-O-MG competed : hence the deduction that configuration at carbons-4 and -3 were not important. Mannose did not compete : hence configuration at carbon-2 was critical. Developing this study, Bowen (1974) found 2-deoxy-D-glucose was taken up at a comparable rate by immature internodal tissue of sugarcane without phosphorylation. D-1-^{14}C-glucose-6-phosphate-^{32}P was dephosphorylated prior to uptake but rephosphorylated (^{31}P) subsequently. Galactose was also recovered as galactose-1-phosphate. U-^{14}C-fructose was taken up and recovered primarily as free fructose and glucose (within 15 s) ; U-^{14}C-glucose was recovered primarily as the phosphorylated hexoses. Pretreating tissue with rabbit anti-invertase antiserum eliminated sucrose transport - evidence that prior hydrolysis of the dissacharide was a requisite of uptake.

Glasziou and Gayler (1972a, b) reviewed their work on sugarcane, which Coombe (1976a) partly summarized as follows:

> While the sugarcane internode parenchyma cells are elongating the accumulating mechanism appears to be: sucrose in the cell wall solution is hydrolyzed by a soluble acid invertase; glucose and fructose traverse

> the plasmalemma by separate, energy-coupled mechanisms; in the metabolic compartment (cytoplasm) the hexoses are phosphorylated and sucrose phosphate is synthesized; the sucrose moiety of sucrose phosphate is transferred across the tonoplast by an energy-coupled mechanism. This can proceed against a concentration gradient. An invertase in the vacuole converts sucrose to glucose and fructose. When extension growth stops and the internode matures there is a large increase in the concentration of vacuolar sucrose. The accumulating mechanism now differs from the preceding in that the wall invertase is a bound enzyme and there is no invertase activity in the vacuole. At all stages there is a neutral invertase in the cytoplasm which inverts free sucrose occurring there.

5.5 Integration of Processes by Cotransport

On the one hand tissue phenomena which are complex are not readily analysed into discrete components. But where separate processes such as specific membrane transport can be described, reintegration of these poses questions concerning regulatory mechanisms. As evident in transfers across the chloroplast membrane, an essential function of cotransport (whether in fact group transport or strictly osmo-osmotic) may lie in the regulatory role of the cotransported species in other processes.

5.6 *In Vitro* Diffusion

Most sugar transport studies reveal a component of uptake linear with time and concentration attributed to passive diffusion (Cameron-Mills and Duffus, 1979; Sokolova, 1979; Willenbrink and Doll, 1979; Delrot, 1980). Reports concentrate upon the kinetics of a saturable process with little regard for this other event. Yet it is dominant at concentrations well below the estimated J_{max} of the mediated process. It is expected to occur at all concentrations and therefore to affect determination of apparent K_T and J_{max} of any carrier. The integrity of the membranes *in vitro* must be questioned, as too the homogeneity of the tissue cells, and the reliability of the tracer to represent flux when retention of endogenous sugars is ill-defined.

Using carrot callus cells, Parr and Edelmann (1976) deduced from washout kinetics that

> the passage of sugars across the plasmalemma occurs mainly, if not entirely, by passive diffusion ... and that the apparent free space includes most, if not all of the metabolic compartment.

Hence other workers' conclusions as to active uptake at the plasmalemma may in fact concern active uptake at the tonoplast with or without prior metabolism. Wyse and Saftner (1979) came to the same conclusion working with *Beta vulgaris* root tissue in which sucrose transport characteristics at the plasmalemma were passive (Q_{10} = 1 ; CCCP insensitive) whilst vacuolar sucrose was ten times the free space concentration (by compartmental analysis). Evidently the concentrative step for sucrose storage was at the tonoplast.

In contrast, Kaiser et al. (1979) concluded that in mesophyll cells of poppy and spinach leaf release of photosynthate through the plasmalemma was extremely low - less than 0.5% of the fixative rate.

Carlier (1974, 1975) described an active uptake system in *Pelargonium* leaf discs which appeared to be stimulated by efflux of the same substrate as an imperfect exchange diffusion.

5.7 Superimposed Processes?

Inflexion in the isotherms of uptake rate versus solute concentration are conventionally interpreted to indicate membranes in series, several transport sites in parallel, transport plus diffusion, or allosteric transitions of the carrier. Bange (1979) argued that such inflexions can result from the movement of substrate particles both in the bound and the free state through a common membrane channel with oscillating binding sites, with negative cooperativity.

Clearly one must question the relationship between non-mediated and mediated transport when observed *in vitro*. With regards to methods:

(i) Is the diffusion an artefact of experimental method? If it is constitutive, has it been increased by the method?

(ii) Even though the two processes may be assessed for the period of incubation, what consequence should be assigned to that diffusion which occurred before and after the incubation?

With regard to the processes:

(iii) Are the two phenomena indicative of two cell types?

(iv) Are the two additive at all substrate concentrations?

(v) Does regulation of the mediated process impinge upon the other?

(vi) How is each process affected by the unstirred layer at the outer face of the plasmalemma and by cyclosis at the inner face?

5.8 Vesicular Transport

In fresh water giant algae such as *Nitella* there are parallel fast and slow phases of chloride flux between the medium and vacuole. MacRobbie (1975) argued that vesicles, derived from ER closely appressed to the plasmalemma, rapidly accumulated the ions then fused with the tonoplast. Salyayev and Salyayeva (1968) coined the term "translosomes". Such a "mini-vacuole" path was also proposed in maize root tip cells (Leigh et al., 1973). Cataldo (1974) envisaged such a "transport compartment" based upon the ER association with plasmodesmata and regulated by cytoplasmic sucrose concentration. Wyse (1979), noting the sites where plasmalemma and tonoplast were appressed in sugarbeet root cells, invoked a similar system for sugar transport. But Cram (1980) argued that on many accounts "pinocytosis does not occur ... and could not occur in plant cells."

Northcote (1972) and Chrispeels (1976) considered transport of extracellular macromolecules. Whilst large scale transfer of solutes by pinocytosis is unlikely our understanding of plant membrane flow is poor. Transfer of macromolecules in animal cells by receptor-mediated endocytosis is receiving new attention (Goldstein, et al. 1979), and this may encourage more exacting investigations of pinocytosis in plants.

CHAPTER 6: THE USE OF PROTOPLASTS IN TRANSPORT STUDIES

THE USE OF PROTOPLASTS IN TRANSPORT STUDIES

According to Vasil (1976) :

> The term *protoplast*, describes that part of the plant cell which lies within the cell wall and can be plasmolyzed, and which can be isolated by removing the cell wall by mechanical or enzymatic procedures. The protoplast is, therefore, only a naked cell-surrounded by the plasma membrane-which is potentially capable of cell wall regeneration, growth, and division.

6.1 Protoplasts as a Tool in Fruit Physiology

In research on the physiology of fruits isolated protoplasts could serve many purposes e.g. :

(i) Properties of the plasmalemma - such as transport characteristics and secretion - may be directly examined without the complications of an ill-defined extracellular space.

(ii) Protoplasts are easily manipulated experimentally and *in vitro* metabolism of tracers may be followed with sequential sampling (and rapid inactivation) from one population.

(iii) The cell is accessible for non-destructive assessment by spectroscopy, radio-immunoassays, binding, etc.

(iv) Compartmental analysis is possible through controlled lysis and recovery of subcellular entities, including cell membranes and preparation of loaded membrane vesicles. Robinson and Walker (1979) elegantly demonstrated the cytoplasmic synthesis of sucrose from labelled products of photosynthesis by separating intact chloroplasts from protoplasts within six seconds of lysis.

(v) One envisages the use of cell sorters to separate subpopulations from heterogenous tissue for separate analyses. Zimmerman et al. (1980), in a single pass through a Coulter

counter, could measure the volume, internal conductivity and dielectric breakdown voltage of cells, thereby identifying different stages of vacuole development.

(vi) Cells may be maintained free of contaminating organisms. Moreover, the relationship between pathogens and individual cells may be examined. One may study the biophysical properties of cells as entities rather than as components of an interacting system.

In short, protoplast technology is a tool for integrating molecular events with complex processes that would be lost in homogenates or confounded in tissue.

However, there are difficulties and limitations in the use of protoplasts which must be borne in mind. For instance, it is simpler to prepare tissue slices than protoplasts. A key component of routine protoplast work is the use of well-defined, uniform starting material. Fruits, as products of a generation or season, are rarely uniform - not even after controlled production or careful selection. Additional specific hazards are: the high osmotic potential of fruits, the large and variable cell size, the inevitable selection of cells by loss during isolation, the modification of membranes with maturation, the presence of deleterious compounds such as phenols, and the high capacity for ethylene production.

Hazards peculiar to uptake studies include the following.

(i) A degree of free-space binding occurs even on protoplasts.

(ii) Metabolism on the exterior surface may still be confused with transport phenomena or cytoplasmic metabolism.

(iii) Data may still be confounded by compartmental intracellular distribution of a substance taken up.

(iv) Particular transport proteins are sensitive to osmotic shock (Ferenci et al., 1977; Guy et al., 1979).

(v) The light spectrum is ill-defined in cultures.

(vi) Cells which have been previously starved of a solute may take up this or related compounds more rapidly than normal cells.

6.2 Is the Physiology of the Protoplasts Representative?

But the primary question in physiological studies is (Burgess, 1978): Does the protoplast behave as a cell without a wall?

In protoplasts one has lost the plasmodesmata; the micro-environment of the plasmalemma against the cell wall; normal variations of turgor; and the spatial relationship between cells - especially where "target" or receptor cells exist in the tissue. The surface area of the plasmalemma has been reduced - by about 40% according to one study (Morris and Thain, 1980a). The plasmalemma is in an artificial medium, of buffering and electrochemical capacity unlike the apoplast. An array of fixed charges in the cell wall has been removed.

During the preparation of protoplasts the tissue is plasmolysed and thereby exposed to water stress. While the cell wall is exposed to high activities of cellulase and pectinase there is normally a spectrum of extraneous activity in these preparations, including proteinase, nuclease, and lipase (Cocking, 1972; Boller and Kende, 1979) and of salts. It is notable that these cell-wall degrading enzymes are often extracted from plant pathogens. It is understandable that laboratories are now, in some measure, purifying these enzymes prior to use.

6.3 Perturbed Metabolism

There have been many reports of perturbed metabolism in protoplasts. Premecz et al. (1977, 1978) found higher proline levels in protoplasts isolated in higher osmotica. Using RNA labelling as a measure of response to stress, they showed that the concentration of osmoticum affected the duration of suppressed RNA synthesis and high ribonuclease activity. Hoffman et al. (1975) found respirations rates typical of a stressed condition in protoplasts from *Petunia*. Fleck et al. (1980) identified a change in protein synthesis in protoplasts relative to tissue : for instance, no ribulose 1, 5-biphosphate carboxylase was synthesized. Photosynthesis in *Chlorella* protoplasts was only 20% of the rate in vegetative cells (Webb et al., 1980).

Ruesink (1973) showed leucine uptake by protoplasts to be much less than by intact cells. Robinson and Mayo (1977), studying this more closely, reported that uptake increased several-fold during the 8 h after isolation and inhibitors of protein synthesis prevented this increased rate of uptake. Taylor and Hall (1976, 1978) found protoplast isolation produced a marked increase in membrane permeability as indicated by the rate of leakage of preloaded ^{32}P and ^{86}Rb. Nevertheless protoplasts took up ^{32}P in a linear manner. The fine structure of protoplasts and tissue was similar, except for the presence of osmiophilic droplets in the chloroplast and cytoplasm of protoplasts. Morris and Thain (1980a, b) also identified in protoplasts many small membrane-bound vesicles (diameter 0.1-0.6μm) both within the central vacuole and within large cytoplasmic vesicles. Yet these protoplasts fixed $^{14}CO_2$ at a rate similar to that in the source tissue.

By maintaining protoplasts of *P. somniferum* in the dark during the "20 h stress period" following incubation the normal photosynthetic capacity was regained (Paul and Bassham, 1977). Any light during these 20 h stopped recovery.

Obviously it is necessary to find attributes (such as O_2 or CO_2 exchange) which may be used to monitor a protoplast preparation simply and thereby to optimize the isolation procedure. In some circumstances a particular attribute may suffice : observation of staining with neutral red, FDA, Evans blue, erythrocin B ; of cyclosis or osmotic-responsiveness.

6.4 Electrical Properties

There is clear evidence that protoplast isolation results in the normal electrical potential (inside negative) of the plasmalemma being converted into a small positive potential (Heller et al., 1974; Racusen et al., 1977). The same phenomenon was observed in plasmolysed tissue. Regeneration of the cell wall by protoplasts was associated with restoration of a negative potential. By contrast, protoplasts of maize which failed to produce a functional cell wall in culture also failed to regain the normal negative potential (Kinnersley et al., 1978).

Heller et al. (1974) also reported loss of membrane potential upon isolation of *Acer* protoplasts, as also loss of electrical resistance in

the plasmalemma (Rona and Cornel, 1979). On the other hand, the surface fixed charge of the plasmalemma (ζ : Theta potential) - due mainly to membrane phosphate groups - is negative and additives in a protoplast suspension can neutralize the charge (Grout et al., 1974). Nagata and Melchers (1978) ascribed clumping of protoplasts to such loss of theta charge, whereas protoplasts of negative theta potential flowed separately in cell electrophoresis.

Just as optimization of protoplast production may lead to improved rates of photosynthesis, so too recent reports have indicated "normal" transmembrane potentials in protoplasts. Rubinstein (1978) used a lipophilic cation, triphenyl methylphosphonium, to determine the potential of oat protoplasts as -62 mV, which was depolarized by sodium azide. Briskin and Leonard (1979) used microelectrodes to measure the following membrane potentials (inside negative) : unplasmolysed tobacco cells, -52 mV ; cells in 0.3M mannitol, -50 mV ; cells in 0.7M mannitol, -49 mV ; protoplasts in 0.7M mannitol, -49 mV.

6.5 "Recovered" Protoplasts

The capacity of protoplasts to recover from the trauma of isolation was demonstrated by Burgess et al. (1978). An 8 h lag preceded visible wall formation on tobacco leaf protoplasts, but subsequent removal of this wall - without additional osmotic adjustment - resulted in a lag period of only 12 minutes.

Nevertheless, the question persists : how comparable is the physiology of "recovered" protoplasts to that of the original cells? In the related field of tissue culture, Pech et al. (1979) observed that pear tissue calli were of a juvenile type (with respect to amylase pattern) whether derived from fruit, stem or petiole. Isolated protoplasts are tools to bridge the common discontinuity between cell biochemistry and cell physiology, or between cell physiology and tissue phenomena. Their use will normally require corroborating work in the larger or smaller dimension.

6.6 The Literature

In the vast literature concerning protoplasts, many reviews of isolation procedures are available. Unfortunately, as single chapters in larger texts, these tend to repeat one another. Furthermore, in the literature one notes diverse manipulations which, in a particular laboratory, proved critical for the isolation of protoplasts from a given species or tissue, yet are unmentioned in the reviews.

Table 6.1 is an annotated list of general references, supplemented with those pertaining to specific techniques which may not be included in the reviews. Ruesink (1980), in a concise but excellent review, can be credited for an elementary but long-awaited table, namely the collation of reported protoplast releases.

Cocking (1960) was the first to use a fungal enzyme to release protoplasts from higher plants, while Takebe et al. (1968) considerably advanced the technology using extracts from *Rhizopus* and *Trichoderma*. Table 6.2 lists the commonly used enzymes for removal of cell walls. The classifications are nominal as each preparation exhibits a broad activity of other enzymes. Recent evidence underscores the need - especially in biochemical or physiological studies - of preliminary purification of these enzymes (Guy et al., 1978; Boller and Kende, 1979).

Only fourteen references to the isolation of protoplasts from fruit tissue have been located (Table 6.3), of which four are directed at the physiology of fruits.

6.7 Protoplasts in Uptake Studies

Table 6.4 lists reports of uptake studies upon isolated protoplasts, including several where incorporation was the essence of the investigation. It does not include uptake by pinocytosis (Willison et al., 1971), via loaded lipid vesicles (Poste et al., 1976; Cassells 1978), or of hormones (IAA : Sõnka et al., 1977). Considering that some of these investigations were essentially studies of incorporation, and that others have not been reported beyond original abstracts, few laboratories have successfully exploited protoplasts for transport studies thus far, contrasting with the use of isolated vacuoles.

Table 6.1: Methods of protoplast isolation - reviews and specific techniques.

Authors	Title	Additional Remarks
Barz et al. 1977	Plant tissue culture and its biotechnological applications	Proceedings; incl. isolation and cultivation (Eriksson), hanging drop multiple-array technique for assessment (Potrykus).
Bhojwani et al. 1977	Protoplast technology in relation to crop plants - progress and problems.	review
Burgess 1978	Plant cells without walls?	critique
Burgess and Linstead 1976, 1977	Ultrastructural studies of the binding of Concanavalin A to the plasmalemma of higher plant protoplasts.	Gold-conjugated ConA to protoplasts from V. vinifera cell culture. Methods for SEM of protoplasts.
Cassells et al. 1976	Environmentally induced changes in the cell walls of tomato leaves in relation to cell and protoplast release.	"soft-plants" - low irradiance - give good yield.
Edwards et al. 1978	Photosynthesis by isolated protoplasts, protoplast extracts, chloroplasts by wheat.	intact, functional (97%) chloroplasts from protoplasts.
Hughes et al. 1976	Scanning electron microscopy of barley protoplasts.	
Kanai and Edwards 1973	Purification of enzymatically isolated mesophyll protoplasts from C_3, C_4 and CAM plants using an aqueous dextran-polyethylene glycol two-phase system.	
Larkin 1976, 1977	Purification and viability determinations of plant protoplasts.	Lymphoprep^R for purification by 2-phase centrifugation; FDA to assess viability by fluorescence.
Maretzki and Thom 1978	Transport of organic and inorganic substances by plant cells in culture.	review : the methods, evaluation and limitations of such data.
Nagata and Ishii 1979	A rapid method for isolation of mesophyll protoplasts.	Pectolyase Y23 to stimulate maceration withi 25'.
Nishimura et al. 1976	Isolation of intact chloroplasts and other cell organelles from spinach leaf protoplasts.	lysis by shearing in a syringe.
Peberdy et al. 1976	Microbial and plant protoplasts.	Proceedings
Perlin and Spanswick 1980	Labeling and isolation of plasma membranes from corn leaf protoplasts.	external labelling with diazotized [^{125}I] iodosulfanilic acid.
Perlman 1979	Use of antibiotics in cell culture media.	comprehensive review and listing.
Reinert and Bajaj 1977	Applied and fundamental aspects of: Plant cell, tissue and organ culture.	techniques (Bajaj); comprehensive table (p48 of plant protoplast cultures to 1976.
Robinson and Walker 1979	The site of sucrose synthesis in isolated leaf protoplasts.	includes technique of silicone oil layer centrifugation.
Ruesink 1980	Protoplasts of plant cells.	Table of published successful protoplast release, with details and enzymes.
Schmidt and Poole 1980	Isolation of protoplasts and vacuoles from storage tissue of red beet.	incl. glusulase in digestion enzymes.
Street 1973 1974	Plant tissue and cell culture.	protocols for isolation (Cocking and Evans).
Thorpe 1978	Frontiers of plant tissue culture 1978.	Proceedings ; protoplast isolation (Eriksson et al.), ultrastructure, membrane transport (Maretzki and Thom).
Vasil 1976	The progress, problems and prospects of plant protoplasts.	
Villanueva et al. 1973	Yeast, mould and plant protoplasts.	
Wagner and Siegelman 1975	Large-scale isolation of intact vacuoles and isolation of chloroplasts from protoplasts of mature plant tissues.	
Watts and King 1973	The use of antibiotics in the culture of non-sterile plant protoplasts.	some antibiotics inhibit amino acid.
Wilkinson and Northcote 1980	Plasma membrane ultrastructure during plant protoplast plasmolysis, isolation and wall regeneration : a freeze-fracture study.	Solanum tuberosum callus ; digestion of prefixed (glutaraldehyde) cells.

Table 6.2: Enzymes commonly used for protoplast release

	Source	Supplier
CELLULASES		
Cellulysin	Trichoderma viride	Calbiochem-Behring, P.O. Box 12087, San Diego, Calif. 92112, USA.
Onozuka (P-1500, 4S, R-10) cellulase	Trichoderma viride	Yakult Biochemical Co. Ltd., Nishinomiya, Japan.
Meicelase	Trichoderma viride	Meiji Seika Kaisha Ltd., Tokyo.
Driselase	Basidicomycete	Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan.
PECTINASES		
Macerozyme R-10	Rhizopus sp.	Yakult Biochemical Co. Ltd., Nishinomiya, Japan.
Macerase polygalacturonase	Rhizopus sp.	Calbiochem-Behring, P.O. Box 12087, San Diego, Calif. 92112, USA.
Pectinase	Aspergillus niger	Sigma Chemical Corp., St. Louis, Mo 63178, USA.
Pectinase	Aspergillus niger	Koch-Light, Colnbrook SL3 OBZ, Buckinghamshire, U.K.
Rohament pectin glycosidase, rhozyme, pectinol		Röhm Gmblt, Darmstadt, Germany.
Colonase pectinase		Wakamoto Pharmaceutical Co. Ltd., Japan.
OTHERS		
Rhozyme (HP-150) hemicellulase	Rhizopus sp.	Röhm and Haas Co., Independence Hall West, Philadelphia, Penn. 19105, USA.
Helicase	Helix pomatia	Industrie Biologique Francaise S.A., Gennervilliers, France.
Glusulase	Helix pomatia	Endo Laboratories, Garden City, N.Y., USA.
Zymolase crude hydrolytic enzyme	Arthrobacter luteus	Kirin Brewery Co. Ltd., Japan.
Pectolyase Y23	Aspergillus japonicus	Seishin Pharmaceutical Co. Ltd., 9-500-1, Nagareyama, Nagareyama-shi, Chiba-ken, Japan.

Table 6.3: Reports of protoplasts from fruit tissue

Reference	Title / remarks
Anderson et al. 1979	"Metabolism of protoplasts from apple fruit tissue." / protoplasts, 188-225 µm ; produced ethylene and metabolised labelled methionine to ethylene.
Brown et al. 1978	"Isolation of vacuoles from V. vinifera pericarp tissue." / including protoplast isolation.
Gregory and Cocking 1965	"The large-scale isolation of protoplasts from immature tomato fruit." / protoplasts from locular and placental tissue with pectinase.
Mattoo and Lieberman 1977	"Localisation of the ethylene-synthesizing system in apple tissue." / as protoplasts form from slices of fruit, ethylene production stops.
Mayo and Cocking 1969, (Willison et al. 1971)	"Pinocytic uptake of polystyrene latex particles by isolated tomato fruit protoplasts."
Mishra and Colvin 1969	"The formation of wall-like envelopes by isolated tomato fruit protoplasts."
Pojnar and Cocking 1968; (Cocking and Pojnar 1969)	"Formation of cell aggregates by regenerating isolated tomato fruit protoplasts." / and TMV infection of protoplasts.
Raj and Herr 1970a	"The isolation of protoplasts from the placental cells of Solanum nigrum L." /pectinase alone was sufficient.
Raj and Herr 1970b	"Isolation of protoplasts from the placental cells of Lycopersicum pimpinellifolium. Mill."
Sharma et al. 1974	——— / protoplasts from placenta of Lycopersicon pimpinellifolium.
Skene 1974, (1975)	"Culture of protoplasts from grape vine pericarp callus." / and regeneration to callus.
Vardi et al. 1975	"Citrus cell culture : Isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos." / plantlets from protoplasts of ovular callus.
Weinbaum et al. 1979	"The influence of ethylene treatment of immature fruit of prune on the enzyme mediated isolation of mesocarp cells and protoplasts."
Willison and Cocking 1972; (Willison 1972)	"The production of microfibrils at the surface of isolated tomato fruit protoplasts."

Table 6.4: Solute uptake by protoplast suspensions

Reference	Title / remarks
Brown et al. 1978	"Isolation of vacuoles from V. vinifera pericarp tissue." / uptake of glucose and sucrose by protoplasts.
Cimino and Johnson 1977	"Fusicoccin-enhanced acidification and Rb^+ uptake in tobacco cell protoplasts."
Dekhuijzen et al. 1978	"The uptake of cytokinins by protoplasts and root sections of Brassica campestris." / cyt. uptake after cell wall regeneration, whilst uridine and adenine taken up by naked protoplasts.
Guy et al. 1978, 1980	"Membrane transport of sugars and amino acids in isolated protoplasts." / P. sativum mesophyll; glucose and amino acid uptake; see text.
Huber and Moreland 1980	"Translocation : Efflux of sugars across the plasmalemma of mesophyll protoplasts." / wheat and tobacco : during $^{14}CO_2$ fixation by protoplasts, efflux of ^{14}C-sucrose and -hexoses into medium, not hexose phosphates.
Lin 1980	"Comparison of ion transport characteristics in corn root segments and protoplasts." / respiration ; H^+ efflux ; K^+ , P_i influx comparable in protoplasts and root tissue.
Mettler and Leonard 1979a, b ; Briskin and Leonard 1979	"Ion transport in isolated protoplasts from tobacco suspension cells." / K^+ , $C\ell^-$, $H_2PO_4^-$ uptake respiration-dependent. Microelectrode for Eoi of protoplasts : -49 mV.
Morris and Thain 1980	"Comparative studies of leaf tissue and isolated mesophyll protoplasts I. O_2 exchange and CO_2 fixation. II Ion relations." / Nicotiana : K^+ influx strongly inhibitied by CN^- , DNP.
Premecz et al. 1978	"Effect of osmotic stress on protein and nucleic acid synthesis in isolated tobacco protoplasts." / both uptake and incorporation of labelled precursors is suppressedby higher osmotica (of mannitol).
Robinson and Mayo 1977	"Changing rates of uptake of tritiated leucine and other compounds during culture of tobacco mesophyll protoplasts." / see text.
Rollo et al. 1977	"Effects of fusicoccin on plant cell cultures and protoplasts." / N. tabacum leaf, S.oleracea leaf, P. tricuspidata cultures ; in protoplasts, FC promoted acidification of the incubation medium, uptake of K^+ and of 3-O-MG.
Rubinstein and Tattar 1978	"Control of amino acid uptake into oat leaves and protoplasts." / α-amino-isobutyric acid uptake by protoplasts.
Strobel and Hapner 1975	"Transfer of toxin susceptibility to plant protoplasts via the helminthosporoside binding protein of sugarcane." / sugarcane leaf protoplasts, the protein also conferred ability to take up ^{14}C-raffinose.
Taylor and Hall 1976	"Some physiological properties of protoplasts isolated from maize and tobacco tissues." / leaf protoplasts, ^{32}P uptake linear 1-4 h.
Zuil-Fodil and Esnault 1976	"Isolement de protoplastes de coléoptile d'avione et étude de leur caractéristiques physiologiques et ultrastructurales." / amino acid uptake by protoplasts and by tissue was similar. Polysaccharide formation observed in protoplasts after 20 h.

Nevertheless, two laboratories have demonstrated the value of protoplasts for transport studies.

(i) Guy and coworkers (1978, 1980) used pea mesophyll protoplasts to study sugar and amino acid transport. Their techniques included the use of dibutyl phthalate layer centrifugation, attention to the light flux upon the suspension during uptake, and a judicious use of double-labelling of substrates which facilitated immediate comparisons (rather than confounding the variability of different preparations). By the latter, they described diphasic uptake of D-glucose during 2 h, while L-glucose uptake was minimal in the same preparation. 3-O-MG uptake was 40 times the L-glucose rate, but only 45% of uptake by tissue slices. Subsequently this uptake of 3-O-MG was shown to be pH dependent, temperature dependent (E_a 20 kcal mol^{-1} : 17° to 27°), stimulated by Mg.ATP and promoted 2.5-fold by light (50 nE cm^{-2} S^{-1}/400-700 nm at surface of the suspension). The diphasic curve represented the approach to flux equilibrium of a small compartment of the protoplasts (probably the cytoplasm) with continued uptake (probably into the vacuole : Guy et al., 1979). From the response of the light- and dark-uptake to CCCP and ATPase-inhibitors, Guy concluded that more than one mode of energy coupling for sugar transport may operate at the plasmalemma of these protoplasts, one energized by the PMF and another directly by ATP or similar high-energy compounds.

Protoplasts also took up the amino acid analogue α-aminoisobutyric acid but this uptake declined after 10 minutes - a phenomenon not observed using tissue slices.

(ii) Mettler and coworkers (1979a, b; Briskin and Leonard, 1979, 1980) encountered three general problems in using protoplasts for uptake studies.

- To regularly obtain a sufficient quantity of relatively pure protoplasts.
- To assure osmotic stability in the protoplasts.
- To completely and rapidly separate protoplasts from the uptake medium. Particularly, they noted that repeated centrifugation with resuspension was too slow and cumbersome; vacuum filtration gave variable results and caused substantial breakage of protoplasts. To use a Ficoll density gradient was satisfactory.

In substantial reports these workers were able to demonstrate in *N. glutinosa* protoplasts : K^+ , $C\ell^-$, $H_2PO_4^-$ influxes which were sensitive to DNP and anaerobiosis ; Ca^{2+} influx which was not coupled to respiration; and similarity of the kinetics of these ion fluxes between protoplasts and cell suspensions. In the presence of Ca^{2+}, transport was selective for K^+ ($^{86}Rb^+$) over Na^+, and K^+ uptake showed negative cooperativity.

Whilst the difficulties and unknowns in working with protoplasts cannot be denied, these two laboratories have demonstrated the advantages of such an approach.

CHAPTER 7: THE VACUOLE OF HIGHER PLANTS AND TRANSPORT AT THE TONOPLAST

THE VACUOLE OF HIGHER PLANTS AND TRANSPORT AT THE TONOPLAST

7.1 Introduction

Five noteworthy articles mark the current interest in vacuoles of higher plants:

Philippe Matile's "The Lytic Compartment of Plant Cells' (1975); Matile and Wiemken's review (1976) of the interaction between cytoplasm and vacuole; Matile's "Biochemistry and Function of Vacuoles" (1978); Quail's "Plant Cell Fractionation" (1979); and Boller and Kende's (1979) thorough execution of vacuole isolation. Coombe (1976a) indicated the potential in studies of water solute flux into the vacuole for an understanding of fruit physiology. This chapter draws much from these reviews, with emphasis upon vacuoles of higher plants. Included is a summary of present literature for the isolation of such vacuoles (Table 7.1), and speculation as to mechanisms of sugar uptake by the vacuoles of fruit cells.

Matile and Wiemken (1976) proposed a terminology as follows:

> ... the protoplast of an eukaryotic cell contains in principle two aqueous phases which are separated by a single membrane. The *matrix* phase comprises nucleoplasm, cytoplasm, and the inner compartments of mitochondria; .... The other phase, the *vacuome*, contains the enchylema (the sap within the ER-cisternae) and the cell sap. It is composed of the spaces encircled by the membranes of the nuclear envelope, rough and smooth endoplasmic reticulum, Golgi membranes, microbody membrane, and the membranes of large central vacuoles and their small precursors or successors. In addition, the vacuome includes the spaces enclosed by the inner and outer membranes of mitochrondria and plastids and the thylakoid compartment of chloroplasts. The vacuome can communicate with the space ... outside the plasmalemma. It is characterized by the absence of ribosomes .... Spherosomes and oil bodies are outstanding in that they originate by inflation of the inner hydrophobic phase of ER-derived membranes.

The term vacuole used in the following text is only operational and its interactions should be borne in mind.

7.2 Tonoplast

The tonoplast is a unit membrane (Matile, 1975) with certain similarities to the plasmalemma but also many distinctions. It is one of the lightest cell membranes, the reported sucrose isopycnic densities (g cm^{-3}) being: 1.07 (ex wheat leaf, Wagner and Mulredy, 1980), 1.088 - 1.100 (ex beetroot, Doll et al., 1979), 1.10 (ex *N. tabacum*, Boller and Kende, 1979) and 1.12 (ex horseradish, Grob and Matile, 1980; ex *Nicotiana*, Briskin and Leonard, 1980). In *Hevea* lutoïds, which are probably atypical, the tonoplast was 80% phosphatidic acid and had ribosomes closely associated (Dupont et al., 1976).

It has been found to have four times the electrical resistance of the cytoplasm (in *A. sativa*, Goldsmith and Cleland, 1978), and is inside positive (in *Lolium*, *Zea*, Dunlop, 1976). Lin et al. (1977a) measured a potential difference across the tonoplast of 10-20 mV at pH8; this potential was increased by addition of Mg.ATP.

Examining freeze-factured tonoplast in the electron microscope, Leigh and Branton (1976) found typical intramembraneous particles of 7 to 12 nm diameter and twice as dense on the protoplasmic face as on the exoplasmic face. In yeast cells lipid bodies enter the vacuole at zones of the tonoplast devoid of these intramembraneous particles (Moeller and Thomson, 1979). The tonoplast does not show affinity for phosphotungstic acid - chromic acid plasmalemma stain (Nagahashi et al., 1978).

Further distinguishing features of the tonoplast and plasmalemma are revealed by the differing stabilities of protoplasts and vacuoles with polycations (Dürr et al., 1975), polyene antibiotics, pH and changing osmolarity. Dimethylsulfoxide (DMSO) acted selectively on the plasmalemma, rendering it permeable to small molecules, at concentrations not affecting the tonoplast (Delmer, 1979). Conversely, the membranes of "lysosomes" from cauliflower inflorescences were particularly susceptible to the alkaloid α-tomatine (Roddick, 1978).

Cram demonstrated the distinctive permeabilities of these two cytoplasmic membranes (1971, 1974) and Raven (1977) has summarized the differences with respect to ion transfer.

No general markers can be designated for the tonoplast (Quail, 1979). Quail comments upon four problems when comparing reports of tonoplast markers from isolated vacuoles : lack of quantitative estimates of contamination in vacuole preparations, inconsistencies between laboratories as to which markers are assayed, gross contamination from extraneous enzymes of the digestive cellulases, and heterogeneity of plant tissue.

An hexokinase particularly active towards glucose was tightly bound to the tonoplast of beetroot (Goldschmidt and Branton, 1977). Moreau et al. (1975) reported NADH cytochrome-C-reductase in lutoïds, and Parish (1975) a membrane-bound perioxidase isozyme in maize root vacuoles. The matter of ATPases will be discussed later: in any case, these are not specific markers.

Membranes of the vacuome system interchange in a manner as yet poorly understood (Matile and Wiemken, 1976). Matile (1975) treats the problem of histochemical and cytochemical work to identify such processes. Buvat (1968, 1969; Buvat and Roberts, 1979) describes the ontogeny of vacuoles in the barley root meristem thus: membrane from Golgi bodies pinches off to form vesicles and tubules (containing hydrolases) which may act as autophagic sequestration vacuoles (Mesquita, 1969, 1972; Moulin-Traffort and Giordani, 1979) and form the "provacuolar apparatus" of Marty (1978) which develop as meristematic vacuoles, and upon cell enlargement these fuse to form central vacuoles.

Microscopists describe processes of endocytosis wherein plasmlemma invaginations appear to be engulfed by sequestration vacuoles (Nishizawa and Mori, 1978). Various workers have proposed that transport from the apoplast occurs by vesiculation (Salyayev and Salyayeva, 1968; Mayo and Cocking, 1969; Fineran, 1973; MacRobbie, 1975, Wyse, 1978), as discussed in section 5.8.

The following three examples indicate the fluidity of both vacuolar volume and membrane.

(i) Constabel et al. (1980), using the precipitation reaction of alkaloid with phenol, demonstrated that in protoplasts from an interspecific fusion (*Catharanthus roseus* with *Haplopoppus gracilis*) the two central vacuoles fused.

(ii) In two thorough reports Craig et al. (1979, 1980) used serial sections to follow the development of protein bodies in developing pea cotyledons. "Results support the concept of a large highly convoluted central vacuole (5-40μm) fragmenting to give rise to the protein bodies (0.5-5μm) seen toward seed maturity." They show this represents an 100-fold increase in the membrane surface area per cell, mostly attained in a four day period!

(iii) Similarly, activation of nyctinastic organs is accompanied by reversible transformation of the central vacuole into many smaller ones, as described in the motor cells of *Albizzia* (Campbell and Gaber, 1980). In *Mimosa pudica* this may be phytochrome regulated (Setty and Jaffe, 1972).

7.3 Functions of the Vacuome

The constituents of the cell sap are of great diversity. The purpose in mentioning a few here is to establish an analytical framework. This shall be based upon theoretical classification as to the functions of the vacuome : namely,

- regulatory
- accumulative, whether storage (thence remobilization) or not
- lytic

and - a defence function.

7.3.a Regulatory function

The principle constituent of the vacuole is water. Because of their capacity to sequester solutes above a concentration which would be deleterious in the cytoplasm, vacuoles are instrumental in attaining a high cell turgor and hence conferring high tissue strength. Turgor is necessary for growth and for cell wall expansion. In turn, as the vacuole volume progressively increases with cell size the ratio of cell surface to cytoplasmic volume does not necessarily decrease (Kazantsev, 1979).

Conversely, cell turgor can affect tonoplast transport. Lüttge and Ball (1974) found loss of malic acid from *Bryophyllum* leaf slices was dependent upon water potential of the medium, and postulated that

increased turgor enhances net malate efflux at the tonoplast. Cram and Laties (1971, 1974) identified a negative feedback of chloride concentration in the cell sap upon influx not only at the tonoplast (where it was active) but also at the plasmalemma. Pressure sensitive sugar transport located in the plasmalemma was also investigated by Smith and Milburn (1980a, b).

In higher plants the osmotic pressure of vacuolar sap varies from 3.5-150 bars and in the vacuole of ripe fruit flesh ranges between 20 and 40 bars (Coombe, 1976a).

The pH of the cytoplasm tends to be precisely regulated (Smith and Raven, 1979) and it is inferred that the vacuole contributes to this buffering process as a source of counter-ions and proton sequestration. Certainly the pH of the cell sap varies much more than that of the cytoplasm. This was elegantly demonstrated with ^{31}P NMR spectroscopy (Roberts et al., 1980) on maize roots : under varied conditions the pH of the cytoplasm shifted from 7.4 to 7.2 while that of the vacuole went from 6.4 to 5.4

Dyes were used in earlier times to assess the pH of vacuoles (Zirkle, 1937) but such methods are approximate at best. Interaction with cell constituents, such as proteins, causes errors. By critical application of such methods Kinzel and Imb (1961) estimated petiolar parenchyma of *Begonia* to have a cell sap pH 1.3-1.4. In tulip petals, Lin et al. (1977) used changes in the absorption spectra of vacuolar pigments to assess vacuole pH : 4 in tissue, 7 in isolated vacuoles (in a medium of pH8). Bowling (1976) reported pH5.1-5.7 for the guard cells of *Commelina*. The juice of fruits, which is predominantly cell sap, is commonly about pH3 (Hulme, 1970).

In certain tissue accumulation of ions in the vacuole is understood as a regulatory function, both affecting cytoplasmic enzymes and clearing deleterious levels of the ion. Whilst vacuolar accumulation of many compounds may, to the investigator, appear as a storage or ecological function, it is possible that such phenomena are best analysed as the consequence of cellular (cytoplasmic) homeostasis.

7.3.b Accumulative function

The accumulation of metabolic intermediates, reserve substances, secondary products of metabolism and inorganic substances is treated by Matile and Wiemken (1976).

(i) Metabolic intermediates

One of the best examples is the diurnal sequestration of malic acid in the vacuoles of CAM plants, an acid which if concentrated in the cytoplasm should attain 7 molar (Buser and Matile, 1977; Kenyon et al., 1978; Kenyon and Black, 1979). Kenyon isolated protoplasts and vacuoles at 3 h intervals from a CAM plant. All malate of the protoplasts could be accounted for in the vacuolar fraction, and these two measures showed diurnal fluctuation, maximal at the end of the dark period and parallel to the total leaf titratable acidity. By measuring electrochemical potentials, Lüttge and Ball (1979) calculated this uptake to be a mediated diffusion of malate^{2-} in a proton gradient generated at the tonoplast, with subsequent Hmalate^{1-} formation at vacuolar pH. The $2H^+$: 1 malate^{2-} stoichiometry of uptake was very tight (Lüttge and Ball, 1980). Hence malate sequestration affects cytoplasmic pH while the effect upon vacuolar pH would be attentuated by the buffering capacity of the malate.

(ii) Reserve substances

- Remobilized reserves : Perennials such as sugarcane (Glasziou and Gayler, 1972a, b; Thom et al., 1980) and beet (Leigh et al., 1979a, b) accumulate sugars in vacuoles and these are no doubt remobilized at later developmental stages. The protein bodies of seeds, considered as specialised vacuoles, are storage entities (Weber and Neumann, 1980). Nishimura and Beevers(1979) have elegantly demonstrated proteolysis in isolated protein bodies.

The accumulation in the grape berry of K^+ and malate, discussed in chapter 1, is inferred to be vacuolar.

One speculates that transfer of sugars across the tonoplast is a regulatory and short-term storage function in many tissues, including developing fruits. Immature tomato fruits can be induced to re-export

accumulated sugars (Walker and Ho, 1977).

- Ecological adaptation : The massive vacuolar accumulation of sugars upon maturation of many fruits is not of storage function but can be considered an ecological adaptation (Coombe, 1976a). The mechanisms of these two separate "functions" (storage and adaptation) may be distinctive, or the latter may represent a modification of the former (such as a mere inhibition of sugar efflux at the tonoplast).

- Secondary products : Secondary products of metabolism accumulate in vacuoles by diverse processes including ion trapping (weak bases become protonated) and adsorption to fixed charges. Tannins are polyanionic and therefore act as cation-exchangers. An example occurs in *Chelidonium majus* in which two particular alkaloids of high affinity for phenols are enriched in the cell sap (Matile, 1976b). These two are deleterious to the membranes of isolated vacuoles, so the phenomenon can be termed detoxification. By contrast, two other endogenous alkaloids are neither sequestered nor deleterious.

It is not clear how much synthesis occurs in the lumen of the central vacuole, probably very little. For instance, the enzymes of anthocyanin synthesis are not compartmented with the pigments themselves (Hrazdina et al., 1978).

Grob and Matile (1980) recently made an interesting observation concerning the high levels of peroxidase in vacuoles of horseradish. This enzyme is involved in chemical modification in a variety of compounds, catalysing oxidative steps in synthesis. If this modification yields an intermediary product which is not subject to transport or diffusion across the tonoplast, "trapping" occurs. The vacuolar location of peroxidases could be key for an understanding of the accumulation of secondary products of metabolism in vacuoles. Parish (1975) also identified a peroxidase in the provacuolar membrane.

It is pertinent to note evidence for the vacuolar location of phenols e.g. in grapes (Harris et al., 1968; Geier, 1980) and in cell cultures (Chafe and Durzan, 1973), though not necessarily in the central vacuole (Baur and Walkinshaw, 1974; Sliwinski and Jaffe, 1977; Parham and Kaustinen, 1977). Some argue that phenols are contained within plastids (Kodryan, 1976), but phenolics may leach during fixation such that

cytochemistry gives deceptive results (Mueller and Greenwood, 1978).

The vacuole may also contain precipitates such as calcium oxalate or potassium bitartrate crystals (Considine, 1978), lipid bodies and other coacervates ... colloidal suspensions separated into two phases (Akers et al., 1977). Other cell sap constituents are mentioned in the reviews and in Table 7.1.

7.3.c Lytic function

The lytic function of vacuoles was enunciated by Matile (review : 1975), stimulated by de Duve's description in animal cells of lysosomes containing a broad activity of degradative enzymes. Matile emphasizes the significance of compartmentation of unspecific hydrolases. He discusses autophagy, and recognition factors such as catabolic inactivation and, conversely, protection of vacuolar proteins by glycosylation. Villiers (1971) demonstrated intense autophagy during the lag period before germination of poorly stratified *Fraximus excelsior* seeds : he determined up to 30 cytoplasmic entities per cell vacuole, a phenomenon absent in cells of stratified seeds.

Integration of the literature concerning hydrolases in higher plant vacuoles suffers the same difficulties noted above by Quail. Many reports of such compartmentation are available (e.g. Matile, 1968; Nishimura and Beevers, 1978; Boller and Kende, 1979; Lin and Wittenbach, 1980), but Leigh (1979) maintains that these only demonstrate catalytic competence, and still poses the question: Do plant vacuoles degrade cytoplasmic components?

The competent research of Boller and Kende (1979) forms a useful model for work on vacuoles: six of their techniques and results are singled out as illustration. The method of isolation was to obtain protoplasts, then to lyse these gently.

(i) Boller and Kende purified the cell wall degrading enzymes. They treated their cellulysin (Calbiochem) with phenylmethylsulfonyl fluoride (PMSF) to inactivate proteinases, desalted in Sephadex G-50, and filter-sterilized the solution. They rechecked for any proteinase activity; and used the sensitivity of tissue proteinase (pineapple leaf enzyme sensitive to ρ-chloromercuribenzoate : PCMB) to distinguish

insensitive proteinase deriving from the degradative enzymes.

(ii) Protoplasts were purified by flotation through Ficoll, an approach superior to pelleting which is deleterious to membrane entities. The vacuoles were similarly purified. In this way they removed much of the contaminating proteinase which associated mostly with the cell wall debris.

(iii) K_2HPO_4 lysis, after Wagner and Siegelman (1975), resulted in less contamination by unlysed protoplasts than did polybase-induced lysis. As protoplasts and vacuoles could not be separated from one another, complete lysis of protoplasts was necessary.

(iv) Activities in different fractions were related to one another, to the original tissue, and to controls with the degradative enzymes. In absence of such summations any data is weak (e.g. Butcher et al., 1977). Furthermore, summation can indicate: (a) residual activity from the cellulases; (b) location of cell wall enzymes (peroxidases and β-galactosidase in Boller and Kende's work); (c) the degree to which assayed protoplasts and vacuoles are representative of cells in the original (heterogeneous) tissue.

(v) As no suitable marker exists for the tonoplast, Boller and Kende used ^{3}H-choline to label membranes before isopycnic centrifugation. They, like other workers, noted heavy contamination of the purported tonoplast by the other membranes.

(vi) Their report concluded that the following acid hydrolases were primarily vacuolar in tobacco cells: α-mannosidase, β-N-acetylglucosaminidase, β-fructosidase, nuclease, phosphatase, phosphodiesterase. A similar composition of acid hydrolases was found in vacuoles of tulip petals, in direct contrast with the work of Butcher et al. (1977). A vacuolar proteinase, sensitive to PCMB, was localized in pineapple leaf vacuoles. No enzyme was identified on the tonoplast which banded at about 1.10 g cm^{-3}.

7.3.d Defence function

Observing that vacuolar enzymes converted yeast cells to spheroplasts, Boller and Kende proposed that vacuoles serve a function of defence against pathogens and predators.

In this sense repugnant or toxic products produced upon damage of tissue are defence mechanisms. Examples are the mustard gas of horseradish root; the proteinase inhibitors of tomato leaf (Walker-Simmons and Ryan, 1977); and the release of hydrogen cyanide upon disruption of certain plant cells as vacuolar cyanogenic glycosides are exposed to β-glucosidase activity (Conn, 1980).

7.4 Techniques for Isolation

For many years the vacuole was studied simply for light- then electron-microscopy using also autoradiography and cytochemistry. The cell sap of large cell algae could be carefully harvested (MacRobbie, 1966) or probed with micro-electrodes. Efflux studies, and especially isotope kinetics, have been very useful (e.g. Robinson and Laties, 1975), including other labelled metabolites suited to photometric tracing (Nakamura, 1973). Currently electron probe X-ray microanalysis is serving to quantify ion distribution in frozen, fractured cell sections.

Eight approaches to the isolation of intact vacuoles are documented in Table 7.1.

Slicing with a razor (class 1) is laborious but yields more vacuoles than one anticipates from their bulk contribution to tissue. The slicing apparatus of Branton (class 5) is more productive but large amounts of tissue are needed and yield is only 2%. It is therefore difficult to accurately relate activities of a vacuolar fraction back to the starting tissue. And the cytoplasm is lost.

Likewise homogenisation of tissue (class 2) causes breakage of most vacuoles and only small vesicles are recovered with an ill-defined selectivity. Prefixing such tissue in glutaraldehyde may find special application (Pihakaski and Iversen, 1976).

Lysis of protoplasts enables recovery of a cytoplasmic fraction and a cell wall fraction as well as vacuoles, provided markers are available to correct for contamination of one with another.

Polybase lysis (class 3) is gentle and can give very high yields - 95% from yeast spheroplasts (Wiemken et al., 1979). [Though Kombrink et al. (1979) criticised this technique as DEAE-Dextran apparently

Table 7.1 : Studies upon isolated vacuoles of higher plants.

The principles of isolation have been classed #1 to #8.

Reference	#	Principle of isolation	Material
Matile 1966	1	plasmolysed tissue, chopped with razor	maize root meristems
Grob and Matile 1979, 1980	1A	plasmolysed tissue, sliced on plexiglass-mounted 10° blade into medium.	horseradish root
Matile 1968	2	homogenised with sand, mortar, pestle	maize root meristems
Parish 1975	2	ground tissue with mortar	maize root meristems
Pihakaski and Iversen 1976	2	tissue prefixed 1% glutaraldehyde, then sliced and ground with mortar	Sinapis alba roots
Roddick 1978	2	homogenised tissue	cauliflower inflorescence
Buser and Matile 1977	3	Polybase DEAE-Dextran lysis of protoplasts	Bryophyllum leaf
Brown et al. 1978	3	DEAE-Dextran lysis of protoplasts	V. vinifera pericarp
Schmidt and Poole 1980	3	DEAE-Dextran lysis of protoplasts	Beta vulgaris root storage tissue
Cocking 1960	4	osmotic lysis of protoplasts	tomato root meristems
Wagner and Siegelman 1975	4	hypotonic K_2HPO_4 lysis of protoplasts, pH8	15 species, including Lycopersicum fruit
Wagner 1977, 1979; Lin et al. 1977a, 1977b; Butcher et al. 1977; Hrazdina et al. 1978.	4	hypotonic lysis of protoplasts	Tulipa leaf and petal; Hippeastrum petal
Wagner and Mulredy 1980	4	hypotonic lysis of protoplasts	wheat leaf
Walker-Simmons and Ryan 1977	4	hypotonic lysis of protoplasts	tomato leaf
Saunders and Conn 1978; Saunders 1979	4	hypotonic lysis of protoplasts	S. bicolor ; N. rustica
Boller and Kende 1979	3;4	protoplast lysis by osmotic shock, or by DEAE-Dextran	N. tabacum suspension cells; Ananas leaves ; Tulipa petals.
Mettler and Leonard 1979; Briskin and Leonard 1980	4	hypotonic lysis of protoplasts	N. glutinosa suspension culture
Kenyon et al. 1978; Kenyon and Black 1979	4	hypotonic lysis of protoplasts	leaves of Sedum telephium
Rouhani et al. 1978	-	————	Ananas, Stipula

	Purification	Comments
	flotation in Urografin with centrifuge	several hydrolases vacuolar, and two oxidoreductases. invertase cytoplasmic
	Urografin sedimentation, then flotation	vacuoles stable 15 h ; d = 1.12g cm^{-3}. Acid PPase and phenols as marker. Non aqueous hexane extraction. Recovery 2-12%. L-ascorbic entirely vacuolar. 70% peroxidase vacuolar.
	centrifugation in discontinuous sucrose gradient	lysosomes 0.1-1.5μ with 9 hydrolases
	sequential centrifugation	one peroxidase isozyme was membrane bound, thought to be a provacuole membrane.
	ultracentrifugation	myrosinase in fractions of dictyosome and S.E.R.
	differential centrifugation	α-tomatine alkaloid lysed "lysosomes" releasing phosphatase. Other membrane entities less labile.
	Ficoll step gradient	malic acid almost entirely vacuolar, not malic enzyme. Neutral Red as marker
	Ficoll and metizamide ultra-centrifugation	uptake of ^{14}C from sucrose and glucose
	Histopaque to purify vacuoles only	photos only
	———	a few vacuoles only
	rinse in mannitol and onto Ficoll	10-15% yield from protoplasts ; 85-90μ ; kept 20 h.
	various: filter through glass wool; flotation through sucrose.	to 70% yield from protoplasts. Used anthocyanins to assess pH. Inside vacuole in vivo pH4, after isolation pH7. Tonoplast potential E_{VC} 10-20 mV, positive inside, at pH8. Glucose, fructose, anthocyanins, 150 mM K^+, 6 mM Ca^{2+} : vacuolar. Hydrolases, enzymes of anthocyanin synthesis : extravacuolar. Mg. ATPase on cytoplasmic face of tonoplast : (+) tartrate inhibits.
	———	Mg. ATPase, ADPase, transphosphorylase associated with vacuoles; isopycnic density of tonoplast 1.07 g cm^{-3}
	flotation in Babcock bottles	impure vacuoles with adhesion of debris to tonoplast occurs in high phosphatase tissue, attributed to surface membrane charge. Immunological identification of proteinase inhibitors I and II.
	Ficoll step gradient; 90000g, 2H, 4°C, swingout	5-10% yield from protoplasts; BSA stops adhesion of impurities. nicotine, acid phosphatase, ATPase, 30% protein associated with vacuolar fraction. The cyanogenic glycoside dhurrin is vacuolar (also by EM) in Sorghum.
	Flotation through Ficoll step gradient: 300g, 20'.	See text. Hydrolases vacuolar.
	Ficoll step gradient	Clumping of vacuoles using K_2HPO_4 lysis, therefore sorbitol. Tonoplast vesicles 1-12 g cm^{-3} isopycnic sucrose density. Acid phosphatase : vacuolar. Malate dehydrogenase, NADH-cyt C reductase mostly extravacuolar. No ATPase with vacuoles.
	Ficoll gradient	Nocturnal storage of malate in a CAM plant. Tissue sampled every 3 h. ; 20% yield from protoplasts. Malate vacuolar. Diurnal fluctuations of tissue acidity, of malate in protoplasts and and in vacuoles were in parallel.
	———	Intending to test theory of diurnal malate storage in vacuole of CAM plants.

CONTINUED ▶

Table 7.1 continued

Reference	#	Principle of isolation	Material
Löffelhardt et al. 1979	4	hypotonic lysis of protoplasts	Convallaria majalis leaves
Granstedt and Huffaker 1980	4	hypotonic and pH lysis of protoplasts	primary leaves of high nitrate barley
Lin and Wittenback 1980	4	hypotonic lysis of protoplasts	wheat and maize leaf mesophyll
Leigh and Branton 1976; Leigh et al. 1979a,b; Marty and Branton 1979; Leigh and Walker 1980	5	multiple-blade slicing in medium	Beta vulgaris storage root
Goldschmidt and Branton 1977	5	slicer	Beta vulgaris storage root
Doll et al. 1979; Willenbrink and Doll 1979	5	slicer of plasmolysed tissue	Red beet storage root
Admon et al. 1980	4;5	by lysis of protoplasts and by slicer	Vicia faba mesophyll ; Beta vulgaris root
Nishimura and Beevers 1978, 1979; Beevers and Nishimura 1978	6	protoplasts and tissue agitated and rewashed in hypertonic 0.7 M medium Released vacuoles separated from many protoplasts.	Castor bean seedling endosperm
Matile 1970, 1976b	7	tapped articulated lactifers	lactifers of Chelidonium majus
D'Auzac 1975, 1977; Moreau et al. 1975; Dupont et al. 1976; Montardy and Lambert 1977; Coupé and Lambert 1977; Marin 1980.	7	lyophilised lutoïds, reconstituted	Hevea brasiliensis lutoïds
Lörz et al. 1976	8	lyse protoplasts by shearing force of a step gradient, 40,000g, 3 h.	Maize shootpith; Phaseolus callus; Calceolaria petals, etc.; wheat, barley, rye, maize, tobacco mesophyll
Guy et al. 1979	8	protoplast lysis by shearing centrifugation	Pisum sativum mesophyll
Thom et al. 1980	8	lyse protoplasts by shearing in step gradient	sugarcane suspension cells
Rona and Cornel 1979	-	few vacuoles in protoplast preparation	Acer pseudoplatanus
Sasse et al. 1979	-	———	Daucus carota cell culture

Purification	Comments
Ficoll step gradient	1-1.5 x 10^6 intact protoplasts or vacuoles from 5 g tissue. Cardiac glycosides vacuolar.
Ficoll gradient	nitrate concentrated in vacuoles.
ultracentrifugation in Ficoll gradient	of proteolytic activity in protoplasts, all associated with vacuolar fraction; also 20% of soluble protein.
metrizamide density gradients, including flotation	1 Osmolar sorbitol to stabilize vacuoles; betanin as marker : 2% yield from tissue; most sucrose, much acid invertase - vacuolar; In aged tissue, vacuolar invertase increased, sucrose decreased; 3 phosphatases : soluble acid PPase, membrane K^+-PPase, partially membrane ATPase not inhibited by molybdate. Marty characterised tonoplast and polypeptide distribution.
———	90% of sucrose in vacuole; hexokinase tightly bound to tonoplast, with K_M 0.16 mM glucose and rates fructose : glucose = 15:100. Aged tissue: tonoplast invertase, sucrose synthase and sucrose synthase in lumen.
Discontinuous Dextran gradient. Silicone-layer-filtering centrifugation	vacuoles' halflife = 4 h (4°C); d = 1.088 to 1.100 g cm^{-3}. ATPase on inside of tonoplast. Cysteine, metrizamide inhibit sucrose uptake of K_M 22 mM, Ea 35 KJ . mol^{-1}; Raffinose is competitive inhibitor. Possible isotope exchange diffusion.
———	Used FDA to test for cytoplasmic debris, and neutral red as positive stain for acidic vacuoles.
Centrifugation (300g, 5') through 40% w/w sucrose; vacuoles pellet.	vacuoles very dense. Demonstrated proteolysis before and during isolation. Sucrose, storage protein : vacuolar. Also acid protease, carboxypeptidase, phosphodiesterase, RNAase, phytase, β-glucosidase
differential centrifugation	up to 1.5 µm. Several alkaloids concentrated in the vacuoles. The 2 alkaloids causing lysis were those which vacuoles could take up, probably by an ion-trap mechanism.
———	Basic a.a., organic acids actively transported. Acid PPase in lumen, NADH-cyt c reductase on tonoplast. Mg.ATPase membrane bound, activated by malate, Cl^- and SH-agents.
purification during lysis	"vacuoplasts" - in EM, plasmalemma still about vacuole with traces of cytoplasm
ultracentrifugation	Vacuoles take up 3-O-MG much faster than L-glucose, especially in acidic media. Added Mg.ATP immediately promoted uptake; protonophore reduced, but countered by ATP. Hence, propose a direct ATP energizing of glucose transport, not via PMF.
purification during lysis	30% yield from protoplasts. Marker enzymes indicate less than 10% contamination by mitochondria, cytoplasm; but much from plasmalemma. Assessed pH (DMO) and PD (tetraphenylphosphonium). 3-O-MG taken up, not accumulated. Vacuoles take up sucrose, unlike protoplasts.
———	electrical resistance of tonoplast 1.4 $k\Omega cm^2$
———	———

inhibited electron transport in chloroplasts, the same authors (Kombrink and Wöber, 1980) subsequently published a method to isolate chloroplasts with this polybase.] Chilling, and use of a sufficient aliquot of DEAE-Dextran to avoid any localized concentration, are necessary details of the method (Nagy, 1979; Kombrink and Wöber, 1980). Problems arise when different tissues and cells exhibit different sensitivity to the lysing agent, so each lysis must be preceded by a trial.

Glucose and a chelator produce metabolic lysis of yeast to yield intact vacuoles. In plant cells hypotonic lysis (class 4), with pH adjustment, has been the most widely used of all these techniques. It is simple, reproducible, yields are good and few protoplasts persist. Mettler and Leonard (1979) improved the stability of isolated vacuoles, and reduced clumping, by the subsequent removal of ionic salts and the inclusion of 0.25 mM EDTA.

For certain tissues hypotonic release of vacuoles from cellulase-treated tissue, with agitation in several aliqots, is appropriate (class 6). However, the principal restriction lies in the need subsequently to separate large numbers of protoplasts and vacuoles. This can be difficult due to the proximity of densities and dispersion of sizes of both entities.

Lysis of protoplasts by the shearing forces of ultracentrifugation through step gradients (class 8) has the advantage of incorporating purification with production, lessening cross-contamination and inactivation.

Some specialised vacuoles are directly accessible, such as the lutoïds of *Hevea* (class 7).

Yield has a qualitative implication. Isolation will be associated with selection of an element of the vacuome, and higher yields imply broader representation of the starting material. The polymorphy of vacuoles and the heterogeneity of tissue has also to be considered.

7.5 Transport at the Tonoplast

What evidence is there for active transport at, or specific transport (permeases) in, the tonoplast?

In characterising active transport systems, Matile (1978) discusses difficulties in assessing true activities in compartments (especially the cytoplasm) where a substantial proportion of the cell water is bound. Furthermore, concentrative phenomena may result from the Donnan charge across the membrane (e.g. arginine accumulation in yeast : Boller et al., 1975; sucrose accumulation in artificial vesicles containing collagen : Veith, 1979).

Moreover Matile (1978) notes that, by analogy with the plasmalemma, the tonoplast must be regarded as an entity of the cytoplasm, and unlike mitochondria and plastids, vacuoles have no metabolism - or energy-generating mechanism - in their own right.

Citrate transport in *Hevea* vacuoles occurs without addition of an energy substrate, yet is concentrative. The permease is sensitive to DNP and enhanced by ATP. d'Auzac (1975, 1977; Coupe and Lambert, 1977) attributed this to a proton/citrate symport, energized by the electrochemical proton gradient across the membrane, with trapping of the transported anion.

Other studies of solute uptake are reported in Table 7.1.

Red beet storage-root vacuoles actively accumulated sucrose, a process stimulated by addition of Mg.ATP and inhibited by raffinose (Doll et al., 1979; Willenbrink and Doll, 1979). Thom et al. (1980), studying sugarcane suspension cultures, reported sucrose uptake by vacuoles but not by protoplasts. 3-O-MG was actively accumulated. Vacuoles isolated from grape pericarp took up ^{14}C more rapidly from sucrose than from glucose (Brown et al., 1978).

Guy et al. (1979) also demonstrated selective, active uptake of 3-O-MG with indications that ATP directly energized glucose transport, not as a secondary active transport. These authors suggest:

> The likelihood that sugar and amino acid transport systems in our cells can be directly energized by ATP (... not as a secondary active pump through PMF ...) is increased by the evidence (Ferenci et

al., 1977) that in bacteria all of the sugar and amino transport systems which are sensitive to osmotic shock i.e. which are dependent on "binding" protein which is removed by shock, use ATP directly. We have reported that sugar and amino acid uptake in leaf cells is indeed sensitive to osmotic shock.

7.6 ATPase

A tonoplast ATPase has been sought for its function in proton translocation and subsequent transport processes. In animal tissue the enzyme ATPase and the transport ATPase are identical : Na^+/K^+ synergistically stimulate, ouabain inhibits.

In plant cells membrane ATPases are poorly characterised, and may easily be confounded with unspecific soluble phosphatases. An investigation may include the following principles (Hodges, 1976) :

- sensitivity to divalent cations : in decreasing order, stimulation by Mg^{2+}, Ca^{2+}, Mn^{2+} ;
- monovalent ion stimulation : generally K^+;
- activation by the purported substrates : Hodges predicts a tonoplast ATPase stimulated by organic acids ;
- pH optimum : generally 7.5-6.5, and predicted higher for a tonoplast ATPase ;
- characteristics of a membrane location
- histochemical identification
- segregation from other reactions by the use of inhibitors : ouabain, Dio-9 (ATPase inhibitors); DCCD and oligomycin (uncouples ATP formation); PCMB (blocks sulfhydryl groups); anaerobiosis (inhibits respiration); P_i, molybdate (inhibits alkaline phosphatase); NaF, molybdate (inhibit acid phosphatase.

In beet storage tissue (Leigh and Branton, 1976) and *Nicotiana* cells (Briskin and Leonard, 1980) no ATPase was associated with a tonoplast fraction, contrary to other reports. Wagner and Mulredy (1980) recovered 20% of vacuolar Mg.ATPase in the membrane fraction. But such phosphatase activity could bind to the membranes during isolation, and need not in any case be a vectorial, proton-translocating ATPase. Leigh and Walker (1980) found ATPase activity partially associated with the membrane, stimulated by K^+, anions and cations. d'Auzac (1977) relied upon molybdate inhibition to discriminate a membrane bound ATPase in *Hevea* lutoïds : it was Mg^{2+} dependent, of optimal pH 7.75-6.5,

activated by chloride and malate, had essential SH-groups, and its temperature dependency was discontinuous at 20° as expected of a membrane bound enzyme.

From petals Lin et al. (1977) recovered vacuoles with 2-3 times more ATPase activity than the stock protoplasts! The ATPase activity thought to be at the tonoplast was Mg^{2+} dependent, K^{+} stimulated, partially inhibited by Dio-9, inhibited by ethyl-3-(3-dimethylaminopropyl carbodiimide) [EDAC], and unlike an ATPase of protoplasts, insensitive to oligomycin.

Doll et al. (1979) determined ATPase activity in beet tonoplasts, but made no attempt to eliminate unspecific phosphatases. This activity was Mg^{2+} and K^{+} dependent, inhibited by EDAC and tartrate, and unaffected by oligomycin. As activity increased 5-fold upon lysis of the vacuole, but was still bound, they concluded the ATPase was located on the exoplasmic face of this membrane.

Despite such investigations, one still questions: Are these specific ATPases, and particularly are they vectorial catalysts? The observations of Guy et al. (1979) cited in section 7.5 are pertinent.

7.7 Comments on Sugar Accumulating Mechanisms

The chapter shall finish with speculation. What are the possible mechanisms for massive sugar accumulation in the highly acid vacuole of fruit (say pH3 relative to a cytoplasm pH7.4), of low transmembrane potential (say 10-20 mV with high resistance and high capacitance by virtue of volume), and with the primary energy supply (e.g. ATP, NADH) in the cytoplasm?

In Fig. 7.1a ten such mechanisms are illustrated. Secondary active transport is represented without regard as to the primary source of an energy gradient across the membrane.

Vesicular transport (#1) may occur, but, it is considered, could not account for such a concentrative process.

Entry of sugars by diffusion (#2) can be moderately concentrative (Vieth, 1979). If the diffused sugar were subsequently metabolised -

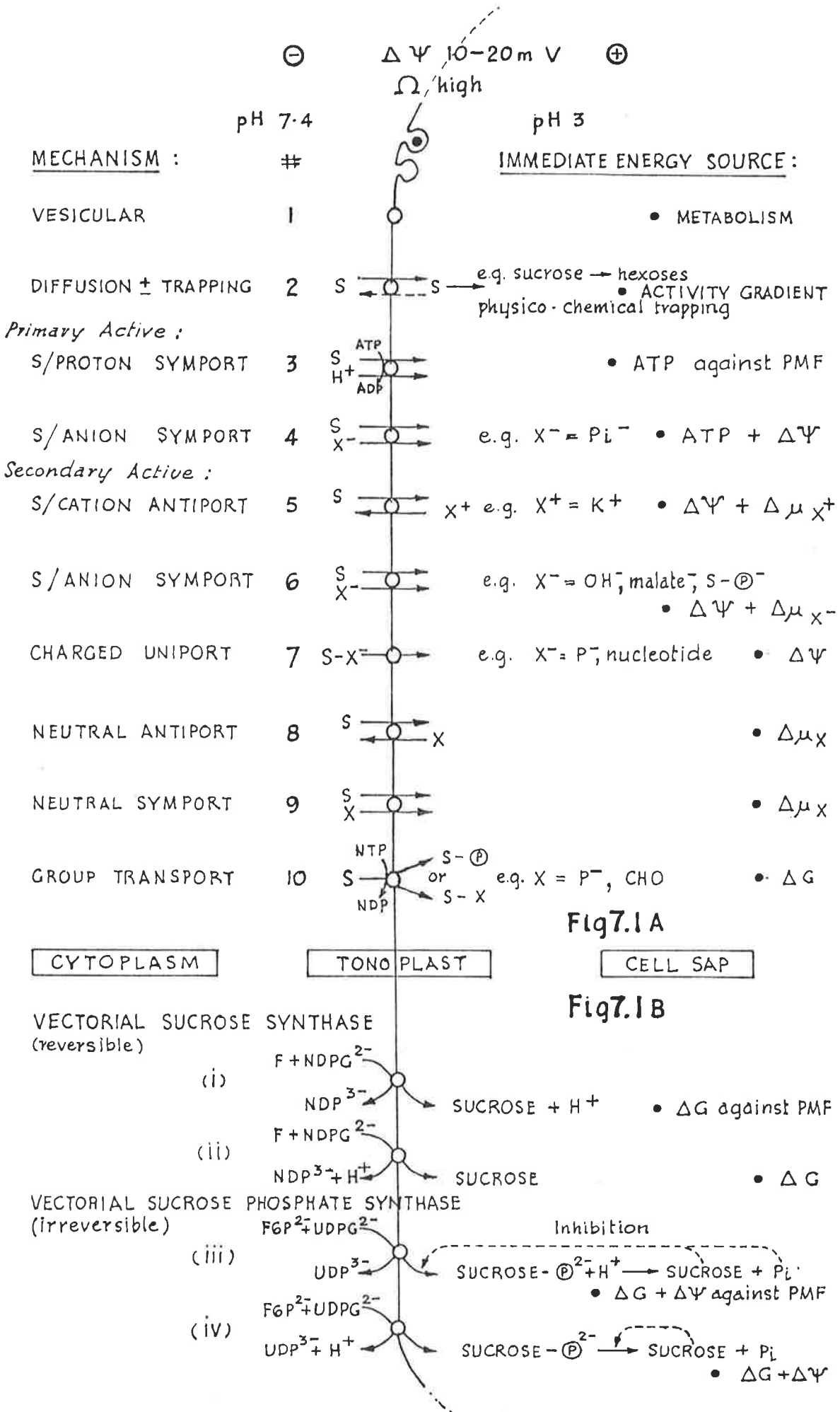


Fig. 7.1 *Possible mechanisms for sugar transport at the tonoplast of a fruit cell.*

such as sucrose being hydrolysed - a massive accumulation of product(s) could result by this "diffusion with trapping" ... provided that the tonoplast was less permeable to the product(s) than the substrate sugar. Hence the diffusion (influx) should be mediated.

A primary active proton-sugar symport (#3) is unlikely to occur against both an electrical and a proton gradient. However, this is not to exclude a proton-translocating ATPase at the tonoplast.

A primary active sugar/anion symport (#4) might be energized by vectorial hydrolysis of a nucleotide without any modification of the sugar. Such a proposition is attractive as the transport of an anion, such as P_i, is electrically favourable and its remobilization could be regulatory (Woodrow and Rowan, 1979).

Consider now a number of secondary active processes (#5,6,7,8,9) :

If a sugar/cation antiport (#5), energized by the electrical gradient, involved protons then the cytoplasmic pH should be perturbed : an alkali ion such as K^+ is more feasible.

A sugar/anion symport (#6) is also favoured by the electrical gradient. If the counter-ion were a weak acid it should become undissociated within the cell sap, and in this more permeable state recycle back to the cytoplasm.

A charged sugar uniport (#7), essentially an electrophoretic mechanism, is unfavourable in that the neutral sugars are generally more permeable.

Electrically-neutral sugar antiports (#8) or sugar symports (#9), driven by a primary activity gradient of X, is improbable to the extent that two uncharged entities are unlikely to associate, without covalent modification, let alone to interact vectorially.

A group transport mechanism (#10), for which there is good precedent in bacteria, may also be electrically favoured. Conceptually it is attractive as energy can be transferred to the vacuole in the form of a covalent bond capable of subsequent polymerisation, such as glycosylation. Fructosan formation in vacuoles is one such case. Indeed one can envisage such a polymer associated with the permease as a trans receptor and activating the transport.

Of all these mechanisms this latter appears most favourable. One finds no precedent for propositions 4 and 6.

Fig. 7.1b presents examples of group transport mechanisms based upon the characteristics of two sucrose enzymes. Of course there is no firm evidence for a membrane-bound form of these enzymes, and a vectorial permease will not be identical to a scalar enzyme. Furthermore the supply of such substrates from the cytoplasm implies complexes of several enzymes.

Sucrose synthase unmasks a proton in the synthesis reaction such that transport would be against the proton motive force, as in Fig. 7.1b(i). However, if this were a two-step reaction (and there is such evidence for this enzyme) the proton liberated in the first step may remain on the cis face while the sucrose dissociates on the trans (7.1b(ii)). Considering the short-term storage function of vacuoles, the reversibility of this enzyme is notable.

Sucrose phosphate synthase also unmasks a proton, but the carrier complex will now be negative and favoured by the electrical gradient.[®] Given the high phosphatase activity of vacuoles, the liberated products may exert inhibition upon dissociation of the carrier complex.

It is concluded that sugar transport at the tonoplast is likely to be a group transport mechanism. In this the synthases show favourable characteristics. Sucrose phosphate synthase would also translocate a phosphate, the recycling of which might be regulatory.

[®]See Fig. 7.1b.iii.

Introduction to the Experimental Section

The juice of a ripe grape berry is predominantly vacuolar sap and contains about 20% sugar in the form of glucose and fructose. The phloem is similarly 20% sugar, as sucrose. By what process do sugars move from the phloem to the vacuole? There are three membranes to be considered in this transport of sugars: the plasmalemma of the phloem, and the plasmalemma and tonoplast of the storage cells.

Sugar accumulation increases markedly at that stage of berry development termed veraison. Skin tissue excised from the berry at or just prior to veraison contains hexoses which will mostly diffuse into an isotonic solution, yet will accumulate glucose *in vitro* (Coombe and Matile, 1980).

What is the compartmentation of sugars in the developing grape berry *in vivo*, and is this affected by shading? Such is the nature of an investigation reported in Chapter 8.

Analysis of compartmentation, of localized metabolism and of membrane processes will be facilitated if isolated, functional protoplasts and vacuoles can be prepared from the relevant tissue. Such methodology is reported in Chapters 9 and 10.

Which sugars are preferentially transported at the plasmalemma and tonoplast of these isolated organelles? What are the characteristics of that transport? (Chapters 11 and 12.) How are these sugar substrates metabolized, if at all? (Chapter 13.)

Of course, such reductionism leaves untouched many matters. For instance, is sugar transfer in the ripening fruit apoplasmic, symplasmic or a combination of both? In either case, however, sugars must move across the tonoplast. Transport across the plasmalemma of a storage cell defines an apoplasmic pathway, while it is relevant to a symplasmic route only as an efflux.

Phloem unloading has not been directly investigated herein.

CHAPTER 8: EFFLUX STUDIES OF HEXOSE COMPARTMENTATION IN THE DEVELOPING GRAPE BERRY AND THE EFFECTS OF SHADING.

EFFLUX STUDIES OF HEXOSE COMPARTMENTATION IN THE DEVELOPING GRAPE BERRY AND THE EFFECTS OF SHADING

8.1 Introduction

Glucose accounts for 85% of sugars in the grape berry in the first growth cycle, but after veraison the ratio glucose:fructose approaches unity (Kliewer, 1967; Harris et al., 1968). This is consequent upon a sudden increase of sugars in the berry, typically 500 µmoles hexoses per berry over 10 days (Ruffner and Hawker, 1977). This phenomenon, and associated changes which characterise veraison (Chapter 2), have led to speculation that the tissue of a grape berry is potentiated to accumulate sugar prior to veraison while actual accumulation follows from de-repression or some primary "triggering".

Coombe and Matile (1980) provided some support for this notion with two observations upon skin segments of berries cv. Pinot noir just prior to the stage of rapid sugar accumulation. Firstly, 80% of hexoses present in the skin were not compartmented. Secondly, this same tissue accumulated sugars *in vitro* at rates higher than those found in whole berries after veraison.

As an approach to the further investigation of these phenomena it was decided to examine the effect of light on compartmentation of sugar in pre-veraison berries. That phytochrome is a regulator of ripening has been proposed by Jen et al. (1977). Moreover, Weisenseel and Haupt (1974) demonstrated a control of fundamental membrane properties (in this case, hydraulic conductivity) by phytochrome (Marmé, 1977). Shading will also reduce photosynthesis in the berry. However, any pronounced effect of shading upon accumulation will not be directly due to this as most of the sugars derive from translocation; an indirect effect, associated with reduced *in situ* photosynthate, is possible.

An experiment was conducted to test the proposition that the low degree of compartmentation of sugars at veraison is light-induced, and that shading will remove this inhibition to compartmentation. The delineation of diffusible and compartmented sugars is arbitrary. However, there is little doubt that two such fractions exist in the skin of the grape (Coombe and Matile, 1980) and is further evident in the qualitative differences between these fractions (herein, and in Chapter 13). In the present experiment "compartmented" sugars were designated as those residual after washing the tissue in a specified medium for 30 min. The amount washed out in 30 min was termed "diffusible" but it is realized that this includes compounds washed from cells damaged during excision.

8.2 Materials and Methods

On 15 January (day 0) four medium sized bunches, neither fully exposed nor shaded, cv. Muscat were selected. On each, eight similar pairs of berries were marked. Berries were judged to be approaching veraison. At 0800 hours one of each pair was wrapped in aluminium foil (60 x 60 mm) with care being taken not to damage the berries or pedicels. The shades were left in position for 16 days.

Twenty-four hours after enclosing the berries (day 1) and at 3 day intervals thereafter until day 16 one pair was sampled from each bunch at 0800. Each berry was weighed. One equatorial and four longitudinal cuts were made in the skin and the eight segments removed so as to avoid attached vascular and fleshy tissue. The skin segments were used for the washing treatment and the remainder of each berry was reweighed to calculate skin fresh weight.

On day 13 and day 16 a slice of about 500 mg flesh was taken and put into 2 ml water at 10°C. The remainder was reweighed to calculate, by difference, the weight of this slice. °Brix of a drop of juice was read using a hand refractometer.

For the washing of the skin the segments were incubated with occasional agitation in 2 or 3 ml incubation medium at 10°C.

The incubation medium contained 100 mM mannitol, 40 mM MES pH 6.5, 5 mM $KC\ell$, 2 mM $CaC\ell_2$, 2 mM $MgC\ell_2$, 1 mM PEG 4000 (all autoclaved)

Fig. 8.1 Protocol for enzymatic sugar assay

Sequential, specific measurement of glucose, fructose, sucrose, glucose-6-phosphate and fructose-6-phosphate is possible by enzymatic methods. Bergmeyer et al. (1974) describe assays for each sugar linked to NADPH formation, measured as extinction at 340 (334, 365) nm. These assays may be performed sequentially in single cuvettes using the compromise pH 6.9 suggested by Jones et al. (1977); the latter authors also give protocols for greater sensitivity using fluorimetry, for which some additional limitations apply.

Principle

$$\text{sucrose} \xrightarrow{\text{invertase}} \text{glucose} + \text{fructose}$$

$$\text{glucose} + \text{fructose} + 2\,\text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{F6P} + 2\,\text{ADP}$$

$$\text{F6P} \xrightarrow{\text{PGI}} \text{G6P}$$

$$2\,\text{G6P} \quad 2\,\text{G6P} + 2\,\text{NADP} \xrightarrow{\text{G6P-DH}} \text{2. 6-P-gluconate} + 2\,\text{NADPH}$$

Reagents

1. Buffer pH 6.90 : imidazole (per Jones et al. 1979) or MES titrated KOH
2. $MgCl_2$ A.R.
3. BSA
4. Dithiothreitol (DTT)
5. ATP . Na_2^+
6. NADP . Na_2^+
7. glucose, fructose, sucrose A.R.
8. G6P. Na_2^+ , F6P.Na_2^+ (or K^+ salt, not Ba^{2+} salts)
9. glycerol A.R. autoclaved
10. glucose-6-phosphate dehydrogenase (G6P-DH) E.C. 1.1.1.49, crystalline, > 140 U mg^{-1}, SO_4^{2-} free (e.g. Baker's yeast)
11. phospho-glucose isomerase (PGI) EC 5.3.1.9, crystalline, > 350 U mg^{-1} , SO_4^{2-} free
12. hexokinase (HK) EC 2.7.1.1 , crystalline , > 140 U mg^{-1} , sulphate free (e.g. ex yeast)
13. invertase EC 3.2.1.26 , crystalline , high specific activity > 400 U mg^{-1} , SO_4^{2-} free (e.g. ex yeast)

Purity of enzyme preparations

The enzymes must be free of hexose, < 0.1% NADPH oxidase, 6-phosphogluconate dehydrogenase or phosphoglucomutase, α- or β-galactosidase, α- or β-glucosidase. HK and G6P-DH must not contain more than 0.1% PGI or 0.01% invertase.

Preparation of solutions

The Reagent Mixture contains

25 mM	MES pH 6.90	5 mM	$MgCl_2$
0.5 mM	DTT	1 mM	ATP.Na_2^+
0.02%	BSA	0.4 mM	NADP.Na_2^+

prepared in freshly distilled deionized water. This mixture is stored at 4°C; or at -5° for several weeks. However, it is preferable to store a stock solution without the last two reagents.

Enzyme solutions are prepared with 25% (w/v) glucerol in 25 mM MES pH 6.90 at the following rates:

	Protein: mg ml^{-1}	Minimal Activity: U ml^{-1} enzyme preparation	Aliquot per assay µl	Minimal Activity: U per cuvette
G6P-DH	0.2	28	5	0.14
PGI	0.4	140	5	0.70
HK	0.4	56	5	0.28
Invertase	8.0	3200	5	16

With glycerol these may be stored at -5°. PGI is the least unstable.

Assay system

dual beam spectrophotometer 340 nm, against air ; controlled temperature (e.g. 23°) desirable.

1.6 ml disposable semi-micro cuvettes or 2.6 ml cuvettes for larger aliquots.

PIPETTE INTO CUVETTES		
Reagent mixture	1 ml	
Sample	up to 200 µl	
Mix		Read E_0
* HK suspension	5 µl	
G6P-DH suspension	5 µl	
Mix, wait for stable end point c. 5 min		Read E_1
PGI suspension		
Mix, wait for stable end point c. 10 min		Read E_2
Invertase suspension	5 µl	
Mix, wait for stable end point < 30 min		
Remix		Read E_3
* For G6P and F6P:		
In another cuvette, repeat the procedure to E_2 but replacing HK with buffer.		Read E_0^1, E_1^1, E_2^1
Final volume = Reagent Mixture + aliquot + enzymes 20 µl		

Calculations

$E_1 - E_0 = \Delta E$ (glucose + G6P)

$E_2 - E_1 = \Delta E$ (fructose + F6P)

$E_3 - E_2 = \Delta E$ (sucrose)

$E_1^1 - E_0^1 = \Delta E$ (G6P)

$E_2^1 - E_1^1 = \Delta E$ (F6P)

$$c = \frac{\Delta E \times V}{\epsilon \times d \times v}$$ µmole ml^{-1} in the sample

or

$$c = \frac{\Delta E \times V \times MW}{\epsilon \times d \times v}$$ µg ml^{-1} in the sample

where ϵ extinction coefficient cm^2 $\mu mole^{-1}$

E extinction

ΔE extinction change

V assay volume ml

v volume of sample used in assay ml

d path length cm

c concentration µmole ml^{-1}, µg ml^{-1}

MW weight of one micromole µg

(glucose = 180.16 fructose = 180.16 sucrose = 342.30

G6P = 260.2 F6P = 260.1)

NADPH has ϵ = 6.22 cm^2 $\mu mole^{-1}$ at 340 nm

Good linearity is observed over the range 1-30 µg hexose equivalents per ml final volume in the cuvette; using a 2.5 ml final volume this range then is 2-75 µg hexose equivalents. The above equations are then applicable. For values greater than 1 O.D. unit a standard curve is necessary.

Limitations

Insufficient purity of the reagent used, particularly the enzymes, results in false values. New stocks should be assessed using sugar standards to confirm stable endpoints. For instance, if PGI is a contaminant of either HK or G6P-DH some fructose will contribute to E_1. This would necessitate a series of readings and extrapolation of E_1 to the time of HK addition for a true E_1. In practice, it is simpler to obtain enzymes of sufficient quality.

In one assay an unstable E_3 value was corrected by addition of 1 mM EDTA to the Reagent Mixture.

The enzymes are susceptible to sulphate inhibition, especially G6P-DH.

The $t_{\frac{1}{2}}$ of reactions should be c. 30 seconds for the first three enzymes and less than 5 min for the invertase reaction to be 98% complete at 30 min. More of a particular enzyme may be added. Deviation from pH 6.90 may cause marked change in reaction rates as the pH dependencies of these enzymes are quite different.

Though HK is non-specific, PGI and G6P-DH are specific for F6P and G6P. Thus the overall assay is specific for each hexose. Invertase (β-D-fructofuranosidase) will hydrolyse saccharides other than sucrose (e.g. raffinose, stachyose) but at a slower rate (Jones et al., 1977). By omitting the PGI one can assay specifically the glucosyl of sucrose (the molar yield of NADPH will be halved) and thereby distinguish sucrose in the presence of other saccharides.

Many plant tissues will contain only trace amounts of hexose-phosphate and saccharides other than sucrose, such that only one incubation to obtain E_0, E_1, E_2, E_3 is necessary.

Plant extracts may be diluted and boiled prior to assay. Deproteinisation with $HCl O_4$ leads to partial hydrolysis of sucrose; alkali and heat will break down reducing sugars. High values of E_0 due to plant material may be reduced by first adding PVP to the extract followed by centrifugation. Spiked samples should be included in the routine.

□

with 2.5 mM SO_2 and 20 µg ml^{-1} oxytetracycline hydrochloride. After 30 min the segments were removed, blotted in tissue paper, submersed in 2 ml water in capped plastic tubes, and placed in a boiling water bath for 15 min. The samples of incubation medium were similarly boiled. All material was stored at -10°C prior to enzymatic analysis for glucose, fructose, sucrose and hexose phosphates.

The enzymatic sugar assay is described in Fig. 8.1. Good linearity with stable endpoints was obtained over the range 1 to 30 µg hexose equivalents. Though the observed extinctions of standard hexose and sucrose solutions were respectively 108% and 111% of theoretical values, calculations were based upon $\varepsilon_{340\,nm}^{NADPH}$. The $t_{\frac{1}{2}}$ of the reaction with glucose-6-phosphate dehydrogenase and hexokinase was 22 sec, with phosphoglucoisomerase 25 sec and with invertase 4-5 min. It was necessary to remix the contents of the cuvette by inversion prior to the final absorbance reading pertaining to sucrose. Recovery, tested by adding 2.5 µg sucrose to every fourth sample, was between 90 and 110%. Sample aliquots were 5 µℓ or 10 µℓ in a final incubation volume of 1 ml, or 5 µℓ of a 1:4 dilution for the flesh samples from days 13 and 16.

As hexose phosphates were only present in trace amounts, 200-400 µℓ aliquots were assayed in 1.4 ml final volume. High background absorbance, particularly in "residual" samples, was reduced by adding 100 mg methanol-washed PVP (Polyclar AT) to these samples, mixing, centrifugation and sampling the supernatant. Centrifugation alone was not adequate.

With the "diffusible" samples extinction slowly increased with time. As spiking with hexose did not alter this it was concluded that the cause of the increase was independent of hexose levels. Reboiling samples for 5 min removed the increase. Aliquots from the "residual" series likewise showed unstable extinction once treated with PVP, so these samples were also reboiled.

8.3 Results

Data from the sugar assays are presented in Table 8.1, Figs 8.2 and 8.3. The °Brix of juice from shaded berries was significantly less than from unshaded berries (Table 8.1). This effect developed

Table 8.1: Effect of shading of Muscat berries for a period of 16 days, beginning just before veraison, on the composition of the skin. Values are averages of samplings during the 16 days of the experiment; the interactions of treatment x sampling date were not significant.

	°Brix	Skin Wt.	Diffusible Glucose	Residual Glucose	Diffusible Fructose	Residual Fructose	Diffusible Sucrose	Residual Sucrose	TOTAL : Hexose & Sucrose	G6P	F6P
	juice	mg			mg g^{-1} F.Wt. skin					μg g^{-1} F.Wt.skin	
CONTROL	8.76	287	11.66	6.07	6.82	2.74	0.44	0.54	28.27	36	55
SHADED BERRIES	7.56	291	8.87	5.59	4.73	2.45	0.52	0.60	22.77	27	39
Level of Significance Anova n = 48 p =	0.005	>0.20	0.005	0.05	0.025	0.125	>0.20	0.20	0.005	-	-

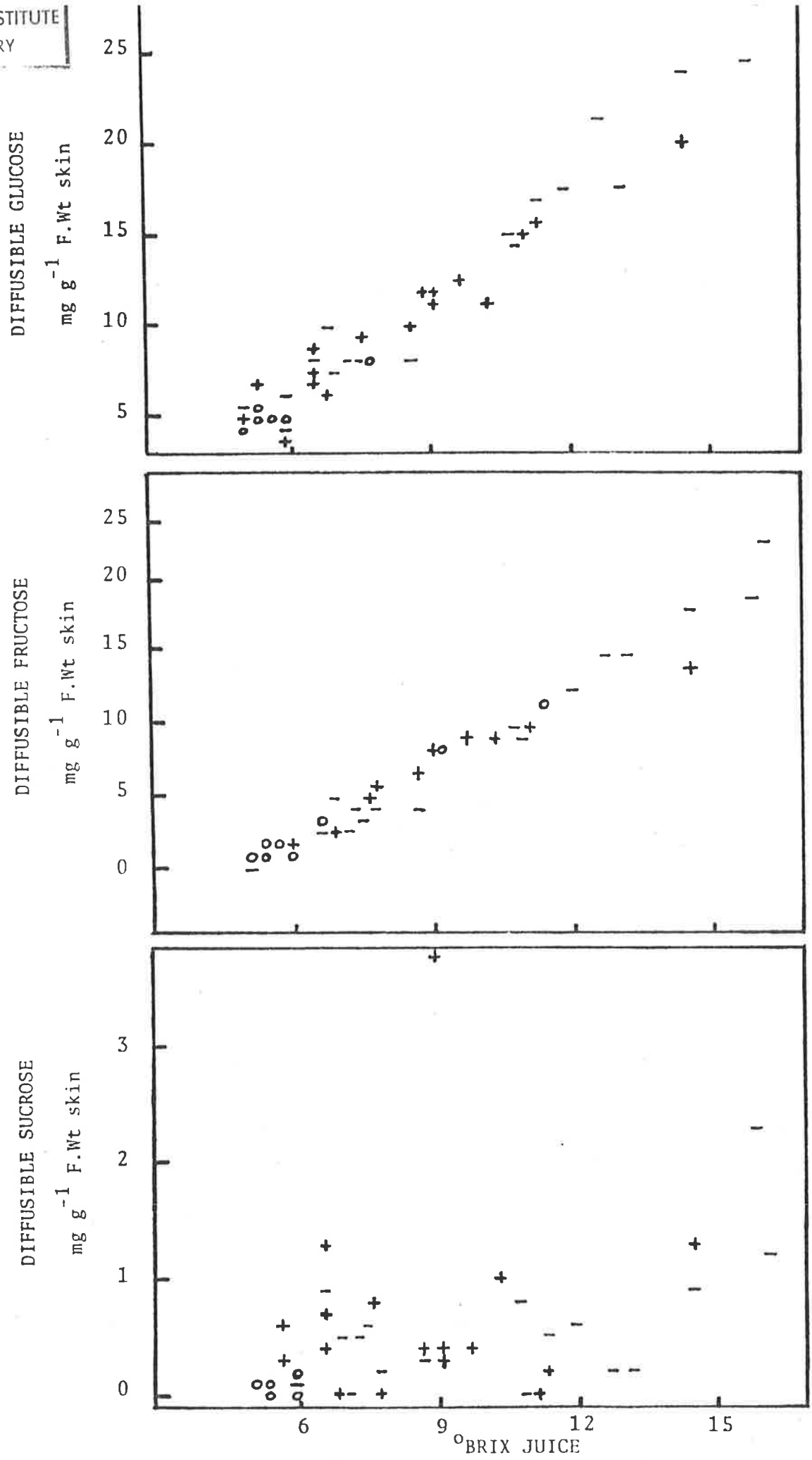


Fig. 8.2 Relationships between °Brix of juice and sugars of skin from Muscat berries: shaded (+), unshaded (-), two data (o) (continued)

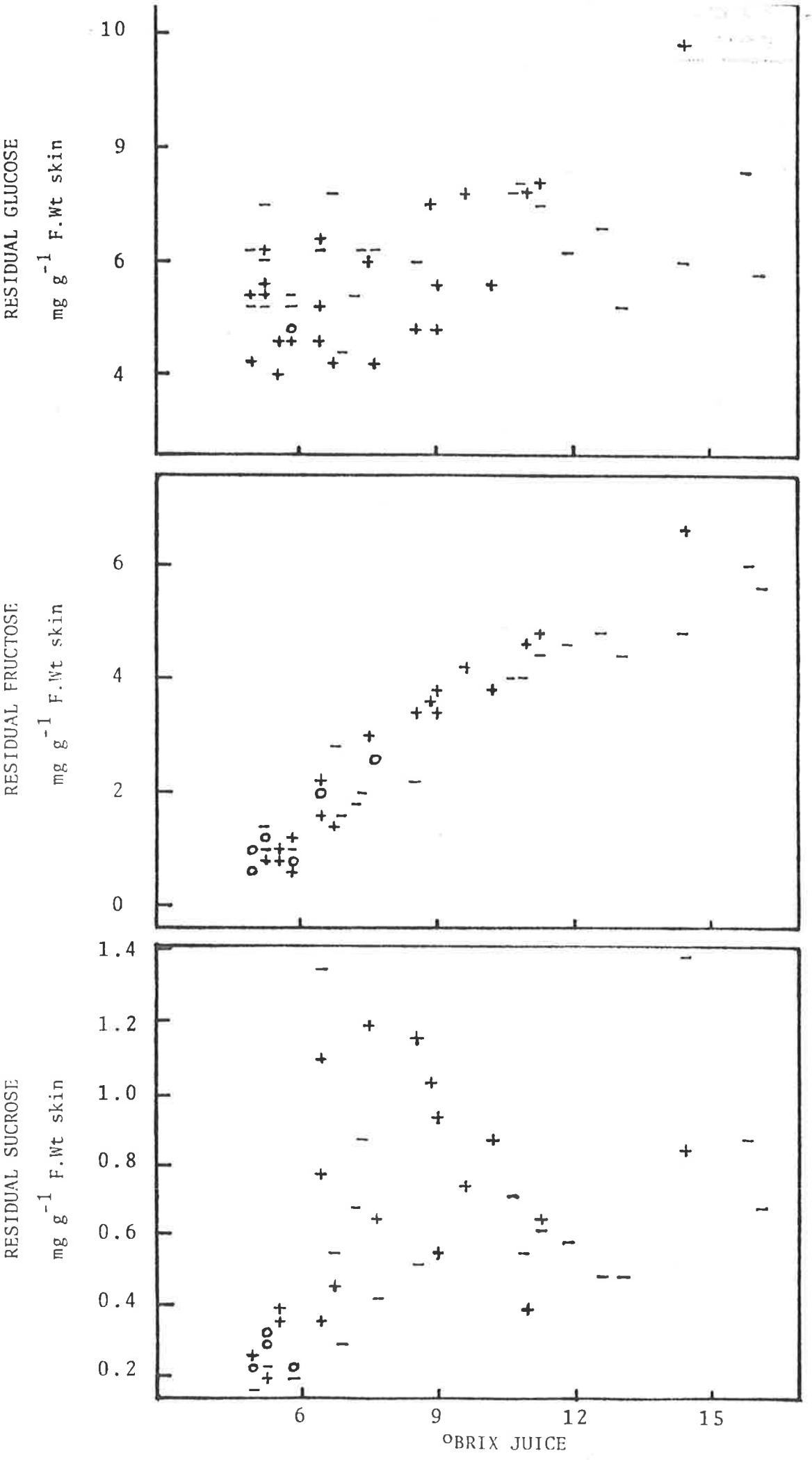


Fig. 8.2 continued. Relationships between °Brix of juice and sugars of skin from Muscat berries: shaded (+), unshaded (-), two data (o)

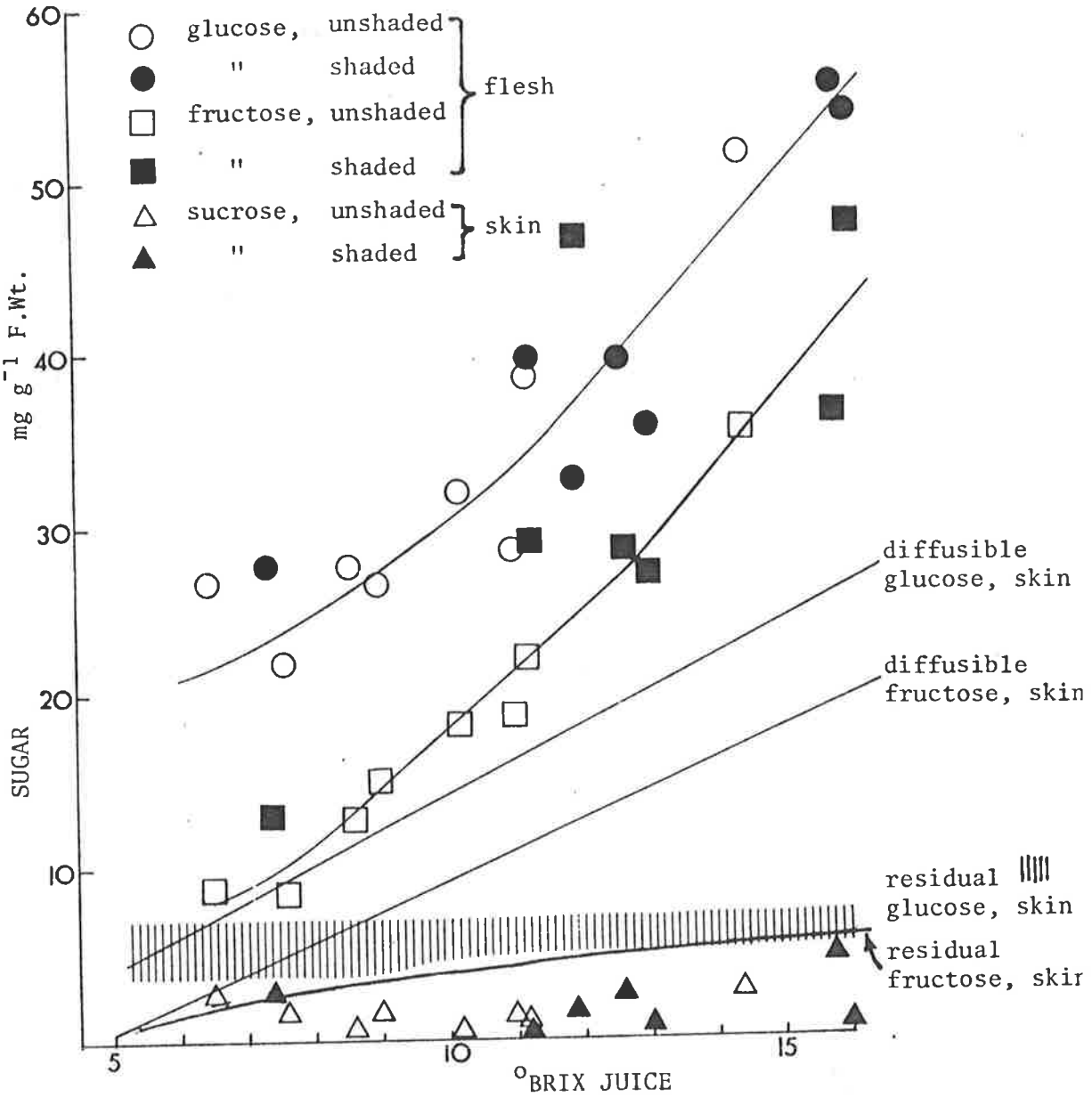


Fig. 8.3 Relationships between °Brix of juice and sugar levels of flesh and skin for Muscat berries.

throughout the period and, at day 16, mean °Brix of four shaded berries was 9.7 compared to 13.4 in unshaded.

Shading also caused lower sugar concentrations in the skin; shaded berries at day 16 had a mean total sugar (hexoses plus sucrose) level of 31.4 mg g^{-1} F.Wt. against 48.9 mg g^{-1} F.Wt. in unshaded. These concentration differences were not explicable in terms of treatment effects on skin fresh weight, since these were unaffected (Table 8.1).

From Table 8.1 it is clear that the skin of shaded berries contained only about a third of the diffusible glucose and fructose levels of unshaded berries. There was also a small reduction in residual glucose but no significant difference in residual fructose.

Shading did not affect sucrose levels which were 3% of total sugars. G6P and F6P were each c. 0.15% of total sugars. One half of the hexose phosphates and sucrose was diffusible.

For the development comparisons it was decided to use °Brix of the juice as the abscissa rather than Date because this parameter increased regularly with time and it unified the otherwise distinct populations of shaded and unshaded berries (see Discussion).

The correlation matrix in Table 8.2 showed the following points:

i) °Brix of the flesh was highly correlated with levels in the skin of diffusible glucose (r = 0.98), diffusible fructose (0.98), residual fructose (0.96); and with the derived values total sugar (0.98), total glucose (0.97) and total fructose (0.99).

ii) Diffusible glucose was correlated with fructose, both diffusible (0.99) and residual (0.94).

iii) Diffusible and residual fructose were strongly correlated (0.94).

iv) Residual glucose did <u>not</u> show such marked correlations with other attributes.

Scatter diagrams of sugar levels (Fig. 8.2) reflect the above-mentioned correlations and show that diffusible glucose, diffusible fructose and residual fructose increased with °Brix. Residual glucose did not relate simply to °Brix: most values were between 4 and 7 mg glucose g^{-1} F.Wt. with much variation between treatments and bunches

Table 8.2: Correlation matrix of attributes of 48 Muscat berries during 16 days from just before veraison.

	°Brix	Wt.	Diffusible Glucose	Residual Glucose	Diffusible Fructose	Residual Fructose	Diffusible Sucrose	Residual Sucrose	Total Sugar	Total Glucose	Total Fructose
	JUICE					SKIN					
JUICE: °Brix	1.00										
SKIN: Wt.	0.24	1.00									
Glucose diffusible	0.97	0.25	1.00								
Glucose residual	0.54	0.23	0.56	1.00							
Fructose diffusible	0.98	0.24	0.99	0.50	1.00						
Fructose residual	0.95	0.23	0.94	0.65	0.94	1.00					
Sucrose diffusible	0.41	0.18	0.42	0.37	0.43	0.45	1.00				
Sucrose residual	0.49	0.05	0.49	0.31	0.49	0.57	0.59	1.00			
Total Sugar	0.98	0.25	0.99	0.61	0.98	0.96	0.48	0.54	1.00		
Total Glucose	0.96	0.26	0.98	0.67	0.97	0.95	0.44	0.49	0.99	1.00	
Total Fructose	0.98	0.24	0.98	0.54	0.99	0.96	0.44	0.51	0.99	0.97	1.00
Total Sucrose	0.48	0.16	0.49	0.39	0.49	0.54	0.95	0.81	0.56	0.50	0.51

on the same day. Mean residual glucose on day 1 was 5.58 mg g^{-1} F.Wt. and on day 16 5.94 mg g^{-1} F.Wt. Over this period diffusible glucose increased five-fold, from 5 to 25 mg g^{-1} F.Wt. These scatter diagrams indicate no differences between shaded and unshaded berries for any given °Brix.

The levels of glucose and fructose in the flesh on days 13 and 16 are compared with glucose, fructose (both diffusible and residual) and total sucrose in the skin in Fig. 8.3.

8.4 Discussion

8.4.1 Sugar in the developing grape berry

At veraison the major sugar in the skin of the Muscat berry was glucose; the ratio of glucose:fructose was 5. However, in the period following veraison fructose accumulated at a rate similar to glucose, such that at 16°Brix the ratio was only 1.2 in both the skin and the flesh. This agrees with observations on whole berries cv. Thompson Seedless (Kliewer, 1966), cv White Riesling (Winkler, et al., 1974) and cv. Delaware (Matsui, 1979). In hypodermic juice samples from Muscat berries (Coombe and Phillips, 1980) the glucose:fructose ratio declined from 1·7 at the beginning of rapid sugar accumulation to unity 14 days later. Conversely, in efflux studies upon berries cv. Pinot noir just prior to veraison, the skin contained more fructose than glucose (Coombe and Matile, 1980).

At 5 °Brix 50% of the hexose in the skin was diffusible while at 16 °Brix about 75% was diffusible. The strong correlation (0.99) found between diffusible glucose and diffusible fructose is predicted if these derive by hydrolysis of translocated sucrose. However, residual glucose varied remarkably while residual fructose values followed a trend. On the one hand this is evidence that residual sugar was indeed a distinct pool in the tissue and not simply a residue of the same pool from which efflux occurred. On the other hand, wherever residual sugars were compartmented the concentration of sugar in the diffusible pool must have been much higher. Coombe and Matile (1980) used a tentative calculation of the volume of solution within the compartmented space of 0.8 ml g^{-1} F.Wt. Using this value (which would in fact change with ripening) estimates have been made

of hexose concentrations in the solutions within the two spaces (Table 8.3).

Table 8.3: Estimates of hexose concentrations (mM) in the skin of Muscat berries.

Solutions in:	°Brix of flesh	Glucose (mM)	Fructose (mM)
Diffusible space	5	150	30
	16	750	600
Compartmented space	5	40	7
	16	40	40

Clearly in the period following veraison the cell compartment became a fructose accumulator. Furthermore there was a substantial concentration gradient between the diffusible and compartmented sugars even two weeks after veraison.

Despite an evident resistance to compartmentation within the cell, the berry accumulated sugar. Thus accumulation is essentially an increase in diffusible sugars, in which the primary process is probably unloading from the phloem into the apoplast rather than eventual compartmentation within the cell.

Berries sampled on days 13 and 16 had higher sugar levels in flesh than in skin. Benvegnin et al. (1947) made a similar observation, which Kliewer (1967) attributed to the temperature gradient across the radius of the berry. This temperature gradient would be greatly diminished in shaded berries, yet the difference in sugar levels persisted.

There are 16 layers of cells peripheral to the dorsal vascular system of the Muscat berry (Considine, 1979), many of which are excised as the skin. The concentration of diffusible sugars in the skin will give one estimation of the diffusible sugar in adjacent flesh, diffusion tending to equalise concentrations. (The extracellular space of the flesh being a lesser proportion, the diffusible sugar content (as mg g^{-1}) is expected in fact to be less.) If this is so, the higher total sugar in the flesh must have been due to more compartmentation in the flesh. Similar compartmentation is constrained in the skin, though it is possible *in vitro* (Coombe and Matile, 1980).

The ratio F6P:G6P in the skin ranged from 1 to 2, unlike the value of c. 0.3 reported for whole berries by Ruffner et al. (1976 : see also Fig. 1.5). However, the amounts assayed were at the limit of sensitivity of the technique and a concentrative step should have been included prior to the assay, or fluorimetry used.

Sucrose levels were 0.03 - 0.5% F.Wt., similar to levels in whole berries (Hawker et al., 1976; Kliewer, 1966; Winkler et al., 1974).

The notable variability of residual glucose, and the singular lack of its accumulation during the 15 days of sampling, were surprising. This variability cannot be ascribed to asynchronous ripening as plotting the data against °Brix compensates for this factor.

Was the variability of residual glucose due to the assay procedures? Residual fructose values showed little variability about the apparent trend, yet these derive from the same samples assayed in the same cuvettes. Another possible source was the different amount of flesh tissue inadvertently removed with the skin, considering the high glucose:fructose ratio of this flesh at lower °Brix. A third explanation for the variability was the washing procedure; a washing medium with an osmolarity of about 200 mM may permit a varying efflux from a compartment especially when the compartment contains solutions of greater osmolarity i.e. in riper berries. The degree of this effect, if any, was not tested.

Variability of residual glucose indicates this pool to be independent of the accumulation process, or that it is interactive with other processes such as diurnal fluctuations of photosynthesis, respiration, gluconeogenesis, and re-export of sugar from the skin. Either glucose was being metabolized in the compartmented space (which therefore includes the cytoplasm) or efflux of glucose occurred. Efflux would be active unloading against a substantial gradient unless a buffering volume of low glucose concentration existed between these two pools.

8.4.2 Effect of shading

Compared with unshaded controls, berries shaded from 1 to 15 days had a mean °Brix of juice 14% lower and a concentration of total sugars in the skin 20% lower (Table 8.1). Such reduction of soluble

solids by shading has been reported by Kliewer and Schultz (1964; Schultz and Lider, 1964 and Kliewer et al., 1967).

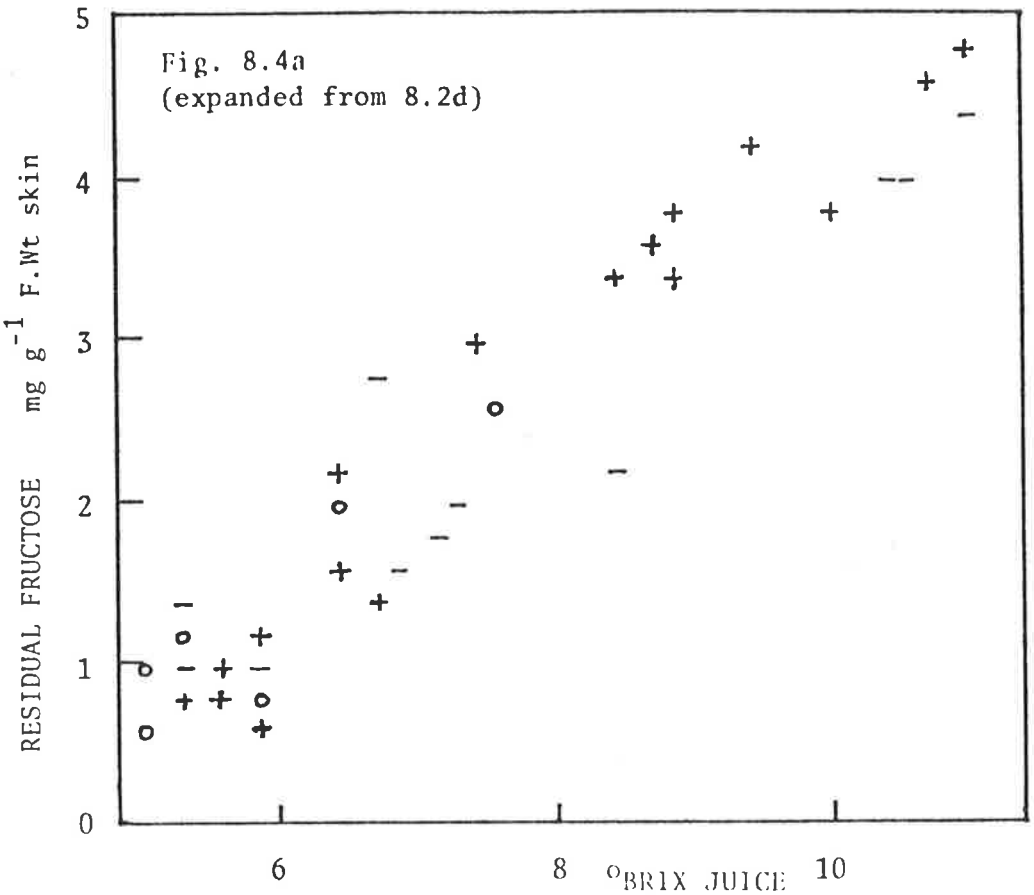
There are other reports of an increased concentration of soluble solids in bunches fully shaded (Kliewer and Antcliff, 1970) or partially shaded (Matsui et al., 1980). Kliewer and Lider (1968) determined that the glucose:fructose ratio of berries was unaffected by shading.

A concomitant reduction of diffusible hexoses and juice °Brix is expected if the major pathway of accumulation of translocated sugar is apoplasmic. Moreover as the concentration gradient between diffusible and compartmented space persisted, uptake was under a control other than simply the availability of hexose substrates. This is further indicated by the less significant reduction in residual sugars (noting Table 8.1) when other processes were perturbed by shading. It appears that unloading of sugars from the vascular tissue into the adjacent free space was more affected by light than was compartmentation.

The original hypothesis concerned the effect of shading upon the relationship of compartmentation and sugar accumulation by tissue, rather than the rate of accumulation *per se*. Plotting data against °Brix not only synchronises berries which entered veraison on different days but also allows comparison of two populations, shaded and unshaded, ripening at different rates. At a given level of soluble solids, was the apparent compartmentation the same in each case?

Examination of Fig. 8.2, with °Brix of juice as an index of ripening, reveals that shaded and unshaded berries formed one population for each recorded attribute.

Diffusible fructose presented the greatest proportionate increase of the measured attributes. Taken as an alternative index of change in the skin during these 16 days, the scatter diagrams of sugar levels against diffusible fructose (not presented) were similar to Fig. 8.2 but with improved linearity in the period immediately following veraison. This improved resolution is demonstrated between Figs 8.4a and 4b with respect to residual fructose.



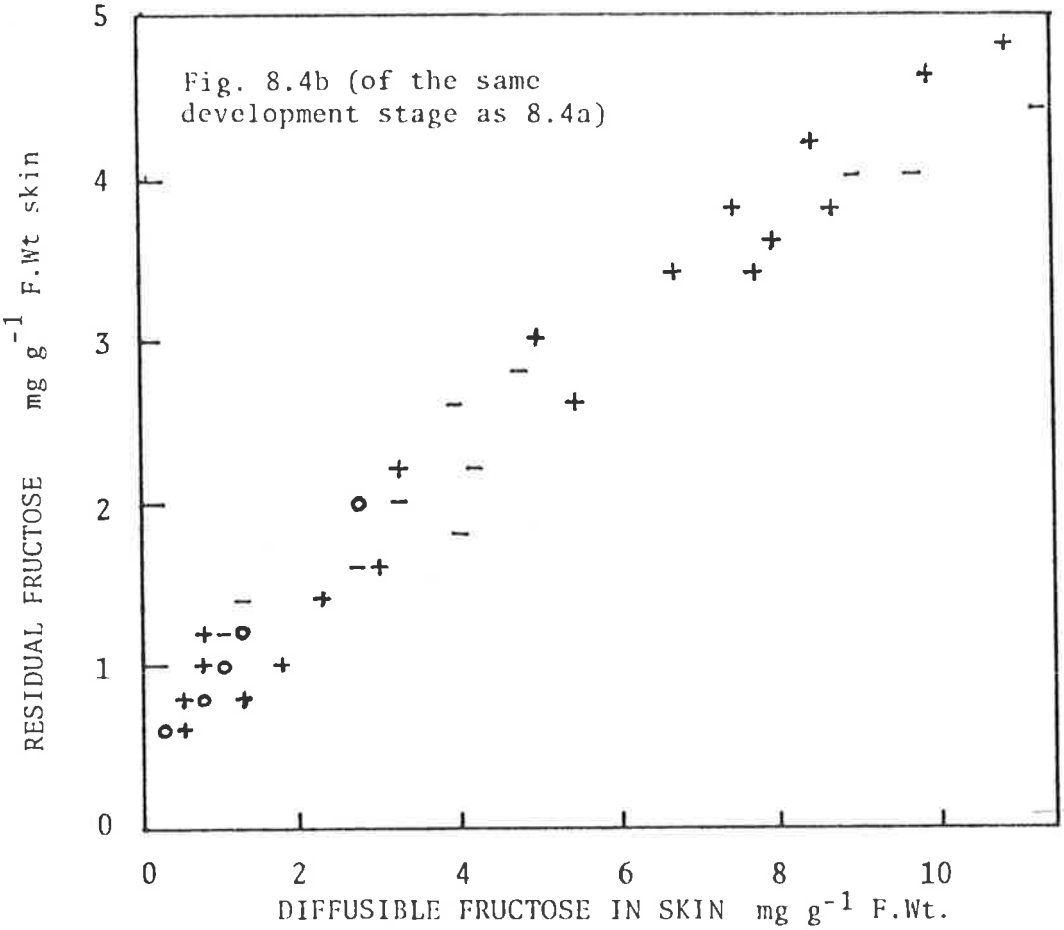


Fig. 8.4 The relationship between residual fructose in the skin and two indices of development in the grape berry; (a) °Brix of juice (b) diffusible fructose of skin: shaded (+), unshaded (-), two data (o).

Table 8.4: Effect of shading of Muscat berries at veraison. Values are averages of all berries with diffusible fructose < 3.9 mg g^{-1} F.Wt.

	°Brix	Diffusible Glucose	Residual Glucose	Diffusible Fructose	Residual Fructose	Diffusible Sucrose	Residual Sucrose
	juice	mg g^{-1} F.Wt. skin					
CONTROL	5.8	6.07	5.60	1.51	1.22	0.23	0.40
SHADED BERRIES	5.7	5.74	5.00	1.65	1.18	0.31	0.40
LEVEL OF SIGNIFICANCE Student's t : n = 22	n.s.	0.10	n.s.	n.s.	n.s.	0.001	n.s.

Analysing berries only in this period (all berries < 3.9 mg g^{-1} F.Wt. diffusible fructose) shading had no appreciable effect on diffusible or residual hexoses (Table 8.4). In the same period there was significantly greater diffusible sucrose in shaded berries. Likewise analysis of all 48 berries in Table 8.1 indicated an increase in sucrose due to shading and contrary to the trend for hexoses. Kliewer and Schultz (1964) also reported an increase of sucrose in green berries shaded to 21% sunlight. Zouaghi et al. (1979) found a phytochrome-dependent transfer of invertase from cytoplasm to the cell wall in radish hypocotyl and indeed the present data suggest that shaded berries have less invertase activity in the extracellular space.

No attempt has been made to differentiate between photosynthetic- and phytochrome-responses, nor between the light and temperature effect of shading. In this last respect, Kliewer and Antcliff (1970) found covered clusters of cv. Sultana were 1-4°C cooler at day and 0.5-3°C warmer at night.

Pairs consisted of similar berries on day 0, each of which developed more or less rapidly according to the treatment, so it is evident that ripening of a berry is not dependent upon its vascular neighbour. This has been shown by Coombe and Bishop (1980).

8.5 Conclusions

During 16 days commencing just before veraison shaded berries ripened more slowly than unshaded controls, as assessed by increase in °Brix of the juice or diffusible fructose in the skin. The interactions of amount and location (diffusible or residual) of glucose and fructose were essentially the same in shaded and unshaded berries - except in this relation to time.

At veraison the skin of these berries contained about 10 mg g^{-1} F.Wt. glucose, half of which was diffusible; but only 2 mg g^{-1} F.Wt. fructose, again half diffusible.

Over the 15 days diffusible glucose and fructose increased by the same amount, at a rate of 1-1.5 mg g^{-1} F.Wt. per day in a linear relation to °Brix. Residual fructose increased in a curvilinear manner to attain the same level as residual glucose which varied

markedly through this period.

At veraison half of the sugar in the skin was freely diffusible but two weeks later 80% (of an increased total) was diffusible. Hence it appears that accumulation is primarily associated with unloading of the phloem into the apoplasm and not loading into the final compartment, the vacuole. This shall be discussed in chapter 14.

Total sucrose in the skin was generally less than 2 mg g^{-1} F.Wt. and was slightly increased by shading.

In the flesh glucose and fructose increased similarly and rapidly. At 15 °Brix the sugar level in the skin was only 60% that in the flesh. For such a gradient to exist, despite the equilibrium expected of diffusible sugars, a substantial compartmentation of sugars in the flesh must occur.

As an index of initial ripening, "diffusible fructose" in the skin (or for simplicity of measurement, "total fructose" in the skin) is more indicative than °Brix of the juice especially in the period prior to veraison when °Brix changes little but total fructose may increase four-fold.

In summary, the original hypothesis - that shading removes the inhibition of compartmentation - was not sustained by the data.

CHAPTER 9: ISOLATION OF PROTOPLASTS AND VACUOLES

ISOLATION OF PROTOPLASTS AND VACUOLES

9.1 Protoplasts

9.1.1 Initial protocol for protoplast preparation

Coombe and Guyer-Huber (pers. comm.) developed a procedure to obtain protoplasts from the skin of pre-veraison berries of *V. vinifera* cv. Pinot noir. The protocol was as follows:

- Select hard green but well grown berries
- Wash in 1% Desogen (detergent) then rinse five times in sterile water.
- Peel the skin with four longitudinal cuts, then slice these sections at 2mm intervals to obtain pieces 10 x 2mm. Place these in an Erlenmeyer flask containing the Protoplast Medium (PM): 350 mM mannitol, 25mm MES pH 5.5 (titration KOH), 5 mM KCl, 2mM $CaCl_2$, 2 mM $Mg\ Cl_2$, 1 mM PEG 4000, 10 mM cysteamine HCl, 20 ug ml^{-1} oxytetracycline HCl. (Total osmolarity c. 400 mosmol.) Skin pieces from five small berries require 10 ml of PM. Change this medium once.
- Put pieces from 5 berries into 10 ml of 2.5% cellulase Onozuka R-10 in PM in a 9cm Petri dish. Shake overnight at 29°C, in the dark, 30 rpm on a 5cm amplitude
- Filter the degraded material through thin cotton wool in an open funnel into a test tube and allow the protoplasts to sediment. Remove the supernatant and add 5 ml fresh PM. Again allow the protoplasts to settle. Repeat several times till the supernatant is clear.
- Remove most of the supernatant and layer the protoplasts on to 3 ml 20% Ficoll 400 in PM in a centrifuge tube. Centrifuge at 2500g for 15 min.
- With a Pasteur pipette collect the purified protoplasts at the top of the Ficoll layer. In a centrifuge tube dilute these five-fold with PM and centrifuge at 1000g for 5 min.
- Remove the supernatant and add 1 ml PM to the pelleted protoplasts.

Poor results were obtained with this protocol using berries of cvv. Pinot noir, Doradillo and Muscat from the Alverstoke and Claremont orchards of Waite Agricultural Research Institute. Yields of protoplasts were low; the presence of many vacuoles indicated unstable protoplasts; and large clumps of tissue remained intact or only partially macerated. Hence it was necessary to re-examine the method.

9.1.2 Losses

Protoplasts were prepared by the original protocol while material and supernatants were assessed and quantified with a haemocytometer. Following the 15 h incubation, a further 9 h wash of skin residues in straight PM doubled the release of protoplasts. Moreover in the purification steps about 40% of the protoplasts, and especially the smaller ones, were remaining in the supernatants such that only 20% of those protoplasts in the original filtrate were being recovered.

9.1.3 Alternative osmotica

Increased molarities and the use of D-glucose, D-fructose or PEG 300 as osmotica were tested as modifications to obtain protoplasts from the skin of post-veraison berries cv. Muscat. Skin pieces from 4 berries were incubated in each medium, as follows:

PM modified to:

1. 0.7 M mannitol
2. 0.7 M mannitol + 0.7 M D-glucose
3. 0.7 M mannitol + 0.7 M PEG300
4. 0.7 M mannitol + 0.7 M D-glucose + 0.7 M PEG300
5. 1.5 M PEG300 omit mannitol
6. 2.1 M PEG300 omit mannitol
7. 0.5 M mannitol + 0.5 M D-glucose + 0.5 M D-fructose
8. 0.7 M mannitol + 0.7 M D-glucose + 0.7 M D-fructose

After 15 h the tissue was only slightly macerated and did not improve with a further incubation of 24 h. When the skin pieces were shaken with forceps some protoplasts were observed in media 1,2,7 and 8 but none with media 3,4,5 and 6. Hence PEG 300 was deleterious to protoplasts; and cellulase in these media did not macerate ripe skin tissue. Medium 1 contained many small protoplasts whereas media with hexoses contained few small protoplasts.

9.1.4 A mannitol series

According to Okuno and Furusawa (1977), in media to isolate protoplasts from cereal leaves there is a crossover between molarities giving low yield and low stability to those giving high yield and stability. This crossover occurred between 0.4 and 0.5 molar mannitol.

The skin of berries cv. Doradillo (4.0 °Brix) was incubated in PM, buffered with 50 mM MES, with twelve levels of mannitol from 0.25 M to

0.80 M, each treatment being in a 5cm Petri dish. These were coded to reduce bias in assessment. The incubates were examined after 15 h, purified by low speed centrifugation without the use of Ficoll, resuspended in 1ml PM, stored at 4°C, and examined 24 h and 48 h later (Table 9.1).

Table 9.1: The effect of osmotic concentration (mannitol) upon production of protoplasts

PM, containing mannitol (Molar) :	After centrifugation at 15 h				Quantity of protoplasts At (15+24) h	Quantity of protoplasts At (15+48) h
	Degree of tissue maceration	Quantity of debris		Quantity of protoplasts		
		large	fine			
0.25	++	++	++	+	0	0
0.30	++	++	++	+	0	0
0.35	++	++	+	+	(+)	0
0.40	++	++	+	+	(+)	0
0.45	++	+	+	+++	(+)	(+)
0.50	++	+	-	+++	+	0
0.55	++	+	-	+++	++	0
0.60	++	+	+	++	++	++
0.65	+	+	-	+	+	0
0.70	+	++	++	(+)	(+)	0
0.75	+	++	-	++	++	(+)
0.80	+	++	++	(+)	(+)	0

Tissue maceration was less at high molarities. Protoplasts were unstable below 0.55 M mannitol despite better initial yields at 0.45 M to 0.55 M mannitol. Fine debris of cytoplasmic origin was an indicator of cell lysis and this increased markedly in the low osmotic media. It was concluded that PM at 0.6 M mannitol gave good tissue maceration and a reasonable protoplast yield and stability. The osmolarity of such a medium would be c. 0.68 due to the additional ions. Spherical vesicles without internal structure, tending to float and not refringent under phase contrast, were observed in 0.35 M mannitol. These were interpreted to be vesicles from resealed plasmalemma, and thus artefact of the incubation.

The trial was repeated and the yield of protoplasts quantified by haemacytometry (Fig. 9.1). Incubates of 0.55 M and 0.65 M mannitol contained notably less debris and both large and small protoplasts. In 0.50 M mannitol 92% of the protoplasts excluded 1.25% Evans Blue.

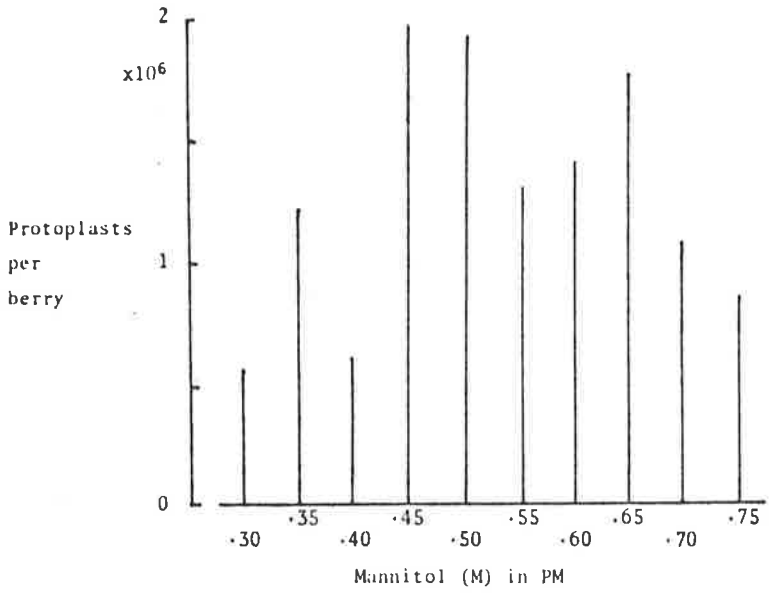


Fig. 9.1 Protoplast yields at different mannitol concentrations from berries cv. Doradillo (4.5°Brix).

9.1.5 Protoplasts from berries in the first growth cycle

Following the original protocol, incubation of skin pieces from berries cvv. Muscat, Doradillo and Seeded Currant in the first growth cycle produced well macerated tissue but few protoplasts. Numerous protoplasts had two central vacuoles with cytoplasm aggregated at the juncture. Intracellular raphides were commonly seen. Replacing cysteamine HCl with 10 mM dithiothreitol decreased the browning of tissue, but resulted in "collapsed protoplasts" - spherical vacuole birefringent in phase contrast within an irregular layer of cytoplasm. These "vacuoles" were not osmotically active. After 15 h the pH of the incubation was 3.45; the buffer was clearly inadequate for such acidic tissue and so was increased to 50 mM MES. In subsequent incubations of pre-veraison berries the incubate did not fall below pH 4.3, or below 5.0 with post-veraison tissue.

9.1.6 Effect of ions and other additives

With post-veraison berries cv. Doradillo, increasing the concentration of K^+ from 5 to 50 mM and of Mg^{2+} from 2 to 20 mM improved the yield of protoplasts. A further comparison between the following modifications to PM confirmed the contribution of ions (Table 9.2).

Table 9.2: Yield of protoplasts from post-veraison berries with modification of the ions in the medium.

#	PM modified to (mM): KCl	$CaCl_2$	$MgCl_2$	Yield of protoplasts (Control = 100)	Amount of debris
1 (Control)	5	2	2	100	+++
2	50	2	20	157	+++
3	50	20	20	430	+
4	10	4	4	210	+++

The control contained some isolated protoplasts, large numbers of cells in sheets of 10 or 20, some single cells, fine and clumped debris presumably from broken cells, intact tissue and the film of cuticle. Most cells were clearly plasmolysed. Others were not, either containing a sap of high osmotic potential or, more probably, having lost membrane function.

The skin of pre-veraison berries cv. Doradillo (c. 4° Brix) was incubated in PM of the following ionic composition:

1. (Control)	5 mM KCℓ	2 mM $MgCl_2$	0.2 mM $CaCl_2$
2.	0.5 mM KH_2PO_4	2 mM $MgSO_4$	0.2 mM $Ca(NO_3)_2$
3.	20 mM KH_2PO_4	2 mM $MgSO_4$	0.2 mM $Ca(NO_3)_2$

Yields of protoplasts were similar, including in each case isolated vacuoles present at a ratio of about 1:7. The incubate was filtered, twice pelleted and rinsed, and kept at 4°C. After 2 days most of the vacuoles had lysed but the protoplasts persisted till a final observation after 15 days whereupon numerous protoplasts were remaining in the medium (#3) containing 20 mM KH_2PO_4.

In yet another trial a medium without salts except 1 mM EDTA. Na_2^+ yielded very few protoplasts whilst a series starting with 1 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $Ca(NO_3)_2$ and increasing in K^+ through 2.5, 10, 25, 100 mM showed a marked yield increase with higher levels of KH_2PO_4 (Fig. 9.2).

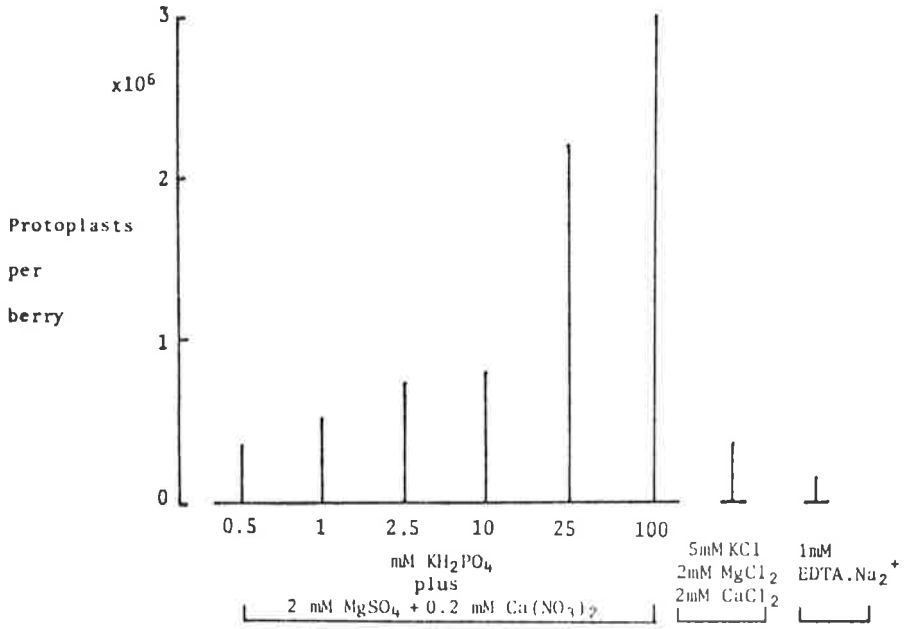


Fig. 9.2 Protoplast yields from berries cv. Doradillo (5°Brix) in media containing mannitol, 50 mM MES, 1 mM PEG 4000, 10 mM cysteamine HCl, terramycin, and the indicated ions: total molarity 575 mOsmol.

100 mM K^+ not only yielded the most protoplasts but these were cleaner and notably free from the browning of tissue observed in media of low K^+. In each treatment the concentration of mannitol had been adjusted so the osmolarity of the medium was unchanged. The dihydrogen phosphate no doubt had a pH effect. Correctly, a K^+ stock should have been prepared by titration (to pH 5.5) of $H_2PO_4^-$ with HPO_4^{2-}.

Inclusion of 0.2% methyl cellulose (B.D.H.) in the PM did not reduce the clumping of protoplasts and in fact reduced the maceration of tissue. Omission of PEG 4000 from the medium resulted in inferior digestion of tissue.

9.1.7 Geometry of the incubation vessel

Protoplasts yields were poor in an Erlenmeyer flask relative to those in a Petri dish. Incubations in a capped plastic cylindrical tube lain on side were superior.

Presumably the flask excessively exposed the incubation to the air, in which regard the tube was the best. Also a mild seiche is desired, and this was readily obtained in Petri dishes and, especially in tubes on side. However, Petri dishes facilitated direct examination of the incubate under the microscope and so were preferred.

9.1.8 Cell-wall degrading enzymes

Skin from almost-ripe berries cv. Doradillo was incubated in different enzyme combinations (Table 9.3), using PM modified to 0.5 M mannitol plus 0.5 M glucose plus 0.5 M fructose. The common enzymes were detailed in Table 6.2; others tested were those often used to clarify must.

Table 9.3: Effect of additional enzymes upon protoplast yield from the skin of almost-ripe berries cv. Doradillo.

ENZYMES	AT 15 HOURS		
2.5% cellulase Onozuka R10 PLUS :	Observation under microscope		
	Protoplasts per field	Remarks	Tissue colour
ALONE (Control)	7-11	More protoplasts upon agitation	green
+ 0.3% Pectinase, Koch-Light	0-3	"Collapsed protoplasts"	dark brown
+ 0.3% Ultrazym 100, Ciba	0-5		green
+ 0.3% Pectinol 10 M	2-5	Improved upon agitation	dark brown
+ 0.3% Clari-phase, Pfizer	1-7	"Collapsed protoplasts"	green
+ 0.3% Panzym rapid, Boehringer	0-2		green
+ 0.3% Panzym super, Boehringer	Few		green
+ 0.3% Klerzyme 200, Industrial Supply	0	Maceration within 1 h. at 15 h, no protoplasts, only cuticle and clumped debris	very brown
+ 0.25% Macerase + 0.25% Pectinase, Sigma* + 0.25% Driselase + 0.25% Rhozyme HP150	0		dark brown

*Medium centrifuged to remove sediment prior to incubation

Clearly cellulase Onozuka R10 alone was the superior enzyme for the release of protoplasts from this tissue.

The release of protoplasts from skin of berries cv. Muscat (9 °Brix) by cellulase Onozuka R10 was not improved upon inclusion of 0.5% cellulysin (Calbiochem) nor 0.25% pectinase (Koch-Light). Nagata and Ishii (1979) reported a "maceration stimulating factor" in Pectolyase Y23, an enzyme mixture from *Aspergillus japonicus* containing pectinlyase and polygalacturonase. 0.1% Pectolyase Y23 with 2.5% cellulase Onozuka R10 in PM caused maceration of skin within minutes but protoplasts were neither isolated nor viable.

9.1.9 Summary and protocol

In summary, a medium including 25 mM KH_2PO_4 reliably produced good protoplasts for one particular season from berries cvv. Doradillo and Muscat of between 4 and 10 °Brix.

In the following season this medium was unsatisfactory and the level of K^+ was inconsequential. K^+ was maintained at only 0.5 mM, supplied as the phosphate buffer pH 5.5. A marked improvement resulted from substitution of reductant using 10 mM dithiothreitol in place of cysteamine.HCL. 0.1% BSA was also included.

In the third season potassium phosphate and BSA could be omitted without consequence upon protoplast yield and either reductant sufficed provided each was fresh. Satisfactory production of protoplasts required a second incubation in fresh medium containing 1% cellulase.

Any reviewer of protoplast technology will stress the prerequisite of consistent plant material preferably grown under "soft" conditions. On the contrary, berries used here were from vineyards subject to varied seasonal effects. A prolonged supply was assured by the following methods.

(i) Late pruning : Vines were not winter-pruned until after budburst, whereupon canes were spur pruned (to remove growing shoots arising at distal nodes) to stimulate the basal (fruitful) buds. This was done upon different vines at 14-day intervals till October 15th.

(ii) Double pruning : Following normal winter pruning and spring growth, shoots were summer pruned to two nodes after formation of new season Anlagen, in December. This forced the new buds which were fruitful despite the lack of a dormant period.

(iii) Vines cv. Muscat, of moderate vigour, produce a second crop on laterals.

(iv) Berries could be stored on the bunch at 4° in sealed bags for up to two months.

Berries were generally selected to be at veraison i.e. when about to soften, or with juice from the flesh below 6 °Brix. In later experiments 2 μℓ hypodermal samples of juice were routinely taken (Coombe and Phillips, 1980) to assay for glucose and fructose. As evident in Chapter 8, berries in the lag phase contain principally glucose while fructose rapidly increases before and after veraison. Thus glucose and fructose concentrations and their ratio provide a good indicator of developmental stage during this period: hexoses increased rapidly after attaining concentrations of 21 g ℓ^{-1} glucose and 12 g ℓ^{-1} fructose.

A revised protocol to obtain protoplasts from grape skin is presented in Fig. 9.3.

The production of protoplasts from grape pericarp is depicted in Fig. 9.5.

9.2 Protoplast Yield and Agglutination

9.2.1 Chlorophyll as an index of yield

To assess the recovery as protoplasts of cells from tissue, four berries cv. Muscat at veraison (hypodermal juice : 15 g ℓ^{-1} glucose, 3.2 g ℓ^{-1} fructose) were peeled and the skin weighed. From a small skin sample c. 10 mg of each berry chlorophyll was eluted into 600 μℓ methanol in a capped Eppendorf reaction tube for several days at 4°. The remainder of the skin was incubated to produce protoplasts which were likewise sampled into 600 μℓ methanol for chlorophyll extraction. The tubes were centrifuged to pellet any crystals (due especially to mannitol in PM) and the supernatant read at 665 nm in a Unicam dual-beam spectrophotometer against air, with subtraction of a pure methanol blank.

Fig. 9.3: Protocol to obtain protoplasts from grape pericarp.

- Select unblemished berries 4-6 °Brix. (1)
- Wash in solution of 5% hypochlorite, 0.1% Tween 20 for 1 min and rinse 10 times in sterile water.
- Make 4 longitudinal and 1 equatorial cuts, peel the skin into cold Protoplast Medium (PM) : (2)

550	mM	mannitol
50	mM	MES pH 5.5 titrated with KOH
1	mM	Carbowax 4000
2	mM	$MgSO_4$
0.2	mM	$Ca(NO_3)_2$
5	mM	dithiothreitol
0.1%		BSA
6	ug ml^{-1}	oxytetracycline (stock 6 mg ml^{-1})

  (This medium excluding the last 3 components has been autoclaved for 10 min maximum.) The skin from 6 berries cv. Muscat requires c. 20 ml in order for the pieces to remain dispersed.

- After 20 min suck off and replace the medium; hold at 4°.
- After a further 20 min, discard the medium and add the pieces to 2.5% cellulase Onozuka R10 in PM in a Petri dish. (3) 20 ml in a 9cm dish is adequate for 6 berries.
- Using a dessiccator attached to a water vacuum pump, vacuum infiltrate the tissue for 5 min.
- Shake 15 h at 26°C, in the dark, 45 rpm. The tissue may be agitated with forceps after several hours to facilitate maceration.
- Gently pour the incubate into a beaker, and use a little fresh, cold medium to rinse out the Petri dish. Gently stir the material to release macerated cells. Filter through coarse nylon net in a funnel to remove unmacerated tissue and cuticle. Allow the incubate to settle at room temperature for 15 min.
- Remove the supernatant and return the sedimented protoplasts (4) and cells to a smaller Petri dish, adding fresh medium and cellulase to 2%. (5) The supernatant may be discarded; or centrifuged at 200g for 20 sec and the pellet resuspended in fresh medium. This contains small protoplasts - or cells, in which case it is added to the incubation. (6)
- After 3 h the material is filtered through 2 layers of nylon mesh (c. 100 µ pore) with rinsing. Allow the protoplasts to settle and remove the supernatant. Add fresh PM. As above, the supernatant may be retained, centrifuged for 20 sec, and the sedimented (small) protoplasts added to the stock.

Comments:

(1) Softer berries may require increased osmoticum.

(2) K^+ may be included. Cysteamine HCl may replace dithiothreitol.

(3) Glassware should be sterile and, preferably, siliconised to reduce adhesion by material.

(4) Protoplast suspensions are handled gently and wide-mouthed pipettes employed, such as a Pasteur pipette broken above the capillary or transfer pipettes with the plastic disposable tip slightly cut back. Air should not be aspired with the suspension.

(5) This second incubation is facultative.

(6) A pellet from centrifugation is without delay gently resuspended by swirling.

Elementary observation indicated these incubations to be mediocre relative to some. Nevertheless the rates of recovery of chlorophyll in the cleaned protoplast suspension per unit chlorophyll in the original skin tissue were 0.27 and 0.31 in the two trials (Table 9.4). These data indicate that two in three cells do not form protoplasts, either lysing or remaining as tissue and macerated cells.

The assumptions are:

(i) The average chlorophyll content of the protoplast population is the same as that of the original cells. — During the incubation a particular class of cells may be lost (larger cells break or smaller epidermal cells are not liberated). The ratio of volumes of protoplasts is a factor of radius cubed, so comparing protoplasts of radii 5 μm and 30 μm the volume ratio is 1:216. From microscopic examination (including natural fluorescence of chlorophyll) it appears that chlorophyll content does not vary by such an amount.

(ii) Chlorophyll is not lost or degraded during incubation. — The layer of cells damaged during excision of the skin though unable to form protoplasts will contribute to total chlorophyll.

(iii) Chlorophyll in debris associated with the suspension is not significant. — Debris was assessed at 8% by volume. Hence chlorophyll yield will be overestimated; however, many contaminants would not be a source of chlorophyll.

(iv) The extinction coefficient of chlorophyll is not affected by other tissue compounds nor, in the case of the suspension, by the presence of 8.5% PM in the methanol extraction.

(v) Other compounds absorbing at 665 nm do not bias the assay.

From 1.091 g of skin 1.39×10^6 protoplasts were produced. (This was about half the yield determined in another experiment from which it is deduced that recoveries may attain at least 60%). Calculated from the yield from four berries, the average cell number in the tissue excised as skin would be 1.2×10^6 cells per berry. Considine (1979) calculated by stereology that the first seven layers of cells in the skin of a berry cv. Muscat contained 2.8×10^6 cells; the epidermal

Table 9.4: Recovery of chlorophyll from grape skin as protoplasts in suspension

	SKIN INCUBATED (mg)	SKIN EXTRACTED FOR CHLOROPHYLL (mg)	ABSORBANCE 665nm (of aliquot)	CORRECTED ABSORBANCE ($\times 10^3$ mg^{-1})	YIELD = chl. in protoplasts / chl. in skin
Trial 1	566		0.091	2.54	0.27
		17.0	0.161	9.47	
Trial 2	525		0.103	2.48	0.31
		17.8	0.144	8.09	

Table 9.5: Distribution of protoplasts 30 min after addition of Concanavalin A.

	PROTOPLAST SUSPENSION containing	FREE PROTOPLASTS per field	CLUMPS per field	PROTOPLASTS per clump	DISCRETE DEBRIS per field
BLANK	no additive	7 22 14 16 12 Total = 71	0 0 1 0 1 Total = 2	- - 2 - 3 $\bar{x}$ = 2.5	3 4 5 5 3 Total = 20
NaCl CONTROL	0.5 mg ml^{-1} NaCl	10 18 17 21 9 = 75	1 0 0 0 0 = 1	3 - - - 1 $\bar{x}$ = 3.0	2 2 5 3 3 = 15
CON A 1	0.25 mg ml^{-1} CON A	3 9 6 5 8 = 31	6 2 3 1 3 = 15	2,2,2,3,5,8 3,7 2,4,12 3 3,3,3 $\bar{x}$ = 4.1	0 2 4 2 2 = 10
CON A 2	0.50 mg ml^{-1} CON A	1 2 4 4 0 = 11	2 4 2 9 8 = 25	2,5 2,3,3,3 2,4 2,4,4,4,4,4,5,5,10 2,2,2,2,2,2,2,7 $\bar{x}$ = 3.5	1 1 2 3 1 = 8
CON A 3	0.75 mg ml^{-1} CON A	1 1 4 4 0 = 10	3 3 4 1 3 = 14	4,8,13 7,14,26 2,2,4,5 4 2,5,7 $\bar{x}$ = 7.4	1 2 0 1 0 = 4

layer comprised 1.2 x 10^6 cells. These values are considerably higher than those quoted by Harris et al. (1968) who found, from a macerated tissue sample, counts of 0.6 x 10^6 cells in the entire pericarp of cv. Sultana.

9.2.2 Concanavalin A with protoplasts

In a preliminary trial it was evident that Concanavalin A (Con A; Sigma grade III ex Jack Bean) caused agglutination of protoplasts in suspension.

Protoplasts were then prepared from berries cv. Muscat at veraison (hypodermal juice : 15 g ℓ^{-1} glucose, 3.2 g ℓ^{-1} fructose), twice rinsed in PM, and filtered through a 2.5 mm long Sephadex column (Para. 10.2). Three levels of Con A and one level of NaCl (a carrier of the Sigma Con A) were tested. Five 45 mm Petri dishes were prepared with a final volume of 1.5 ml containing about 5 x 10^4 protoplasts in PM and the treatments indicated (Table 9.5). These were shaken at 40 rpm at 25° in the dark. After 15 min clumping was apparent; at 30 min this was quantified by counting material in 5 fields (magnification x 150) at the centre and four cardinal points of each dish. 18 h later all dishes still contained numerous viable protoplasts (Fig. 9.6A,B).

Results are presented in Table 9.5. As the number of agglutinations increased the count of free protoplasts decreased. At 0.75 mg ml^{-1} Con A, on average, seven protoplasts formed a single clump. Similarly the varied contamination of the suspension (chloroplasts, membrane pieces, broken cells) also agglutinated with Con A. Sodium chloride alone did not induce agglutination.

This work was not repeated with purified vacuoles. Travis and Berkowitz (1980) reported that Con A did not bind to purified "tonoplast" of Soybean. Might not Con A therefore serve to remove contaminating protoplasts from a vacuole suspension? The system envisaged would use Con A Sepharose (Brunner et al., 1977). Beads could be mixed into the suspension, left at 4° for 15 min, then filtered through a very coarse mesh to remove the free-flowing vacuoles from the protoplasts binding to the beads. Furthermore the present trial indicates that debris and cells would also be removed.

9.3 Vacuoles

9.3.1 Vacuole isolation

The protocol to obtain vacuoles by polycation-induced lysis of protoplasts from grape pericarp (Fig. 9.4) was developed from preliminary work by Guyer-Huber and Coombe (pers. comm.) on berries of cv. Pinot noir.

9.3.2 Counts and yields

A haemacytometer was used to quantify protoplasts and vacuoles in suspension. When a sample was introduced at the side of the haemacytometer, as is the convention, the medium tended to flow ahead of the cell material especially where large protoplasts or cell debris were present. An alternative would have been to use a haemacytometer of depth 0.2mm rather than the usual 0.1mm. However, reproducible counts were obtained by an unorthodox procedure of sampling a drop directly on to the etched grid and carefully lowering the cover-glass. Certain haemacytometers have a surface coating which appears to absorb heat and rapidly causes vacuoles to break.

Lysis of ten different protoplast suspensions yielded on average 64% vacuoles by number, ranging from 88% to 41%. This average does not include one preparation of vacuoles from protoplasts in a high $[K^+]$ medium. These protoplasts contained several vacuoles and lysed to yield 147 vacuoles per 100 protoplasts. In an aliquot of the original suspension 16 days later large numbers of protoplasts were still intact.

Vacuole preparations were labile, requiring gentle handling. Normally their number declined during succeeding hours, so any experiments were conducted without delay. If vacuoles were purified by ultracentrifugation the decline was more rapid. In one case the recovery from a Ficoll gradient (ref. Chapter 10) was only 5% and these were labile, whereas vacuoles in the same lysate persisted, uncleaned, at 4° for at least 7 h without loss.

9.3.3 Key variables of the protocol

In the protocol are four key variables: the time between DEAE-Dextran and Dextran-sulphate additions (the "lysis time"), the density

Fig. 9.4: Protocol to obtain vacuoles by polycation-induced lysis of protoplasts from grape pericarp.

- Dilute a protoplast suspension five-fold with the following cold Vacuole Medium (VM):

  0.7 M mannitol
  10 mM HEPES pH 7.3 titrated with KOH

- Centrifuge at 200*g* for 20 sec, allow to settle 3 min, then discard supernatant and resuspend the protoplasts in VM. Adjust to c. 4 x 10^5 protoplasts per ml ; proceed without delay.
- Using a wide-mouth pipette (e.g. a transfer pipette with the disposable tip cut on an angle to an orifice c. 2mm), gently place 1 ml aliquots in test tubes in ice. ①
- Add 200 µℓ 20 mM EDTA.Na_2 in VM then 100 µℓ 0.1 mg ml^{-1} DEAE-Dextran in VM with gentle but immediate mixing. ② Transfer to a 35° water bath with reciprocal shaker (60 rpm).
- After exactly 40 sec add 100 µℓ 0.2 mg ml^{-1} Dextran sulphate.Na_2 in VM. Swirl gently and leave to incubate a further 2 min.
- Examine a droplet under the microscope; return to the tube to ice.
- The aliquots are bulked and fresh medium is added containing cysteamine HCℓ to give a final concentration of c. 5 mM ; and cysteamine is included in VM forthwith.
- Either

  (i) the preparation is left to settle at 4°C, the lytic agents and cytoplasmic material being only partly removed as the supernatant. Many vacuoles will not settle, while centrifugation is deleterious, or

  (ii) the vacuoles are purified

  e.g. layered over a linear gradient of metrizamide 2 to 20% w/v in VM. This is centrifuged at 8000*g* for 10 min at 5°C in a swing-out rotor and the vacuoles recovered from within the gradient using a Pasteur pipette (see Fig. 10.5).

Comments:

① Routinely, an 0.5 ml aliquot is lysed using half volumes to ascertain the period giving maximal lysis of protoplasts in a particular suspension. The time may be adjusted between 20 and 60 seconds; or larger aliquots of poly-ions are used.

② In one season increased concentrations were required, namely: 1 mg ml^{-1} DEAE-Dextran and 2 mg ml^{-1} Dextran Sulphate. In any event the ratio of these is kept at 1:2 (w/w). Localized concentrations of reagents must be avoided.

of the suspension, the volume of DEAE-Dextran, and its concentration.

If the lysis time was protracted the vacuoles gradually lysed also. If lysis was brief, protoplasts persisted.

The more significant variables concern the quantity of DEAE-Dextran per unit plant material. There appeared to be a broad optimum affected no doubt by the physiological state of the tissue and protoplasts and the degree of contamination.

There was a broad optimal density of suspension which generally resulted in even and quite complete lysis of protoplasts. At lower densities excessive lysis of vacuoles as well as protoplasts occurred unless the time interval was brief and precise. At higher densities one observed many persistent protoplasts.

A larger aliquot of DEAE-Dextran increased the percent lysis of protoplasts if this was incomplete. As noted, a more concentrated stock of DEAE-Dextran was necessary in one season.

The pH of the suspension at lysis and presence of ions are important as indicated by poor lysis of protoplast when in PM. A preparation in VM was titrated with 0.5 M MES buffer to a range of pH : 5.7, 6.0, 6.25, 6.5, 6.75, 7.00. Only partial lysis occurred at the lower pH while the more alkaline lysis was clearly superior.

Addition to VM of 0.2 mM Ca^{2+}, thought to be necessary for membrane stability, made no perceptible difference to lysis of protoplasts or persistence of vacuoles.

9.4 Microscopy

9.4.1 Observations of lysis

Observing lysis under the microscope (Fig. 9.7) one typically saw the plasmalemma rupture along an arc. The vacuole extruded progressively through the aperture as the protoplast diminished. Some cytoplasmic material was liberated into the medium while some, including the membrane, retracted (Fig. 9.7.A). Commonly this material remained loosely attached to one point of the naked vacuole which was then termed "non-discrete". A vacuole appearing as an isolated sphere was termed "discrete" (Fig. 9.7.C).

The vacuole population included the range of sizes as found with protoplasts, up to 70 µm diameter, and typically a large number of small vacuoles or vacuoles which rapidly contracted. Some preparations consisted largely of very small vacuoles less than 4 µm diameter.

Following the same protocol one obtained sometimes entirely discrete vacuoles and other times many non-discrete vacuoles with such variation even through a series of lyses from one protoplast stock. Non-discrete vacuoles were poorly purified by simple sedimentation and rinsing, tended to clump, but otherwise produced discrete vacuoles upon density gradient centrifugation or Sephadex filtration (Para. 10.2).

A remedy for this problem was not found. Most probably the cause was the loss of similar (repulsive) surface charges in protoplasts and vacuoles (Nagata and Melchers, 1978). A recent report emphasized the need to remove ions in order to avoid clumping of vacuoles and proposed the use of 0.25 mM EDTA (Briskin and Leonard, 1980). Presumably divalent cations are particularly inducive to clumping. As my procedure included EDTA one concludes that removal of vacuoles from the lysate (and hence from endogenous ions) is necessary.

Protoplasts which persisted after lysis of a suspension were viable in VM and were not separated from the vacuole preparation (Chapter 10). Hence, the objective was to maximise percent lysis. Some preparations were lysed to vacuoles with no contaminating protoplasts (and recovery of 50 vacuoles per 100 protoplasts lysed). Twelve preparations during different seasons produced a ratio of vacuoles: unlysed protoplasts of 100:6 excluding an unacceptable lysis where this was only 3:1.

On the other hand, some vacuoles appeared during tissue incubation to release protoplasts. The ratio of protoplasts to "natural" vacuoles varied markedly, from 2:1 up to 14:1, especially when investigating alternative incubation media. Those media giving higher yields, cleaner and viable protoplasts, also contained fewer vacuoles. Thus, these vacuoles indicated protoplast instability and were themselves labile in PM. By purification of protoplasts the ratio improved to 69:1 in one case and 56:1 in another. Remaining vacuoles were probably lost during DEAE-Dextran lysis.

9.4.2 Micrographs and fluorescence

The photomicrographs in Figs 9.5 to 9.10 were prepared with a Leitz Orthomat Photomicroscope (black and white); a Zeiss Photomicroscope II (colour) with facilities for fluorescence (HBO 100 high pressure mercury vapour lamp; Zeiss exciter filter BG 12, 330-540 nm; Zeiss barrier filter 47, 460 nm); and a Jeol JEM 100CX electronmicroscope. Film was Kodak. ASA 80 and Ektachrome daylight ASA 400.

Under phase contrast the tonoplast is strongly refringent (Fig. 9.8.A). In some manner birefringence relates to size, the smaller vacuoles and protoplasts both being refringent. A small percentage of such entities were not refringent (Fig. 9.6.C) even though accumulating Neutral Red which indicated a pH gradient across the membrane.

Certain protoplasts appeared to lyse naturally about a contracted vacuole but this structure neither excluded Evans Blue nor accumulated Neutral Red. Though the vacuole appeared intact pH equilibrium had occurred; presumably solute efflux causes this decrease in volume. Similarly while some protoplasts lysed (with acid or hypotonic media) to only plastids and membranes, others collapsed about a resistant sphere which stained darkly with Neutral Red (not by accumulation but by fixation). This is attributed to a vacuolar matrix appearing *in vitro* by some condensation or precipitation.

Larkin (1976) developed the use of fluorescein di-acetate (FDA) to assess viability of protoplasts. Fluorescence depends upon permeation of the substrate; esterase activity in the material; and temporary trapping of liberated fluorescein behind a membrane. It also depends upon pH, as fluorescein is colourless and non-fluorescing below pH 4 and fluorescent above pH 4.5 (DeMent in Weast, 1976). Using this method it was evident (Fig. 9.8.B) that a layer of cytoplasm always separated the tonoplast and plasmalemma and that the vacuolar sap of normal protoplasts was below pH 4.

Using other fluorescent indicators it was possible to distinguish those cells with atypical pH. For instance, protoplasts and vacuoles in Erythrosine B (fluorescent above pH 4.5) showed strong fluorescence of the cytoplasm but not of the vacuole. Examining debris taken out of

a Sephadex cleaning column (see Para. 10.2) "vacuoles" within collapsed protoplasts were shown to fluoresce strongly, indicating that these had equilibrated towards the pH of the medium (pH 5.5). Magdala Red (Fig. 9.9.C), which fluoresces above pH 4.0, was also used as a dye to discriminate intact acidic vacuoles (purple colour in bright field illumination) from the fluorescent cytoplasm and cell sap of higher pH. Fig. 9.10.B shows debris from a Sephadex column in both Neutral Red and FDA which appear as complementary stains for the vacuole, the former being positive and the latter negative.

Some of these results were obtained with protoplasts stored for 16 days at 4°, indicating that the pH gradient was being maintained over such a period.

Of these dyes Neutral Red was the simplest to use and FDA gave the most rapid and clearest result of the fluorescent indicators. Admon et al. (1980) used these two dyes to assess the purity of isolated vacuoles. As fluorescein must concentrate behind a diffusion barrier and is only a negative test for vacuoles, Neutral Red is still the superior dye (Fig. 9.6.D). Indicators such as Acridine (green fluorescence below pH 4.9), though untested, might be developed as positive tests of the vacuolar sap. Indeed fluorescent indicators could find application in revealing gradations of pH in cells, thereby to permit cell sorting.

9.4.3 Electronmicrographs

Glutaraldehyde 2.5% was added to a protoplast suspension (from berries cv. Muscat) for 30 min at 4°, centrifuged 20g for 10 min, the supernatant discarded, the pellet rinsed once in PM and finally added to liquid 2% agar in a small, warm Petri dish. The material was post-fixed in 1% buffered osmium tetroxide for 1 h at 4°, rinsed three times with buffer, and examined in an electron microscope.

The electronmicrographs (Fig. 9.11) are dominated by electron-dense concentrations presumed to be condensed phenolics. Aside from the knife marks on the sections, a certain texture is evident in these concentrations which generally lie against the tonoplast. It is evident that this material can redistribute during fixation.

The ability of the tonoplast to form vesicles has been mentioned. Also, Morris and Thain (1980b) calculated the surface area of the plasmalemma in a protoplast to be only 60% of that same cell. Under the light microscope one often observed a line of vesicles or membrane folds at the limit of the clumped cytoplasm, between the plasmalemma and tonoplast. This was conveniently termed a "necklace effect". Some membrane apparently in-folds or breaks off as small vesicles to produce this effect. Such membrane tends to be "relaxed" i.e. it is not refringent. Fig. 9.11.A indicates not specifically the "necklace effect" but as least the formation of such vesicles or membrane folds which are attributed to *in vitro* manipulation of these cells. In a second micrograph (Fig. 9.11.C) this membrane is put into relief by the electron dense material in a manner consistent with endocytosis.

Finally the spatial relationship between the two cytoplasmic membranes is evident in Fig. 9.11.B. Some authors (Para. 5.8) have speculated upon the consequence of two membranes approaching in this manner, separated nevertheless by ground cytoplasm with few mitochondria, no plastids, but with cyclosis evident under the light microscope. But this "proximity" does not substantially alter the relationship between membrane transport and diffusion in the cytoplasm anticipated for less-vacuolated cells. For instance, the elements of solute transfer would be on the following scales : the solute molecule, several nm; the membranes; c. 5 nm each; the cytoplasm, c. 400 nm.

Fig. 9.5.A: The action of cellulase upon excised skin of a berry cv. Muscat from the hypodermal face toward the cuticle. Cells are highly plasmolysed. Protoplasts (arrow) are being released from the epidermal layer to leave a bare cuticle (diamond) with imprints of cells.

Fig. 9.5.B: Protoplasts from grape pericarp. Note the polar cytoplasm about a large vacuole, a distribution similar to the localized cytoplasm in the tissue cells (but unlike mesophyll protoplasts). There is a large range of size in cells and protoplasts. Cell and wall debris is evident at top right.

Fig. 9.5.C: Protoplasts kept in PM at 4° for 16 days. One large protoplast contains a raphide of potassium bitartrate (arrow).

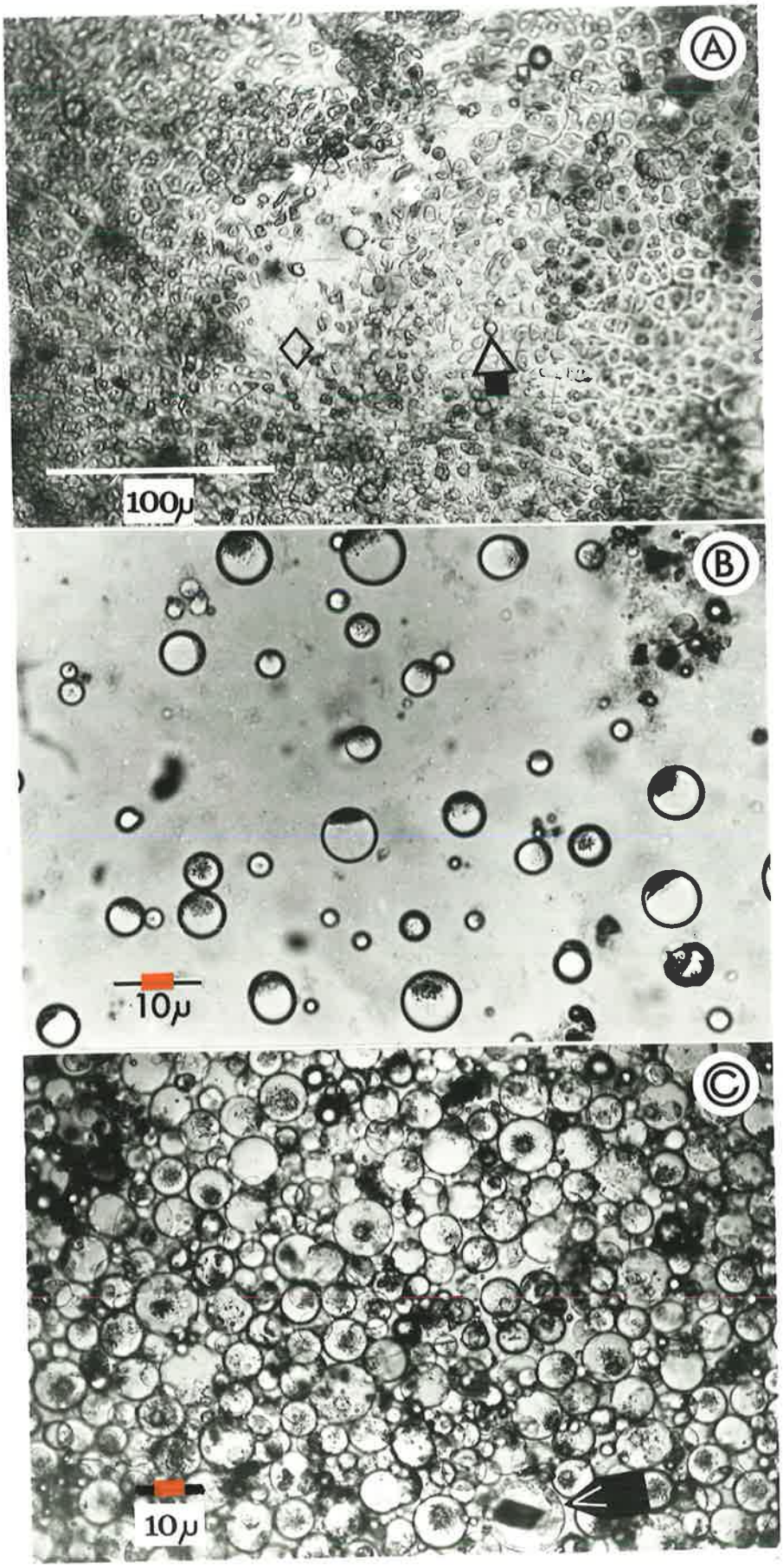

A
100μ
B
10μ
C
10μ

Fig. 9.6.A & B: Effect of Con A ex Jack bean upon protoplasts in medium after 30 min (Table 9.5).

A = Blank : no additives

B = Treatment 3 : 0.75 mg ml^{-1} Con A. Note the agglutination of protoplasts.

Fig. 9.6.C: Protoplasts under phase contrast showing typical birefringence. As occasionally observed, one large protoplast (marked) is not refractile.

Fig. 9.6.D: An isolated but "non-discrete" vacuole accumulating Neutral Red indicative of an acidic sap and functional membrane. The external debris stains a deeper crimson.

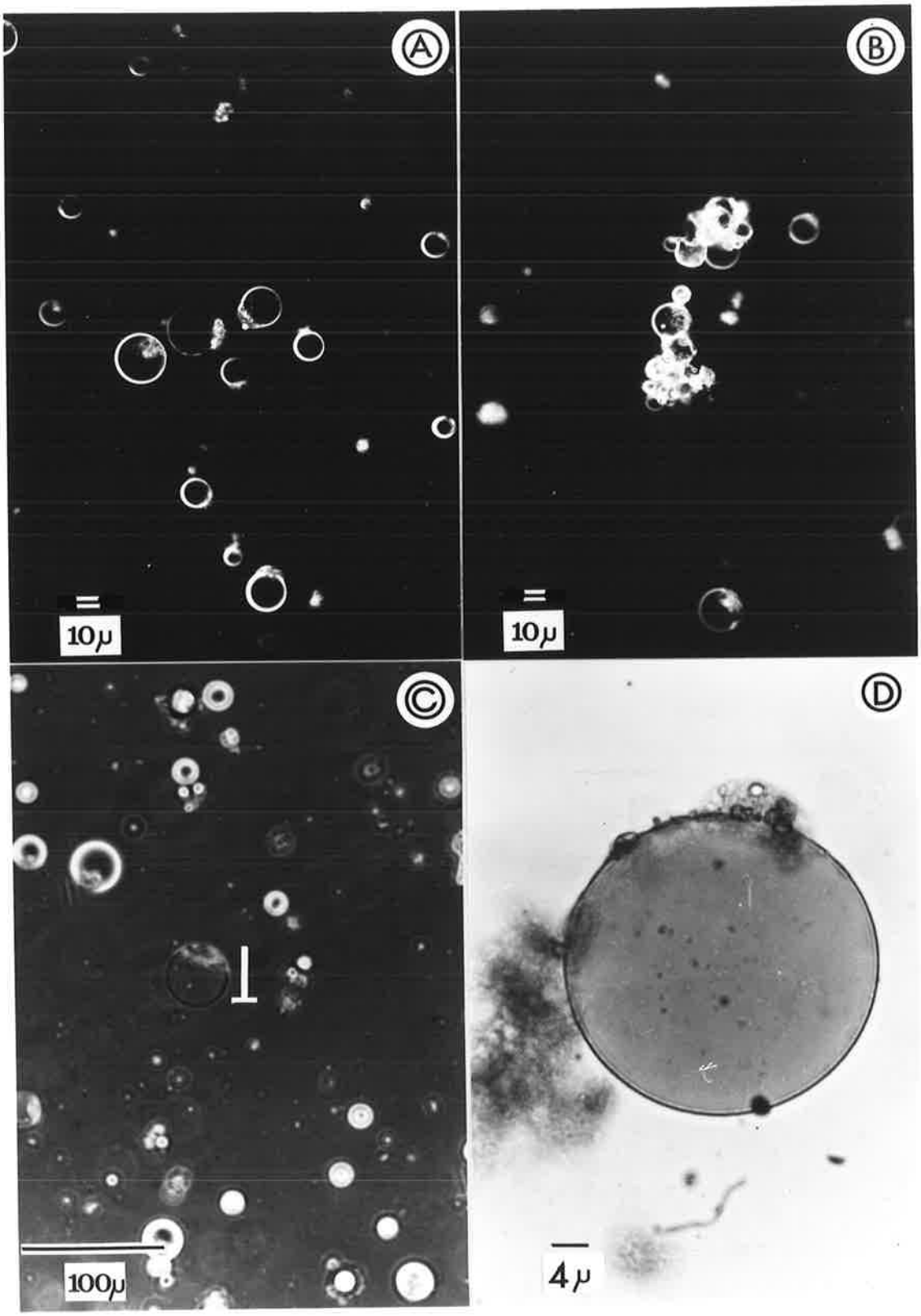
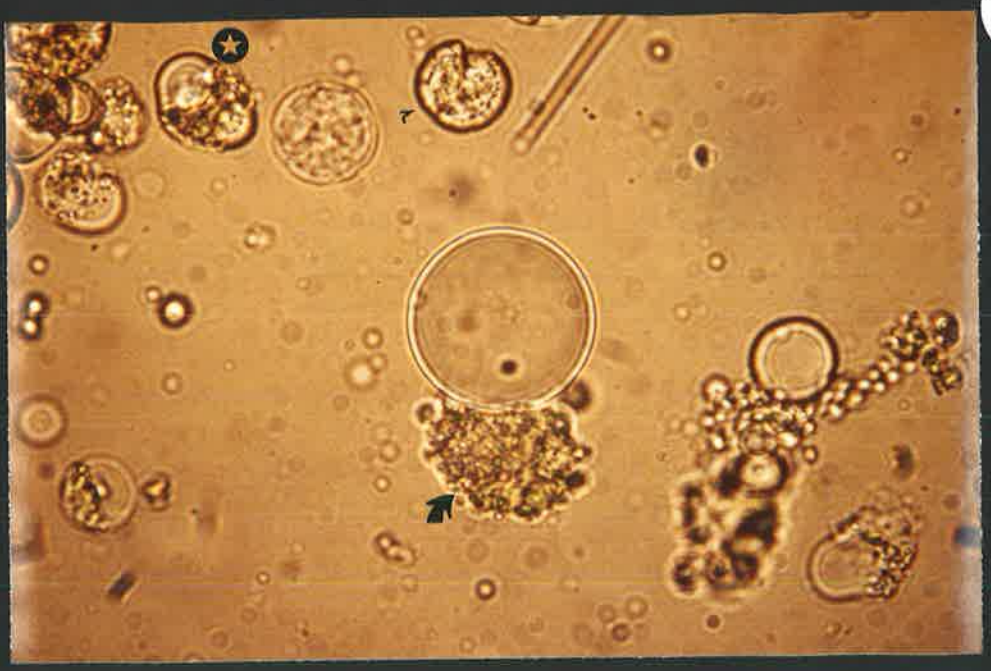
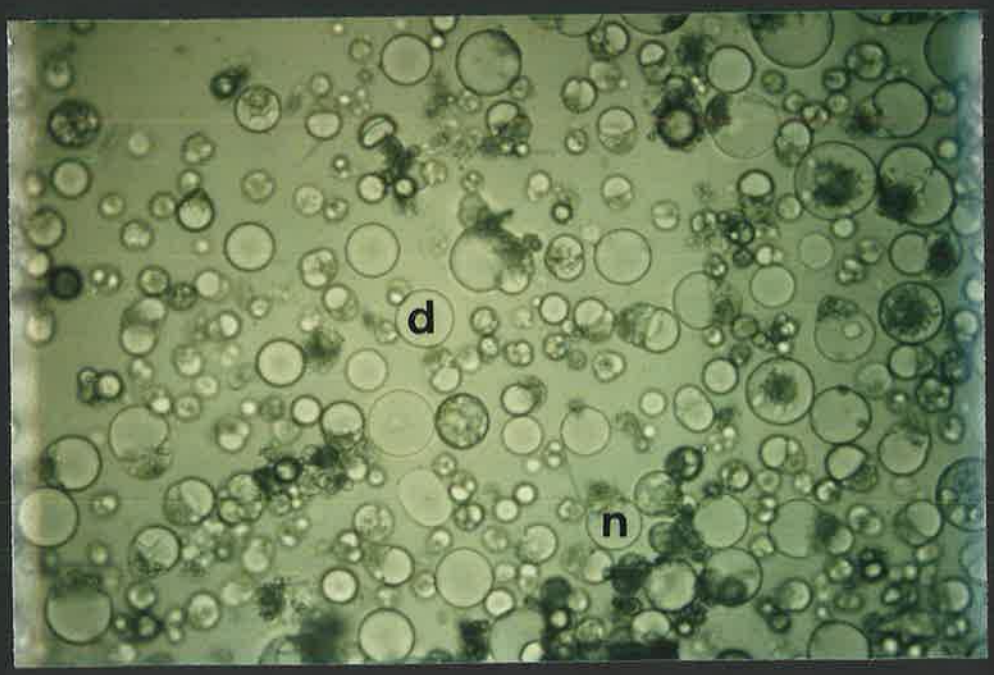

A
10μ
B
10μ
C
100μ
D
4μ

Fig: 9.7.A: Lysis of a protoplast induced by DEAE-Dextran. As the vacuole is liberated cytoplasmic material is released into the medium or substantially trapped within the crumpled plasmalemma (arrow). Smaller protoplasts are judged about to lyse (☆). A single protoplast containing two large vacuoles is indicated (✪). x 710

Fig. 9.7.B: Lysis of protoplasts. Some vacuoles are "discrete" (d) others "non-discrete" (n) with cytoplasmic debris still attached. x 110

Fig. 9.7.C: Isolated vacuoles from protoplasts of different sizes. External contamination by cytoplasmic material, plastids, etc. is evident. x 710




d
n

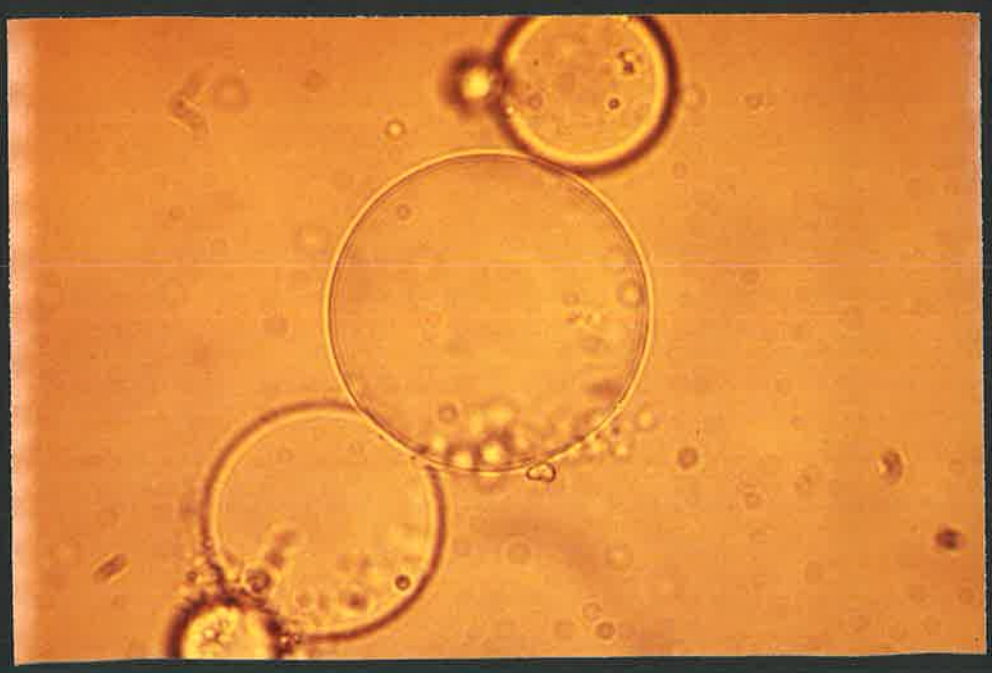


Fig.9.8.A: Protoplast viewed under phase contrast to reveal the refringent tonoplast, the less refringent plasmalemma (P) and the clumped cytoplasm. x 1125

Fig. 9.8.B: Protoplast in FDA (Sigma : stock solution 0.5% in acetone) viewed by fluorescence. The cytoplasm is active, including an entire, thin layer between the tonoplast and plasmalemma. A weak area in the fluorescence is the nucleus (nu). The large central vacuole absorbs light and does not fluoresce though fluorescein probably penetrates the tonoplast as it evidently leaks through the plasmalemma.

x 710, oil

Fig. 9.8.C: Protoplast viewed under weak bright-field illumination (green filter) such that the autofluorescence of chlorophyll is evident. x 710, oil

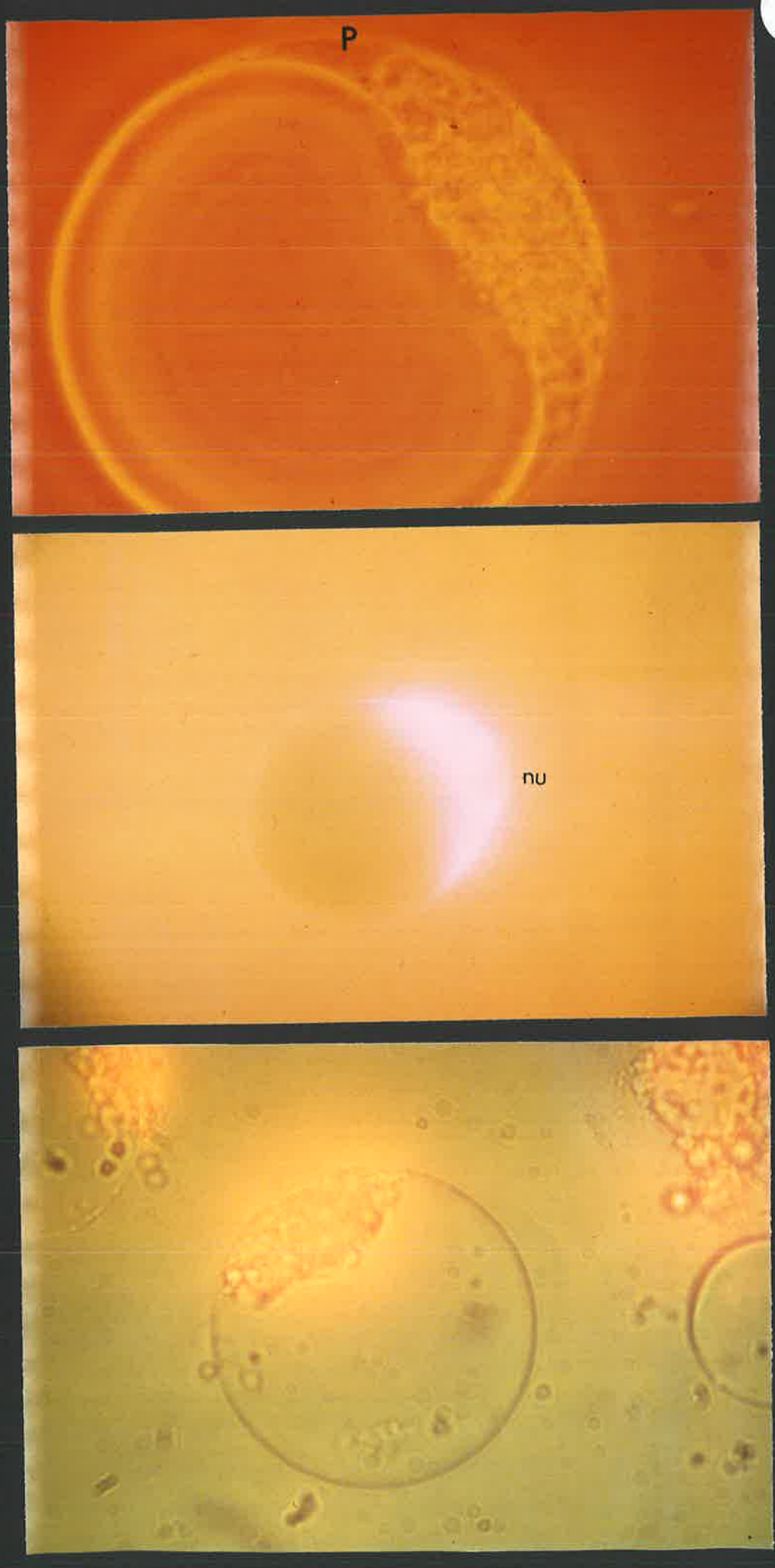
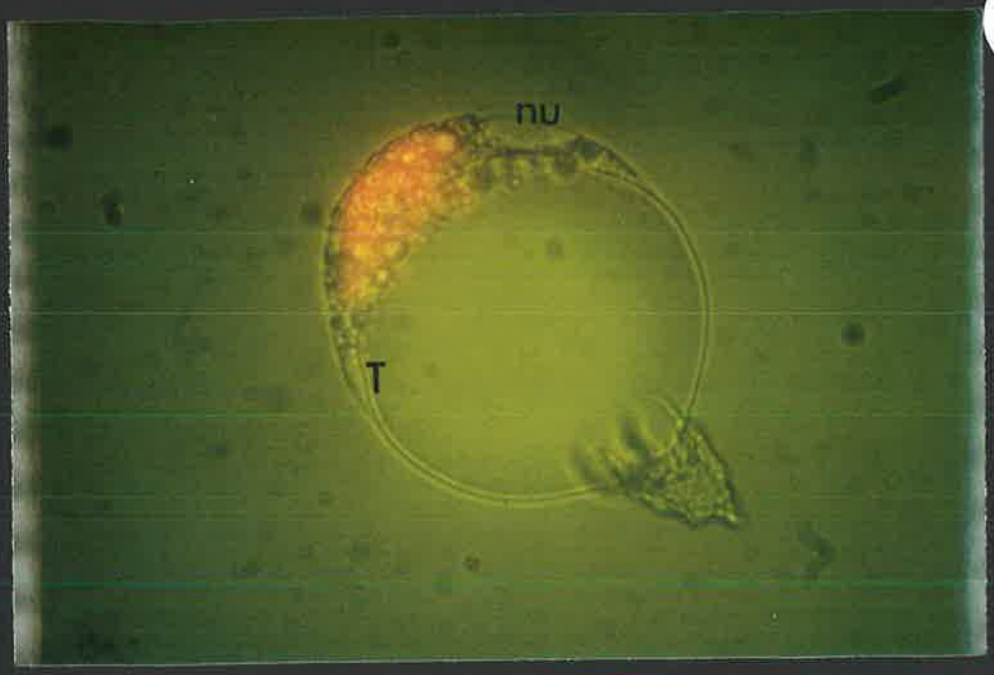

P
nu

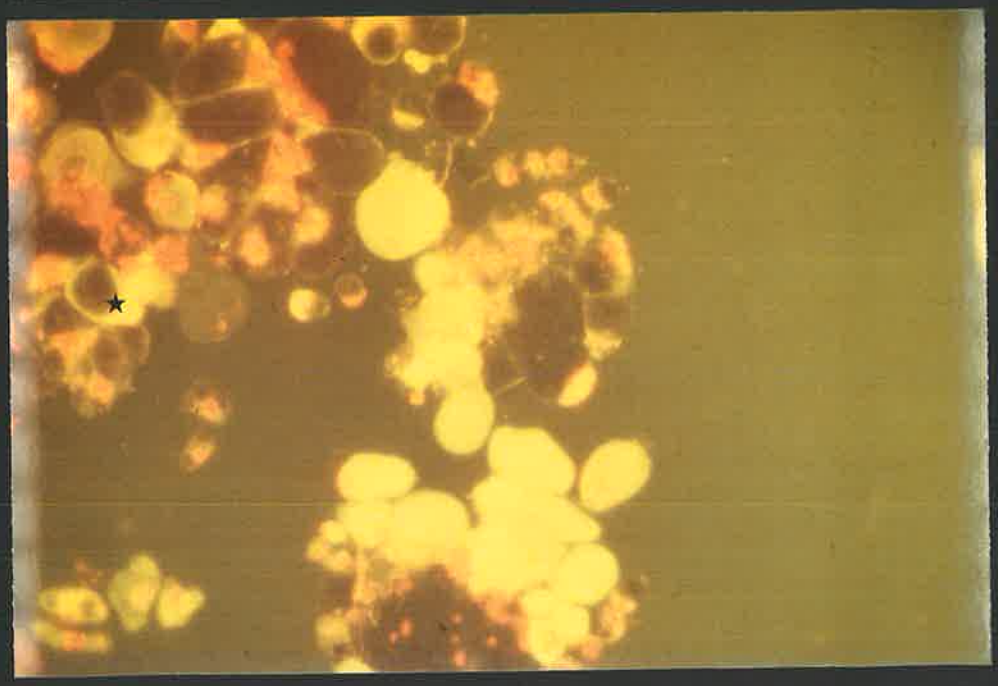
Fig. 9.9.A: Autofluorescence of chlorophyll in a protoplast superimposing upon weak bright-field illumination. The plasmalemma is intact though adhering to the coverslip. Note the tonoplast (T) and the large nucleus (nu) observed in some protoplasts only.

x 710, oil

Fig. 9.9.B: Debris from macerated tissue off a cleaning column stained with Neutral Red (stock : 1%aq.) and FDA (stock : 0.5% in acetone) and viewed by fluorescence. Intact vacuoles within protoplasts have accumulated Neutral Red and absorb scattered light. Strong fluorescence about these same vacuoles indicates an intact active cytoplasm (*); weak fluorescence with chlorophyll (red) dominating indicates degenerate cytoplasm. Protoplasts showing complete fluorescence are evidently above pH 4.5, no doubt as a result of the medium (pH 5.5), though the membranes retain fluorescein. Lack of absorbance in the same indicates no, or little, accumulation of Neutral Red, a process dependent upon a pH gradient. x 180

Fig. 9.9.C: Fluorescence by protoplasts and vacuoles in Magdala Red (Stock : 0.5%aq.). After 10 min, intact acidic vacuoles absorb light (pH below 4.0); cytoplasm fluoresces. Differences in intensity and rate of colouring were evident within both protoplast and vacuole populations. x 110


nu
T


★

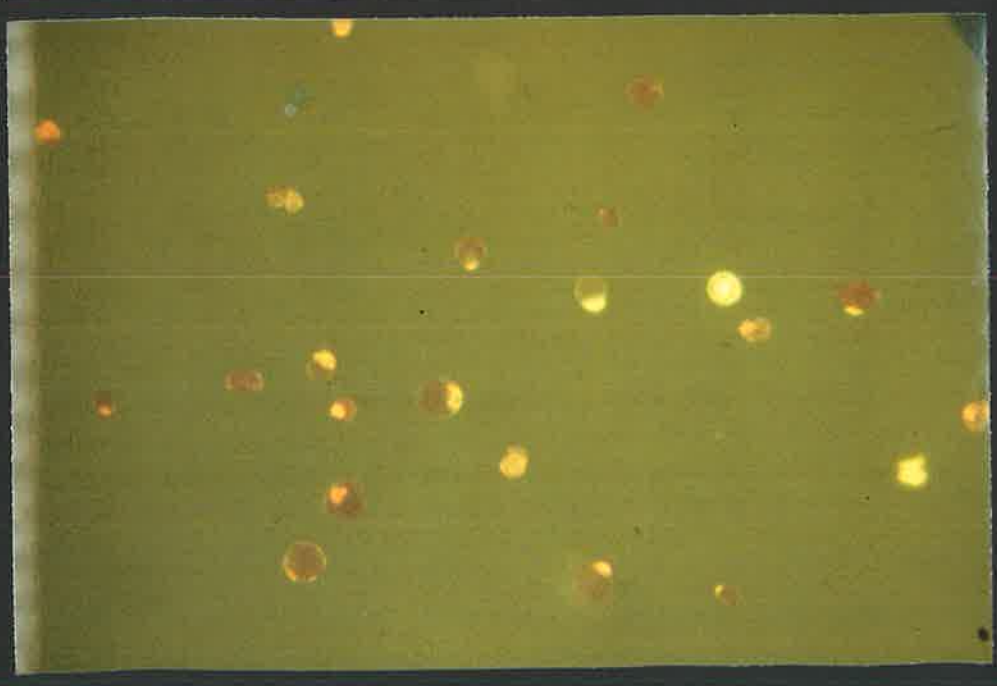
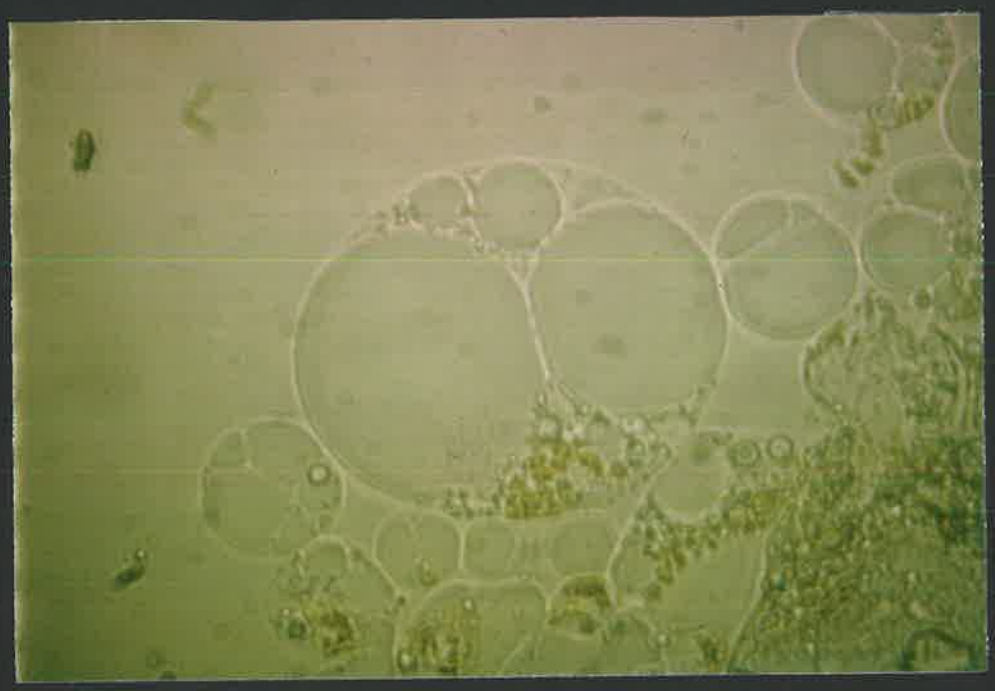


Fig. 9.10.A: Cells and protoplasts, removed from an incubation in cellulase, left under the heat of the microscope without coverslip. Though these multivacuolar cells are artefactual they demonstrate the dynamic property of the tonoplast. Multivacuolar protoplasts have been kept for 16 days at 4°C as have "normal" protoplasts (Para. 9.3.2). x 710

Fig. 9.10.B: Coarse Sephadex beads after passing a crude protoplast suspension through the column. Note the debris collected in the interstitial space, the frequency of cells and the absence of protoplasts. x 110



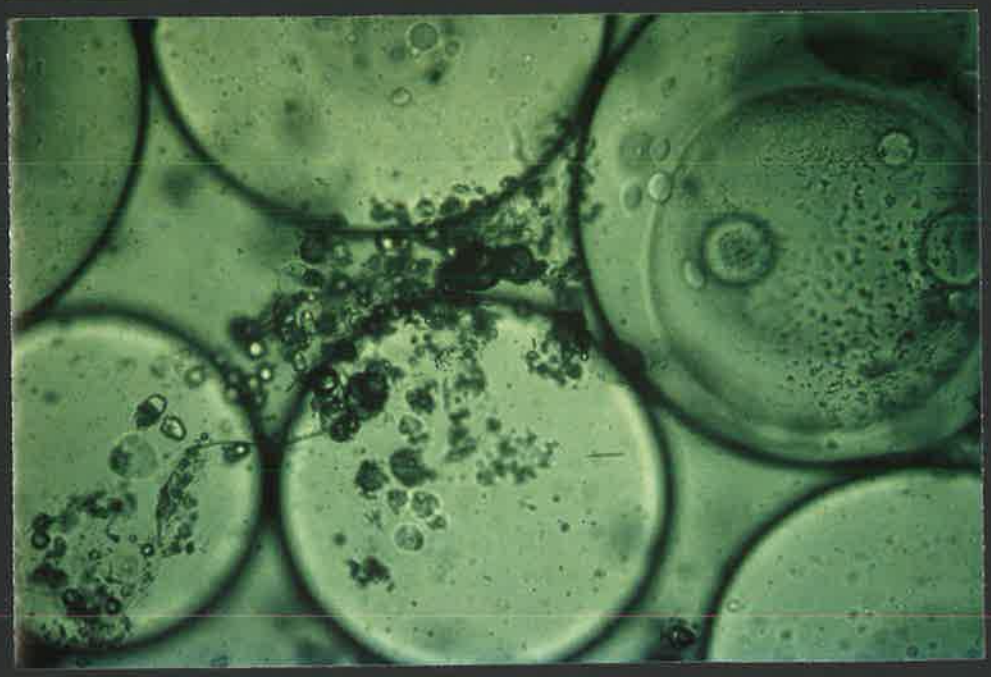
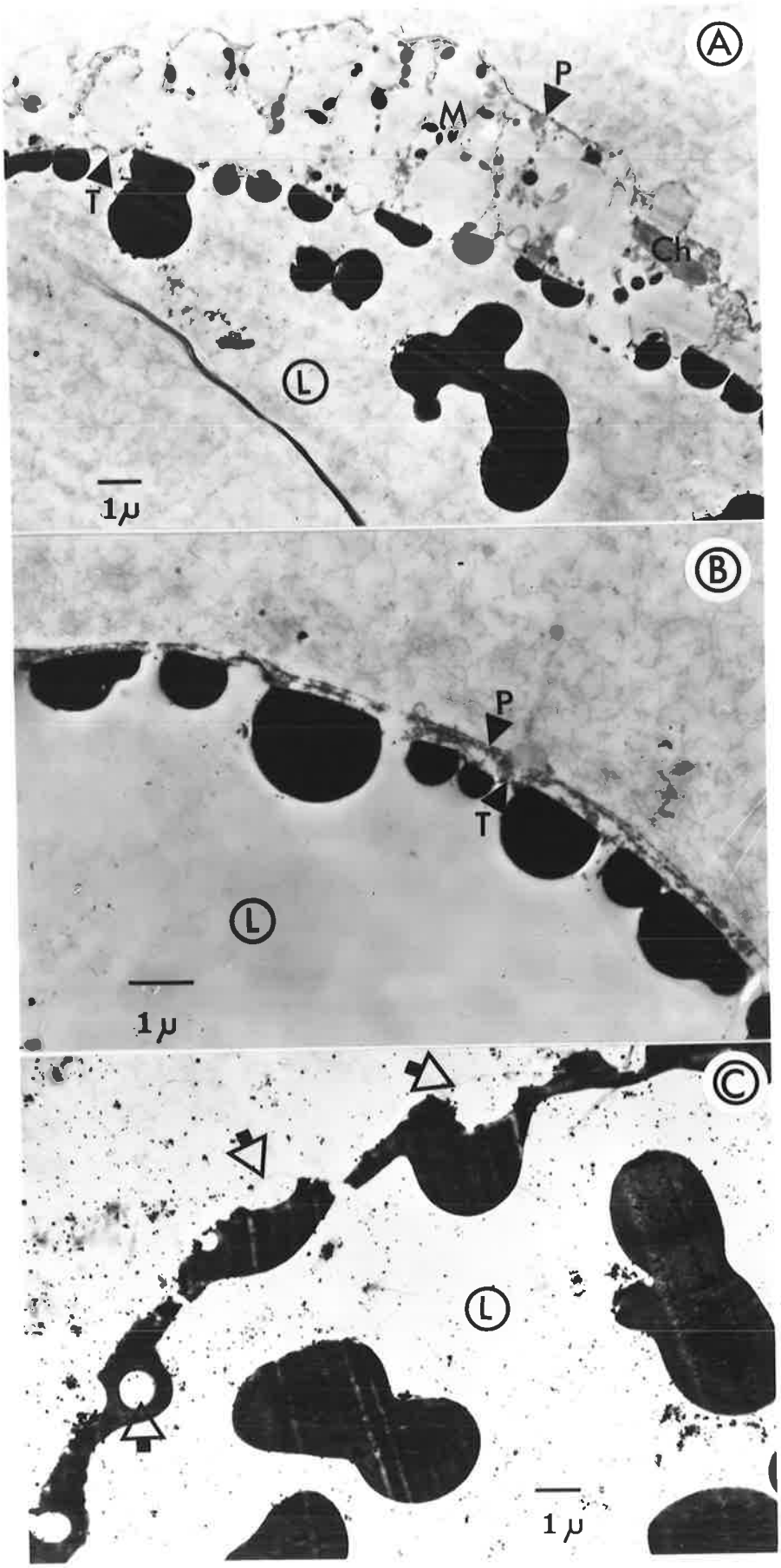


Fig. 9.11: Electron micrographs of protoplasts from grape pericarp of a berry at veraison cv. Muscat; fixed in glutaraldehyde, post-fixed in osmium. The cells are highly vacuolated with a predominant lumen (L) of the vacuole. The plasmalemma (P) and tonoplast (T) are evident.

9.11A: Membrane folds or vesicles - characteristic of some protoplasts - trapping chloroplasts (Ch), mitochondria (M) and ground cytoplasm. Dark osmiophilic material is presumed to be phenolic; different forms are observed in various micrographs - sometimes globular, other times dispersed - but normally associated with the membrane. The reactivity of phenolics predisposes to artefacts and leakage during fixation.

9.11B: The tonoplast appressed against the plasmalemma with a layer of ground cytoplasm between containing occasional mitochondria.

9.11C: The osmiophilic material places in relief vesicles or membrane folds associated with the tonoplast rather than the plasmalemma.


A
P
M
T
Ch
L
1μ
B
P
T
L
1μ
C
L
1μ

CHAPTER 10: PURIFICATION OF PROTOPLASTS AND VACUOLES

PURIFICATION OF PROTOPLASTS AND VACUOLES

10.1 Introduction

The purification of protoplasts is qualified by the use to which the preparation is to be put. For instance, substantial absence of "other material" as assessed under the microscope may suffice if the preparation is to be lysed, although the interaction of ions in such lysis is ill-defined. Biochemical criteria of purity will require removal of cellulytic enzymes and factors released from the apoplasm or from broken cells, which may affect metabolic studies. A more exacting criterion is to execute purification without loss of protoplasts of a sub-group in the population, or conversely, to retain protoplasts of only a particular cell-type.

The purification of protoplasts derived from grape pericarp in the present experiment proved difficult. It was concluded that this was due in part to the autocatalytic deterioration consequent upon cell degradation due probably to the high phenolic activity.

10.2 Filtration

One observed a diameter range of protoplast from 4 μm to 70 μm, an apparent range of density within and between preparations, and the interaction of osmotic adjustment at membranes with density and size. Contamination as seen under the microscope appeared to be from plastids, released protoplasm, membrane pieces, individual cells and cell wall material. Also contamination by components of the digestion medium could be expected. The debris and cells tended to clump, especially when abundant. The fragility of protoplasts and their tendency to clump when concentrated under force, as in a pellet or at an interface, indicated limitations to centrifugation methodology.

Nylon mesh (poresize 110 μm) and silk bolting cloth (80-90 μm) when folded in double in an open funnel removed large aggregates of cells or debris, but not single cells with intact walls nor even clumps of small cells. Reducing the pore size retained many of these cells and likewise the large protoplasts. Miracloth (Chicopee Mills Inc. N.Y.) has a mat of fibres presenting irregular and deep pores of about 40 μm (in plan only). The surface properties are such that cell debris was preferentially retained and better purification resulted but again most large protoplasts were lost.

Such filters were rapidly overloaded with debris, whereas if the filtration were through a deep medium this overloading would be less acute. Wellburn and Wellburn (1971) used Sephadex to purify etioplasts. The bead diameter, and not the pore size within the beads, defines a macro-sieve. With spheres of radius R the smallest interstitial radius r is 15%R. In a column where packing is not perfect this minimum is seldom attained. Sephadex beads have advantages over glass beads in that reasonable flow rates are maintained, and surface properties are not deleterious.

Coarse grade Sephadex G50 (dry particle diameter 100-300 μm) was prepared with normal swelling and with particular attention to decant off the fines. It was autoclaved and stored at 4°C in 0.02% sodium azide. A 20 x 10 column was poured over a nylon grid (0.5mm mesh) held in place by a cotton mesh (1.0mm mesh). The column was thoroughly rinsed with water then medium. A protoplast or vacuole preparation was applied to the column by pipette with disturbance of the upper layers. Initially the material flowed rapidly at about 4 ml min^{-1}. This slowed as overloading commenced, whereupon more medium was added, with gentle agitation, to rinse more protoplasts out of the column. The Sephadex was discarded.

An incubate was purified in batches if necessary, depending less upon the total volume and more upon the amount of debris. Using a 25 x 25mm column the capacity was markedly increased. The procedure was surprisingly effective in removing all large and much fine debris without retaining too many protoplasts, as indicated in Fig. 9.10.B. In particular it was the only technique that separated large and small protoplasts away from large amounts of debris. Large protoplasts and

vacuoles passed through the column as rapidly as small ones. The flow might possibly be characterised as a series of major and minor streams with the irregular shaped debris becoming trapped, and in turn trapping more, while the spherical protoplasts transfer readily to other streams.

It was important to recognise the onset of overloading; pressure could not be applied to assist flow without causing protoplasts to break. In agitating the column care was taken not to disturb the lower 10mm which could force beads through the support.

10.3 Phase Distribution

Kanai and Edwards (1973) purified protoplasts at the interface of two phases resulting from a mixture of polymers in medium. Albertson (1971) described the conditions for such phase formation. This approach was assessed for the separation of vacuoles from protoplasts remaining after an incomplete, polycation-induced lysis.

The polymers used were Dextran T500, Dextran sulphate, Ficoll 400 (all Pharmacia) and PEG 4000. The vacuoles and protoplasts were in 0.8 M mannitol with 10 mM HEPES pH 7.3. 8% w/w stock polymer solutions in 0.52 M mannitol, 6 mM HEPES pH 7.3, were used to prepare the systems:

1. PEG 4000:Ficoll 400 :vacuole suspension 8:10:10
2. PEG 4000:Dextran SO_4 :vacuole suspension 8:10:10
3. PEG 4000:Dextran T500:vacuole suspension 8:10:10
4. PEG 4000:Dextran T500:Dextran SO_4:vacuole suspension 8: 7: 1:10

Other configurations using 0.8 M mannitol or sucrose were abandoned as the phases formed not at all or too slowly.

Each tube was gently inverted several times then left to settle for 30 min at 4°C. Systems 1 and 2 formed two phases in suspension without any separation of cellular material. Systems 3 and 4 formed two phases which separated incompletely in 30 min and did not resolve after several hours. Chloroplasts were in the bottom of system 3 (essentially the Dextran T500 phase) but in system 4 were also at the interface - indeed, they were concentrated upon the surface of droplets in suspension. Protoplasts and vacuoles did not separate from one another.

Kanai and Edwards' system was reproduced, namely, in 6 ml final volume of PM : 5.5% w/v PEG 6000, 10% w/v Dextran T40 and a protoplast suspension. At 4°C the phases separated with protoplasts at the interface and no cleaner than the original suspension. The approach was abandoned. This has proved to be an unwise decision as the technique has since been widely cited (review : Walter, 1978).

10.4 Centrifugation

10.4.1 Materials

Techniques so far discussed had the object of removing particulate matter while soluble contaminants are only diluted. The systems which follow, based upon centrifugation, were constructed with the object of moving protoplasts or vacuoles out of the original incubation medium, through a washing step and, ideally, concentrating them. About 50 configurations of gradients were assessed of which only some are reported.

The following materials were used for density-gradient centrifugation : Ficoll 400 (Pharmacia), Metrizamide (Nyegaard and Co. A/S, Oslo), Ludox HS40 (Allied Petrochemical Pty. Ltd., Melbourne) and Percoll (Pharmacia). Fig. 10.1 indicates some colligative properties of these materials, with sucrose included for reference.

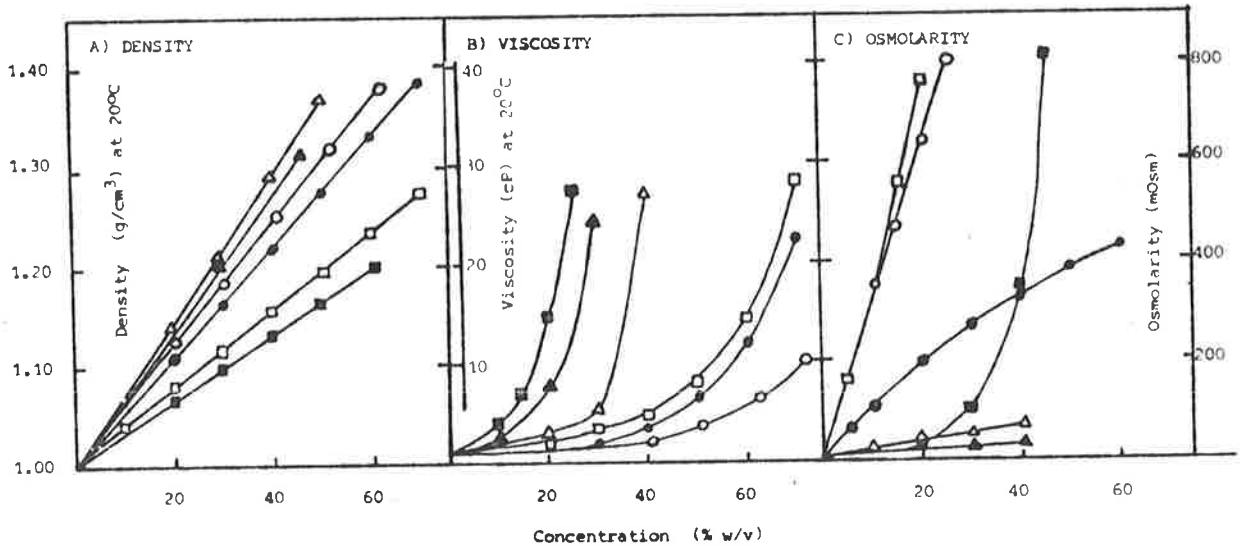


Fig.10.1 Properties of density-gradient media. The increases in (A) Density, (B) Viscosity and (C) Osmolarity with concentration are shown. The data are for sucrose □, Ficoll ■, sodium metrizoate ○, metrizamide ●, Ludox HS △ and Percoll ▲. Curves for Percoll and Ludox HS derive from silica % w/v, Percoll being 20% silica & Ludox 30% silica. (from Rickwood 1978)

Ficoll, a synthetic copolymer of sucrose and epichlorohydrin, has a MW of about 400,000 daltons. Its viscosity and osmolarity increase rapidly above 15% concentration. Metrizamide, a tri-iodinated benzamido-derivative of glucose with a 2-deoxy-D-glucose moiety, will form dense media of low viscosity but of appreciable osmolarity: a 40% w/v solution is c. 350 mOsmolal. It cannot be autoclaved and is unstable in U.V. light, liberating iodine. It can bind reversibly to proteins (Hinton et al., 1974) and has been shown to interfere with sugar uptake (Doll et al., 1979). Ludox HS40 is a silica sol, 40% silica, which was cleaned by stirring in Dowex 50W-X8 AR cation exchange resin (H^+-form) and activated carbon granules, then filtering off the silica sol - a tedious and inefficient step. Percoll is also a silica sol, 20% silica, modified by addition of polyvinylpyrrolidone which coats the silica to render it non-toxic. It has low osmolarity, low viscosity, will spontaneously form a gradient during ultracentrifugation, and now supersedes Ludox.

The theory of centrifugation is treated in many texts (e.g. Rickwood, 1978) wherein Stokes' equation defining the frictional coefficient of a sphere is transformed to indicate that the sedimentation rate of a sphere in a fluid of given viscosity is proportional to (i) the square of the radius (ii) the difference in densities between the particle and the medium and (iii) the applied gravitational field. The sedimentation rate decreases as the viscosity of the medium increases, and is zero when the density of the particle is equal to that of the surrounding medium (as observed in isopycnic centrifugation).

In the following section relative centrifugal forces are calculated as R_{av}, the midpoint of the tube. Ultracentrifugation was with 13 ml cellulose nitrate tubes in a Beckman L2-65B machine. Low speed centrifugations were in 10 ml glass tubes in an MSE Multex.

10.4.2 Differential pelleting : Ficoll

When a crude protoplast suspension was left to settle (1g) at 4°C in a test tube for 40 min the pellet contained protoplasts, discrete cells, clumped cells and debris. The supernatant contained plastids, nuclei, cytoplasmic particles, membrane pieces and the few "light" vesicles thought to arise by resealing of membrane. It also contained about 40% of the protoplasts, especially smaller ones. Centrifugation

at 250g for 20 sec pelleted most of these protoplasts. If the supernatant was replaced with fresh medium, the pellet gently resuspended, and the process repeated, a substantial amount of the original medium and fine debris was removed.

When a protoplast suspension was layered over a more dense medium, e.g. 20% w/v Ficoll in PM, and centrifuged at 2500g for 15 min, much of the larger debris pelleted through the Ficoll while the protoplasts remained at the interface and much of the fine debris was still in the less dense phase (Fig. 10.2). The position of the protoplasts would be determined by their buoyant density and size and by their surface properties; particles tended to remain at an interface. This "cushioned" centrifugation proved satisfactory for many preparations providing these contained little debris, otherwise protoplasts were apparently pulled through the interface into the pellet. By gently rotating the layers prior to centrifugation the interface was made diffuse and its capacity increased.

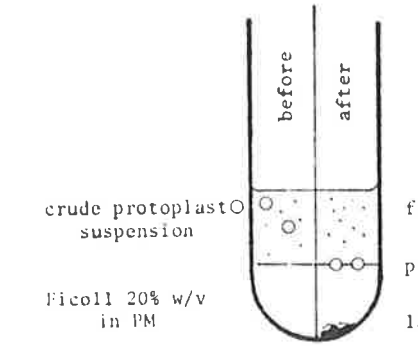


Fig. 10.2: A single step sedimentation : 2500g for 15 min

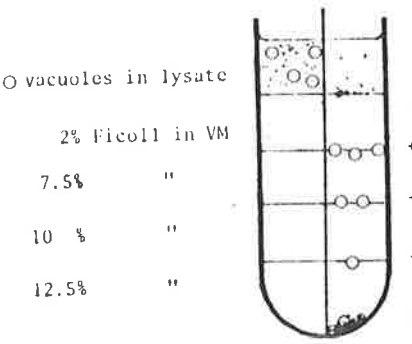


Fig. 10.3: Purification of vacuoles by ultracentrifugation in a Ficoll step gradient: 8000g for 10 min, Rotor SW27.1

Quite often, and unpredictably, the protoplasts moved through the 20% Ficoll into the pellet where, at 2500g, they rapidly deteriorated. When the Ficoll was purified by dialysis the problem persisted. Either these protoplasts were adjusting osmotically, becoming more dense, and sedimenting; or their surface properties were such that the interface was not favourable. When Ficoll was increased to 25% or 30% some proto-

plasts still pelleted while the interface retained more debris. Similar problems were encountered using two-step Ficoll gradients and various centrifugal forces.

A four-step gradient of 12.5% Ficoll successively overlaid with 10, 7.5 and 2% w/w Ficoll each in VM, with lysed protoplasts layered on top, was spun at 8000*g* for 10 min (Beckman rotor SW27.1, 10,000 rpm). This purified vacuoles in the desired manner (Fig. 10.3). Cell debris and broken protoplasts pelleted, vacuoles and persistent protoplasts collected at the interfaces away from the lysing medium and fine debris. The faster sedimentation of small vacuoles may indicate (i) greater density relative to other vacuoles (ii) response to the osmotic gradient presented by Ficoll, or (iii) a lack of attraction to interfaces.

There were four disadvantages to this gradient:

- no separation of protoplasts and vacuoles was achieved.
- the cleaned vacuoles recovered from the interfaces with a Pasteur pipette contained sufficient Ficoll that the vacuoles would not subsequently sediment at 1*g*. These could not be concentrated at low speed without loss.
- ultracentrifugation was time-consuming and subjected membrane to high hydrostatic and shearing forces.
- certain preparations yielded protoplasts and vacuoles which unpredictably moved through the Ficoll layers, even through 40% Ficoll in VM with only 10% recovery at the interface.

Ficoll was purified by dialysis for 24 h followed by determination of refraction to indicate the stock concentration. A Knauer Freezing-Point Depression micro-osmometer was used to assess the osmolality of Ficoll 400 (likewise mannitol and metrizamide).

Lysate was layered over an 8 ml continuous gradient, 2-20% Ficoll in VM, and spun at 8000*g* for 10 min (Fig. 10.4.). Vacuoles, protoplasts and cells were dispersed through the upper two-thirds of the gradient. The dense third contained much fine debris indicative of burst cells. Recovery was low.

As with protoplasts vacuoles would settle onto a Ficoll "cushion" (500*g* for 4 min) but where a step gradient was introduced no separation of vacuoles from cells or debris resulted.

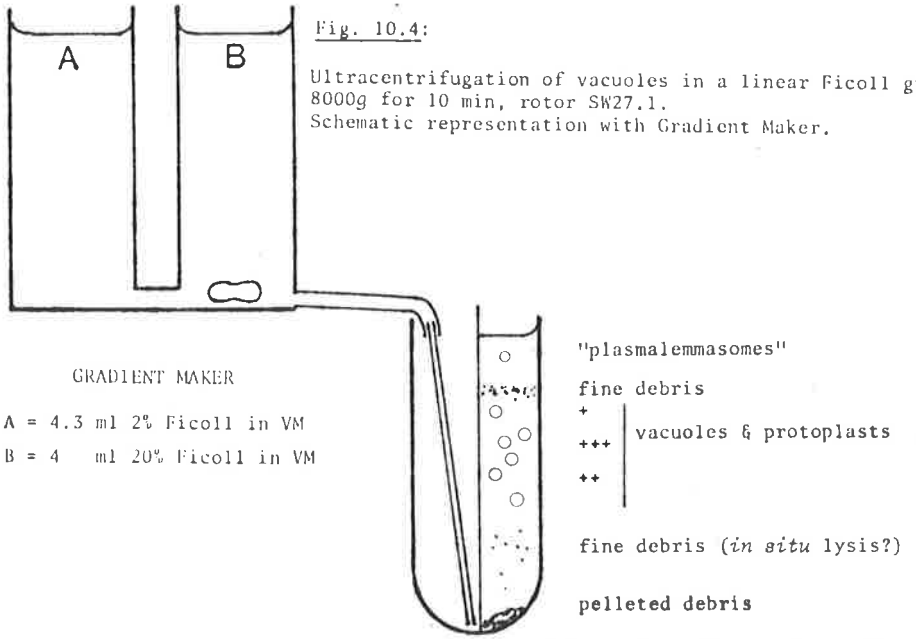


Fig. 10.4:

Ultracentrifugation of vacuoles in a linear Ficoll gradient: 8000*g* for 10 min, rotor SW27.1.
Schematic representation with Gradient Maker.

Vacuoles have been successfully purified by flotation through Ficoll step gradients: overlayering a 5% Ficoll suspension of vacuoles with 3.75, 2.5 then 0% (w/v) Ficoll in medium (300*g* for 20 min) (Boller and Kende, 1979). The flotation systems examined in the present work were not successful.

10.4.3 Metrizamide

Bench centrifugation (2500*g*, 15 min) of protoplasts over 20% w/v metrizamide in PM concentrated the protoplasts at the interface while the debris pelleted.

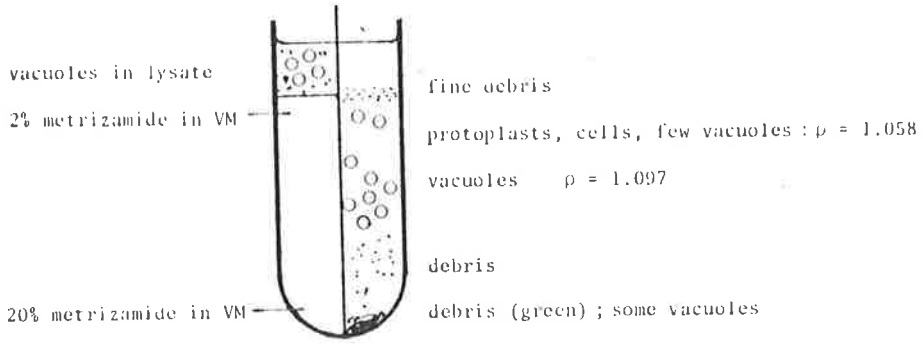
An 8 ml continuous linear gradient, 2-20% metrizamide in VM, with 2.8 ml of lysate overlaid (Fig. 10.5) was centrifuged at 8000*g* for 10 min (rotor SW27.1). This is a gradient of both density and osmolarity, 20% metrizamide adding c. 200 mOsmol to the 780 mOsmol of the medium. Fine debris remained in the top 5mm of the metrizamide; protoplasts, macerated cells and some vacuoles were diffuse in a volume (ρ = 1.058) below this; most vacuoles were at the middle third of the gradient (ρ = 1.091 to 1.097) and were clean; slight debris was observed down to the pellet which contained much debris.

The densities were determined by measuring the refraction of the relevant samples and calculating as follows:

VM is η = 1.3516 which is a mannitol solution of ρ = 1.0437 kg ℓ^{-1}. The $\Delta\eta$ attributable to metrizamide allows calculation of ρ attributable to metrizamide by the equation $\rho = a.\Delta\eta - b$ (where a = 3.350 and b = 3.462 at 20°C ; p.212 in Rickwood, 1978). The two components of density are assumed additive, such that for any given point, ρ gradient = ρ metrizamide + ρ mannitol.

These densities were taken as indicators, considering that the vacuoles were dispersed over a range and probably were not at their isopycnic positions. This was the first gradient in which vacuoles moved away from protoplasts and cells. Moreover these vacuoles subsequently compartmented U - ^{14}C-sucrose.

Fig. 10.5: Ultracentrifugation of vacuoles in a linear metrizamide gradient: 8000*g*, 10 min, rotor SW27.1



When the 20% metrizamide stock was prepared in only 600 mM mannitol (to reduce the osmotic gradient) the vacuoles were recovered over a smaller range in the middle of the gradient.

Attempts to use self-formed metrizamide gradients for isopycnic centrifugation failed. For instance, 4 ml of 40% metrizamide was overlayered with 4 ml of 5% metrizamide in 0.6 M mannitol; a glass rod was swirled once in the tube to disperse the interface; the tube was spun at 10,000*g* for 30 min (Rotor SW27.1) to preform a steep, linear gradient. But the appropriate media will only preform gradients in fixed angle rotors in the force-field of which a column is very short and sedimentation of density material is rapid. When the rotor stops the column reorientates to a long density gradient. The tube is then removed to a swing-out rotor, with the material loaded, for the separation step.

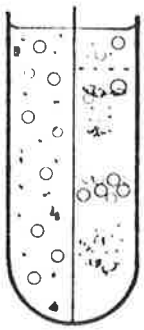
Alternatively a gradient may be generated with the material (to be separated) *in situ* in a fixed-angle rotor. As this would subject the vacuoles to yet higher forces for longer periods it was not tested. Also debris will pellet then tumble back into the gradient (somewhat) upon reorientation.

10.4.4 Ludox and Percoll

It was possible that metrizamide affected metabolism or membranes sensitive to osmotic and hydrostatic forces, whereas the silica gradient materials Ludox and Percoll would not; these materials have negligible osmolarity and form gradients spontaneously which can then be used at low speeds. Purified Ludox HS40, neutralized with HCl to pH 7.5, was however found to form a fine film in contact with a lysed protoplast suspension which contained the polyanion Dextran sulphate. It also was toxic to protoplasts and vacuoles, though it was noted that vacuoles moved ahead of protoplasts in Ludox gradients.

To establish their buoyant densities, 2 ml of vacuoles and protoplasts were added to 10 ml 65% Percoll in PM and spun in a fixed-angle rotor 65 at 40,000*g* 30 min (25K). A duplicate tube contained material prestained with Neutral Red to facilitate analysis. The distribution of material is indicated in Fig. 10.6. Small vacuoles banded distinctly at ρ = 1.082 and 1.075. Protoplasts and cells were distributed at the top of the gradient (ρ c.1·055). Some large vacuoles were at this density: these may represent a subpopulation or indicate lysis of some protoplasts after centrifugation, though such was not observed. Both accumulated Neutral Red and were osmotically active. In many gradients, including isopycnic centrifugations, the smaller vacuoles were at higher densities than the larger. In the supernatant was a band of unstained vesicles and cells which by their low contrast under phase and bright field were interpreted to have degenerate membranes. This isopycnic banding confirmed that such vesicles and cells, which form only a few per cent of the population, had equilibrated with the medium.

Fig. 10.6: Ultracentrifugation of protoplasts and vacuoles in 60% Percoll, in media, for an *in situ* self-generated gradient: 40,000g, 30 min, rotor 65



2 ml suspension + 10 ml Percoll in PM/VM

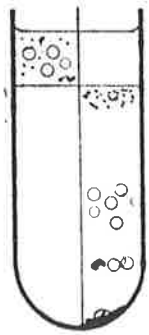
clear phase with vesicles and "light" cells

protoplasts and cells, clumped; some large vacuoles $\rho = 1.055$

distinct band of small vacuoles: tube I $\rho = 1.075$
tube II $\rho = 1.082$

debris

Fig. 10.7: Centrifugation of (i) protoplasts (ii) vacuoles in a linear Percoll gradient : 900g, 20 min



(i) 10% Percoll in 0.5 M mannitol
(ii) 10% Percoll in 0.7 M mannitol

(i) Protoplasts, (ii) vacuoles

clumps protoplasts and cells

(i) 50% Percoll in 0.5 M mannitol
(ii) 60% Percoll in 0.4 M mannitol

pellet

To avoid subjecting cell material to high force fields such a gradient (but with 50% Percoll in 0.5 M mannitol) was preformed in the ultra-centrifuge then a protoplast suspension layered on top and spun at 500g for 30 min. A clean suspension of protoplasts was obtained from the upper portion of the gradient. This was diluted with 5 volumes of PM and the protoplasts sedimented at 200g after 2 min. The supernatant, with most of the Percoll, was removed and the pellet immediately resuspended.

Percoll gradients were also constructed with a gradient maker (Fig. 10.7). For protoplasts the starting density was 10% Percoll and the finishing solution 50% Percoll (total including medium : ρ = 1.095) each in 0.5 M mannitol; for vacuoles, 10% Percoll in 0.7 M mannitol and 60% Percoll in 0.4 M mannitol respectively. With the suspension layered on top the columns were centrifuged at 900g for 20 min. Protoplasts and vacuoles were recovered in one diffuse band.

Media composed of Percoll with PM caused more clumping of material than Percoll with buffered mannitol. Also the lysing chemicals DEAE-Dextran and Dextran sulphate destabilize Percoll, producing a membrane gel at the interface. Vacuoles prepared at more than 1000g were "discrete", freed of any loosely attached cytoplasm or membrane

deriving from protoplast lysis. Percoll gradients did not separate protoplasts from vacuoles.

Percoll has a silica content 20% w/v and density 1.13 g cm^{-3}. If *in situ* separations upon self-forming gradients are desired, average starting density (including dilutions with medium and suspension) must be greater than the density of vacuoles. Otherwise some vesicles will be pelleted, and thereby broken, before the gradient has formed.

10.5 Discussion

The purification of grape skin protoplasts was neither simple nor always reproducible, given the unpredictable variability of (apparently) critical properties of the protoplasts. Protoplasts derived from fruit tissue appear more sensitive and variable than mesophyll protoplasts reported in the literature. Major compromises were necessary: speed and simplicity were obtained at the expense of incomplete removal of fine debris (e.g. Sephadex column purification : Section 10.2); higher purification was obtained at the expense of yield and longevity (e.g. metrizamide gradients : Fig. 10.5) and with introduction of gradient material.

Similar problems appeared when purifying vacuoles in which longevity declined with increasing manipulation.

Protein vacuoles of castor bean endosperm are particularly dense and were simply separated (Nishimura and Beevers, 1978). Saunders (1979) used a discontinuous Ficoll gradient (90,000*g*, 2 h) to purify vacuoles from tobacco, but grape vacuoles did not survive long runs. Boller and Kende (1979) were unable to separate persistent protoplasts from vacuoles but by flotation they not only removed debris but also recovered vacuoles at an interface i.e. in a concentrated form.

In conclusion, the use to be made of protoplasts and vacuoles will determine the choice of purification methods. Emphasis is to be placed upon obtaining high yields of good protoplasts in the first place. Thereafter successive sedimentaions under low *g* forces, filtration, or metrizamide gradients may be necessary. Silica sols tend to be unstable in some media and thereby affect cell viability. The use of density gradients is confused by the apparent tendency of

vacuoles to modify under the influence of density or hydrostatic and osmotic gradients. Nevertheless density gradients will be applicable in many cases for biochemical and membrane analyses. Vacuoles were recovered at ρ 1.09-1.10 in metrizamide and at about ρ 1.08 in Percoll; protoplasts were, surprisingly, less dense (cf. Saunders, 1979).

In subsequent chapters the particular method employed to purify protoplasts or vacuoles is indicated. As stated in the Protocols (Paras. 9.1.9 and 9.3.1) protoplasts were prepared for lysis simply by filtration, successive sedimentation at 1*g* or, where considerable debris was present, by use of the Sephadex system. Vacuoles were often used after simply diluting out the lysing reagents and spinning at 200*g* for 20 seconds. For more thorough purification the lysate was layered over a continuous gradient of 2%/20% metrizamide in VM but this markedly reduced longevity.

CHAPTER 11: MEMBRANE TRANSPORT STUDIES WITH ISOLATED PROTOPLASTS AND VACUOLES

MEMBRANE TRANSPORT STUDIES WITH ISOLATED PROTOPLASTS AND VACUOLES

11.1 Introduction

Isolated protoplasts have been used to study transport at the plasmalemma (Table 6.4). Guy and coworkers (1978, 1980) studied sugar and amino acid transport in pea mesophyll protoplasts. D-glucose uptake was diphasic, representing an approach to flux equilibrium in a small compartment of the protoplasts (probably the cytoplasm) with continued slower uptake (probably into the vacuole: Guy et al. 1979). These protoplasts also took up α-aminoisobutyric acid but the rate declined after 10 min, a phenomenon not observed with tissue slices from which the protoplasts were derived.

Isolated vacuoles have been used to study transport at the tonoplast (Table 7.1). For instance, Guy extended his studies of pea mesophyll protoplasts to vacuoles isolated therefrom, to demonstrate active uptake of 3-O-MG with indications that ATP directly energized glucose transport.

The phenomenon of compartmentation in tissue of the grape berry has been demonstrated (Chapter 8). The delineation of such a compartment will be a membrane. In the skin tissue, glucose accumulates prior to veraison with a change at veraison favouring fructose accumulation. From the concentration of diffusible hexoses in the skin and the sugar content of the flesh it was deduced that a greater compartmentation of both glucose and fructose proceeds in the fleshy pericarp cells.

Some progress has been made toward description of sugar transport across membranes of higher plant cells (Chapter 5). Much emphasis has been given to the proton-sugar symport mechanism described for Chlorella. However a group transport mechanism is more likely for the transfer of sugar into the vacuole of fruit cells (Paragraph 7.7). Possibly several mechanisms function, at the same or different membranes, or at different stages of development. Moreover, changes during ripening in physical properties of membranes are as yet poorly understood. Thus it is difficult to integrate measurements of membrane flux and compartmentation with other elements such as changes to the cell wall, turgor and metabolism; this difficulty has delayed the unravelling of the ripening process.

The initial hypothesis stated below, for the present investigation upon grape berries, was derived from studies of accumulation in sugarcane internode parenchyma cells (Paragraph 5.4 and Coombe 1976a). The description of sucrose phosphate synthase and sucrose synthase in the grape berry, along with other enzymes involved in the metabolism of carbohydrate (Figs. 1.6 and 1.7) strengthened the following hypothesis:

> The pathway of carbohydrate movement during the accumulating stage of ripening in the grape berry is in the first place apoplasmic. Sucrose is unloaded from the phloem into the intercellular space where transfer through the tissue by diffusion, and inversion, occurs. Glucose and fructose traverse the plasmalemma; sucrose (or sucrose phosphate) is synthesized in the cytoplasm and transported at the tonoplast; glucose and fructose are produced in the vacuole by the action of an invertase.

The following riders are evident:

(i) mediated transport of sucrose at the tonoplast would be a sufficient condition for other transfers in this pathway to function passively (i.e. to be energetically favourable);

(ii) sucrose (or sucrose phosphate) synthesis may occur vectorially at the tonoplast such that the apparent substrates for transport are NDPGlucose and fructose (or F6P).

It was decided to investigate the selectivity of the plasmalemma towards sugars by studying the behaviour of protoplasts and vacuoles isolated from the skin of the grape berry. The aim was to characterise the transport mechanism, using analysis of the radiochemical products (Chapter 13) after incubation of protoplasts and vacuoles. This information would help distinguish transport at the plasmalemma from the effects of subsequent metabolism or compartmentation.

To quantify comparisons between protoplast and vacuole suspensions, the phenolic content of each was used as a common base with the assumption that all phenols were vacuolar. Thus one phenol unit equivalent of vacuoles is theorectically derived from one phenol unit equivalent of protoplasts. Deficiencies arise in practice: the concentration of phenols in the berry changes with ripening; large differences exist between cultivars and seasons; the assay reacts with HEPES buffer (of VM) and amino phenols. Inclusion of BSA in PM complicated analysis, even where the protoplasts to be assayed were cleared of that medium,

for it was not known to what extent the albumen associated with the membrane exterior.

An alternative - comparison by numbers as counted with a haemacytometer - was not possible where pelleted material had to be assessed, and takes no account of differences in volume distribution of the two populations.

Comparison on the basis of enzyme activity was not attempted. It would be necessary to account for activation or deactivation in the two populations and media, especially as phenols will interact with enzymes.

11.2 Materials and Methods

Only the general methodology is presented here. Each section of "Results and Discussion", or the relevant figures, are prefaced with an indication of the tissue used and of any modifications to the general method. Cross-references are given to results reported in other chapters.

Protoplasts and vacuoles were prepared according to the protocols indicated in Figs. 9.3 and 9.4.

Incubation was in a 1.5 ml plastic Eppendorf reaction tube inverted and modified as indicated in Fig. 11.1, a technique developed by Boller (1977).

The incubation volume was 1 ml. The incubation cups were prepared with 500 µl PM (or VM) containing the desired components (additives) at double final concentration. Total osmolarity was kept constant with aliquots of 1 M mannitol. For instance, an incubation in 50 mM sugar was prepared by adding, to 450 µl PM (or VM), 50 µl from a 1 M sugar stock containing 1 µCi radiochemical. In Controls, 50 µl 1 M mannitol replaced the sugar. At time zero, 500 µl protoplasts or vacuoles were added and the tube gently inverted twice to mix. In Blanks, 500 µl medium replaced the protoplasts and vacuoles. Temperature was 23°C.

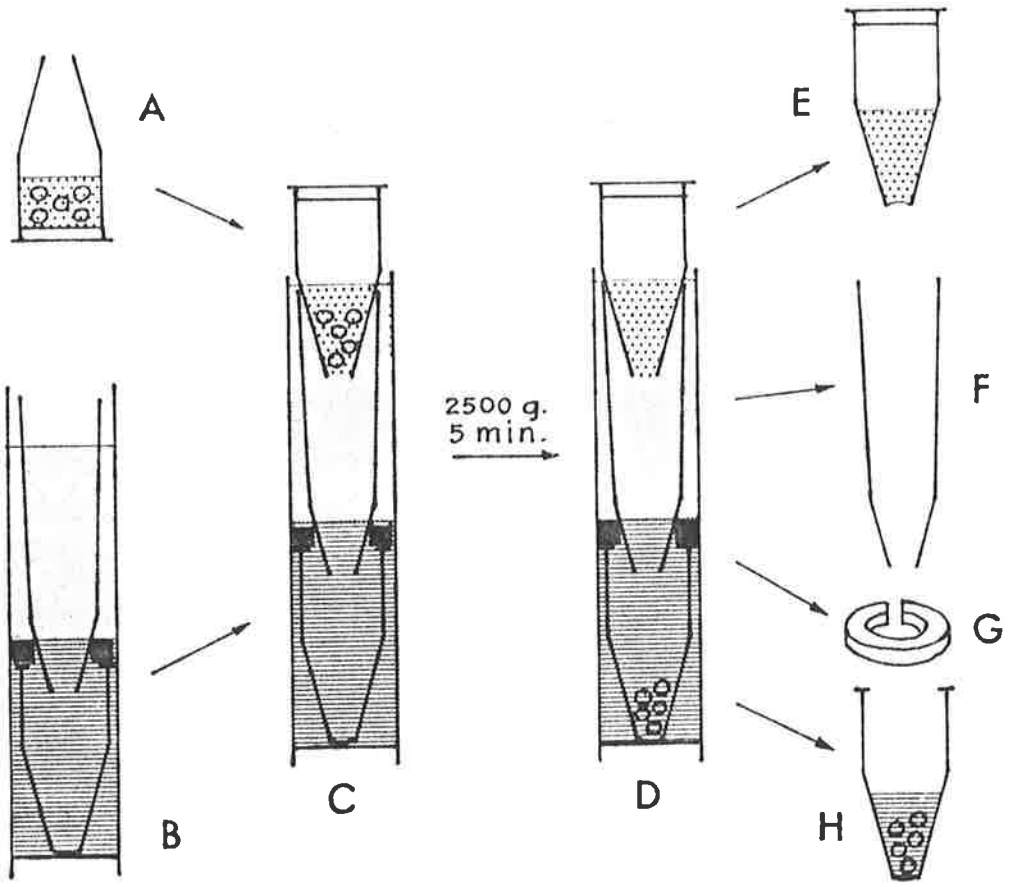
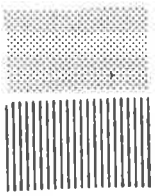


Fig. 11.1 Incubation and centrifugation module to recover protoplasts or vacuoles from their respective media. (after Boller 1977)

A. Incubation cup, an Eppendorf reaction tube cut at tip, with protoplasts or vacuoles (o) in medium and radioactive sugar ( ).

B. Cleaning column, comprising plastic support column (F) and C-ring (G) above the premarked receiving cup (H), containing



7% Ficoll in PM or VM

10% Ficoll in PM or VM

BSA, oxytetracycline and reductant are omitted from PM here.

C. Incubation cup inverted upon the column. In practice, the top lip of the cup rests upon the support.

D. Incubation completed by activation of centrifuge, and protoplasts or vacuoles sediment.

E. Medium is recovered in incubation cup. Ficoll is removed down to the receiving cup by aspiration.

H. The receiving cup - an Eppendorf tube without cap - is removed, containing the protoplasts or vacuoles. Gradient material is reduced to a premarked level (50 µl); the pellet resuspended; and 200 µl water added for lysis.

The same system may be used with incubation cups of 0.6 ml Eppendorf tubes.

During incubation the cups were inverted once every five minutes. Two minutes prior to termination the cups were inverted onto Ficoll gradients precooled to 4°C in pre-balanced centrifuge cradles. At full-time the centrifuge was actuated for a run of 5 min at 2500 g with the brake off.

The incubation cups containing residual medium were carefully removed from the top of the column: slight dilution occurred from the Ficoll gradient. Using a Pasteur pipette attached to a vacuum pump, the inner, top column of Ficoll was removed with the outer (clean) Ficoll entering out the C-ring. The support column, ring, and receiving cup were removed and the Ficoll therein reduced to a pre-marked 50 µl. To the receiving cup 200 µl water was added and the pellet resuspended using a vortex mixer and agitation with a pipette tip. Where the radioactive solution was to be chromatographed the sample was rapidly frozen in liquid nitrogen.

An 100 µl aliquot was counted in 2 ml xylene/triton/PPO/POPOP scintillation cocktail (ACS: Amersham Australia Ltd.) in a plastic disposable vial. To quantify the protoplasts or vacuoles a 25 µl aliquot was assayed for phenolic compounds using the Folin-Ciocalteu reagent (Fig. 11.2).

All radiochemicals were supplied by Amersham Australia Pty. Ltd. Stock solutions were subsampled in order to determine specific activity.

Where data are presented in cpm correction has been made for differences of specific activity. Uptake is generally expressed as nmole equivalents of substrate, without implying that the substrate (e.g. sucrose) is necessarily transported or accumulated without modification. That presented as "per unit phenol (Gallic acid equivalents)" is based upon an assay of each receiving cup; that as "per 10^6 protoplasts (or vacuoles)" is calculated from counts of the stock suspension and assumptions that (i) equal (500 µl) aliquots were added to each incubation and (ii) any losses thereafter were insignificant.

As it was difficult to subsample aliquots from the pellet, the entire 250 µl in the receiving cup was counted for radioactivity if other assays were not required. The sample was transferred to a scintillation pot, the cup was rinsed with an additional 250 µl water, 10 ml ACS was added to the pot, vigorously mixed, and counted.

U-^{14}C-F6P.Na^+ was prepared by incubating, for 15 h: 20 µCi U-^{14}C-fructose in 10 mM HEPES pH 7.6, 0.5 mM ATP.Na_2, 5 mM $MgCl_2$ and 2 units hexokinase. The products were separated by electrophoresis for 30 min at 1500 V in ammonia/ammonium bicarbonate buffer pH 9.4. The zone corresponding to F6P was eluted and desalted through a 1 ml column of Amberlite IR-120(H) Analytical Grade resin. Elution proceeded till no more radioactivity appeared; the eluate was adjusted to pH 5.5 with 0.1 N NaOH and concentrated under dry N_2 and warm air. Aliquots with and without phosphatase treatment were electrophoresed in borate buffer to confirm the identity of F6P.

Invertase was assayed by adding 50 µl protoplast suspension to 2 ml phosphate buffer pH 3.0 containing 50 mM sucrose. An aliquot was also added to buffer without sucrose. Tubes were incubated at 37^{o} and 100 µl subsampled at 1, 2, 5, 10, 20 min into the Somogyi copper reagent of the Somogyi-Nelson assay for reducing sugars (modified from Paleg 1960). These tubes were placed in a boiling water bath for 15 min, 400 µl arseno-molybdate reagent added and mixed. A further 2 ml water was added before reading absorbance at 560 nm. A standard curve was constructed using 1 - 10 ug glucose. (N.B. Ethanol and citrate inhibit this assay.)

Fig. 11.2 Protocol for Phenolic assay using Folin-Ciocalteu reagent

The method involves oxidation of the phenols by a yellow molybdotungstophosphoric heteropolyanion reagent and colourimetric measurement of the resultant molybdotungstophosphate blue at 765 nm. A standard procedure is here modified for reduced volumes (Singleton and Rossi, 1965; Slinkard and Singleton, 1977).

Reagents

1. "Folin-Ciocalteu" reagent : prepared according to (Folin and Ciocalteu, 1927; Slinkard and Singleton, 1977) or purchased, contains sodium tungstate, sodium molybdate, orthophosphoric and hydrochloric acid, bromine and lithium sulphate. It should be bright yellow without any green tint. A stock may be reconditioned by boiling, in a fume hood, with a few drops of bromine till the latter is fully removed. Store at 4°C in the dark and avoid contamination of the stock by deposits from the lip of the container.
2. Sodium carbonate A.R. : at 75 g ℓ^{-1}, distilled water.
3. Gallic acid : dissolve in a few drops of 90% dist. ethanol, then add water to volume for a standard of 1mg $m\ell^{-1}$. Store at 4°C in the dark and for longer periods at -5°C.

▶

Assay system

spectrophotometer 765 nm; test tubes, if a flow-cell is available, or cuvettes; vortex mixer ; stopwatch

PIPETTE INTO TUBES INCLUDING THOSE FOR STANDARDS AND BLANKS :			
water	1325	µℓ	
sample	50	µℓ	
Folin-Ciocalteu reagent	125	µℓ	(Final dilution 1:20)
Mix immediately. Start timer			
At t = 4 min,			
add Na_2CO_3 75 gℓ^{-1} with mixing.	1000	µℓ	(Final conc. 30 g ℓ^{-1})
Vortex each tube			
Final Volume 2.5 mℓ			
EITHER:			
Leave at room temperature for 2 h			
OR:			
Place all tubes simultaneously at 50°C. After 10 min, remove and cool.			
Read A_{765} of sample against the blank			

Reference Standards : 5-30 µg gallic acid per tube
Water Blanks : 50 µℓ water replacing the sample

Calculations

Good linearity is observed over the range 1-50 µg gallic acid equivalents (G.A.E.) in a final volume of 2.5 ml. A reduced final volume (e.g. 1 ml) can improve sensitivity and increased volumes extend the range provided that the Folin Reagent is not diluted below 1:20 and Na_2CO_3 is c. 30 g ℓ^{-1}.

From the standards derive the increased extinction per unit gallic acid, designated A cm^2 μg^{-1} G.A.E.

Then

$$c = \frac{\Delta E \times V}{A \times d \times v} \quad \mu g \text{ G.A.E. } m\ell^{-1} \text{ in the sample.}$$

where V is final volume and v is aliquot volume.

The method is generally applicable to phenols, giving for example 1 molar equivalent phenol with monophenols, 2 equivalents with pyrocatechol, 3 with catechin. It is obviously empirical as applied to a complex mixture of phenols. Any phenolic group will react, including those in proteins and secondary metabolites.

Limitations

The colour development is dependent upon partial reduction of a complex which is in turn dependent upon phenolate ion formation due to the alkaline state. The process is subject to many variables : hence, reference standards are run with each batch and routine methodology observed. For instance, the sample must be properly diluted prior to addition of Folin-Ciocalteu reagent, mixed in a consistent manner, and rendered alkaline > pH9 after a constant interval (between 0.5 and 8 min). Individual reactions giving excessive absorbance cannot simply be diluted and values corrected algebraically; such analyses must be repeated at greater dilution with new reference standards. If pH is not greater than 9 after adding Na_2CO_3, more solid Na_2CO_3 should be added.

Furthermore, the process is subject to interference at different steps, notably by reductants ($Na_2S_2O_3$; ascorbic-acid ; SO_2 ; Fe^{2+}) and sugars (Slinkard and Singleton, 1977). Controls, such as medium minus phenols, are required so that any interference can be deducted from E; controls of spiked medium will indicate a recovery rate in the presence of such interference. Spiking may indicate synergistic interference, such as reported for SO_2 (Somers and Ziemelis, 1980) for which it is particularly difficult to correct. □

11.3 Results and Discussion

11.3.1 Protoplasts: Uptake of glucose and sucrose

Protoplasts prepared from the skin of the grape berry cv. Muscat (10^{o} Brix) were cleaned by ultracentrifugation in Percoll such that the suspension appeared microscopically free of cell-wall material. These protoplasts accumulated ^{14}C from uniformly labelled D-glucose and sucrose, each 20 mM. At 62 min protoplasts in D-glucose had taken up four times as much label as those in sucrose (Fig. 11.3). Uptake from each sugar

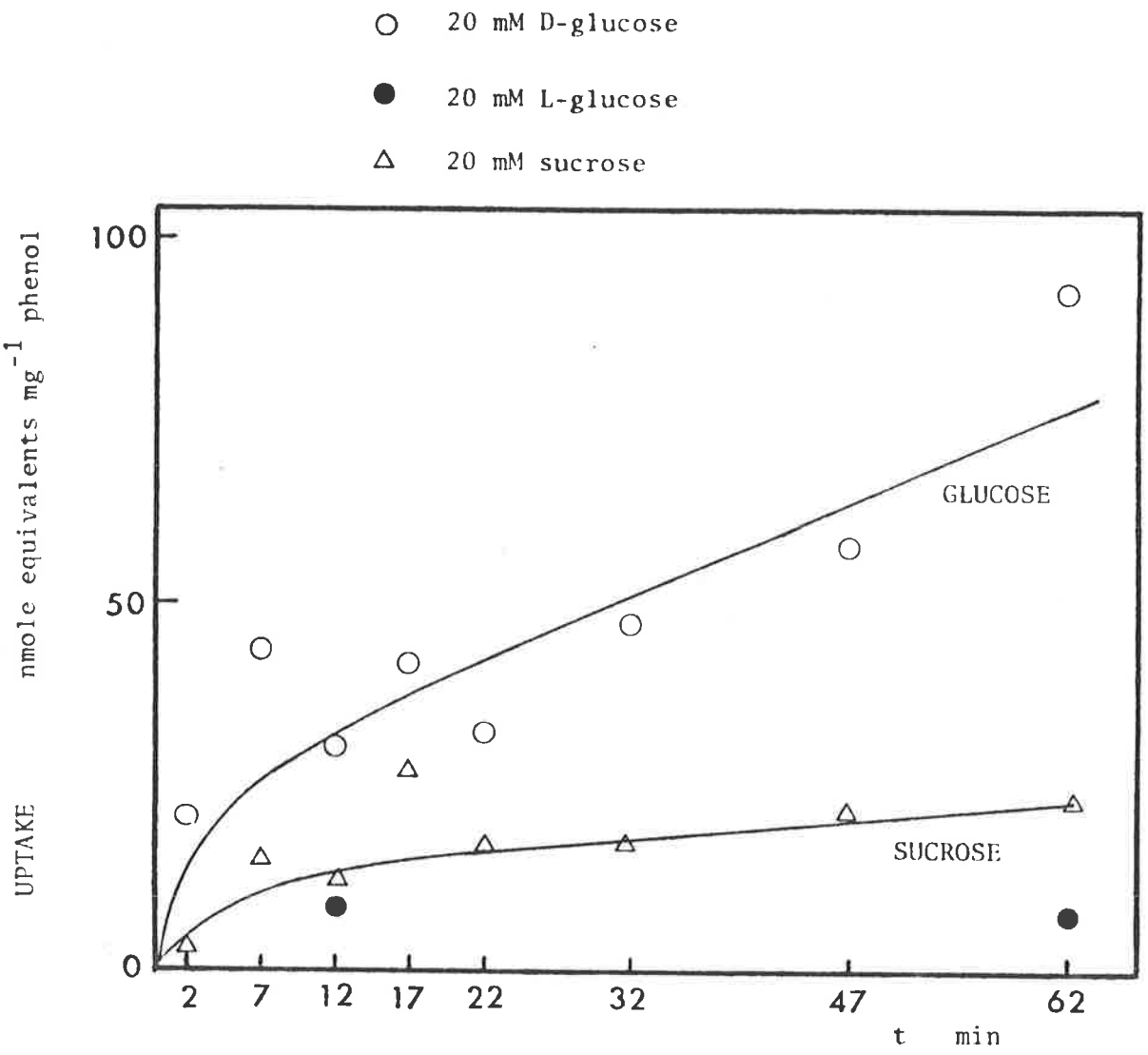


Fig. 11.3 Uptake of ^{14}C from D-glucose, L-glucose and U-^{14}C-sucrose by protoplasts from the skin of grape berries cv. Muscat (10^{o}Brix). Refer chromatography: Figs. 12.9 & 10.

Points are means of duplicates.
Medium included 3mM DTT. 10^{6} protoplast ≡ 48 µg phenol

was diphasic with the initial rapid phase occupying less than 2 min for glucose and 7 min for sucrose. The intercept on the Y axis is termed the "zero time uptake". Uptake from L-glucose did not increase from 12 to 62 min at which time it was only 10% of uptake from D-glucose.

In contrast, in other experiments using protoplasts prepared, stored and incubated in two different media, accumulation of radiochemical from 50 mM U-^{14}C-sucrose was about equal to that from 50 mM U-^{14}C-D-glucose (Fig. 11.4). One medium (PM-I) included 25 mM KH_2PO_4 and 10 mM cysteamine HCl; the other (PM-II) included 10 mM DTT, 0.1% w/v BSA and no P_i. In PM-I the rate of uptake was double that in PM-II. Moreover, there were similar zero-time uptakes despite this difference in rates. The protoplasts had been stored for 15 h at 4^{o}, and the results contrasted with a preliminary trial upon these protoplasts before storage (Fig. 11.4 Insert) in which no zero-time uptake was apparent and uptake from glucose exceeded that from sucrose at each time sampled. Also after storage in PM-II the rate of uptake was only half that observed in freshly prepared protoplasts.

These experiments showed that uptake of D-glucose and sucrose was more than that attributable to passive diffusion (indicated by L-glucose) and was diphasic. Uptake of both sugars was similarly affected by changes to the medium.

11.3.2 Competition by deoxyglucose but not raffinose

Upon addition of 10 mM 2-deoxy-D-glucose, uptake by protoplasts of ^{14}C from 10 mM U-^{14}C-D-glucose was reduced by 42% and 45% at 30 and 60 min respectively (Fig. 11.5). This preparation of protoplasts, when lysed, exhibited invertase activity at pH 3.0 of 3.4 nmol sucrose hydrolysed $(min)^{-1}(10\ protoplasts)^{-1}$. Hence invertase activity in these protoplasts, at pH 3, was sufficient to form free glucose from sucrose at a rate exceeding uptake (although pH- and concentration-dependence would substantially affect such a comparison).

Similarly in another experiment (Table 11.1) 2-deoxy-D-glucose reduced ^{14}C uptake from D-glucose by protoplasts.

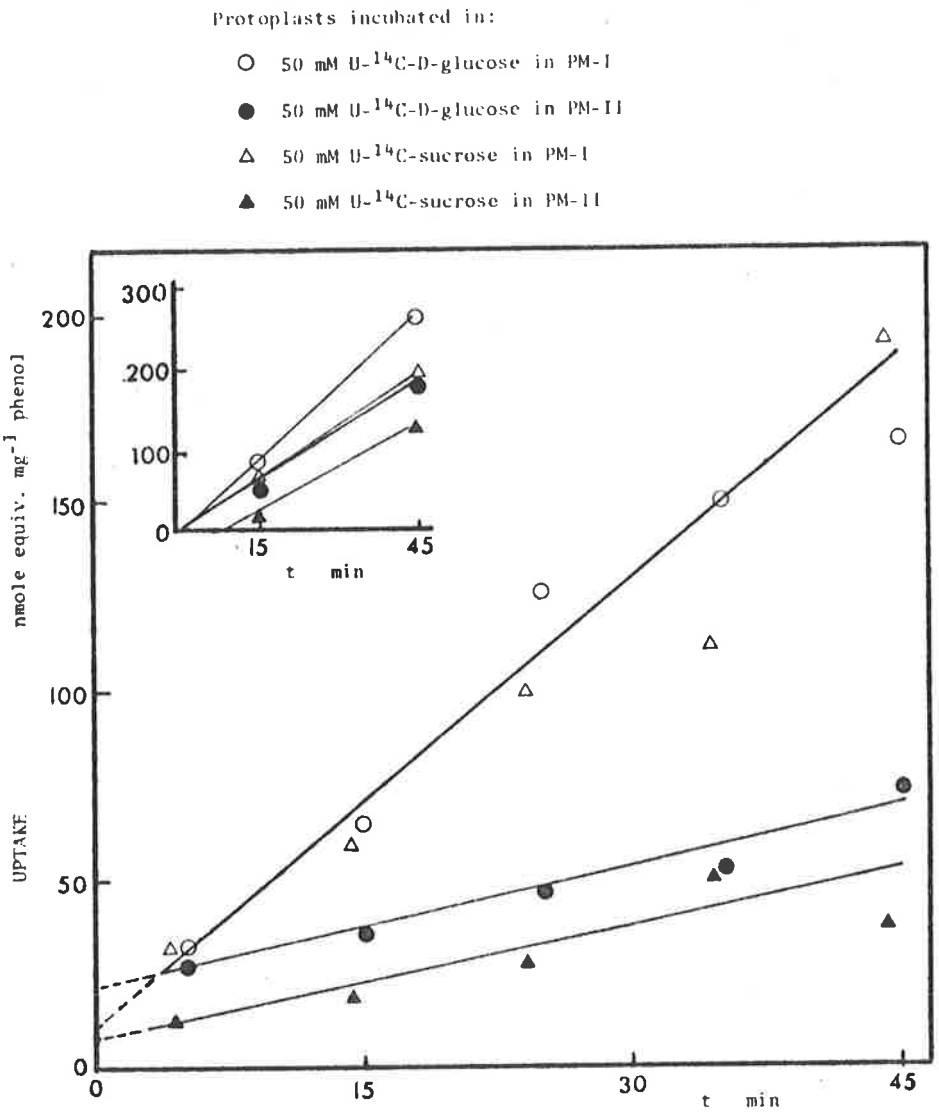


Fig. 11.4 Uptake of ^{14}C from D-glucose and U-^{14}C-sucrose by protoplasts in different media.

Insert: A preliminary trial of the same, in which there was no positive Y-intercept ("time zero uptake") contrasting with the latter experiment.

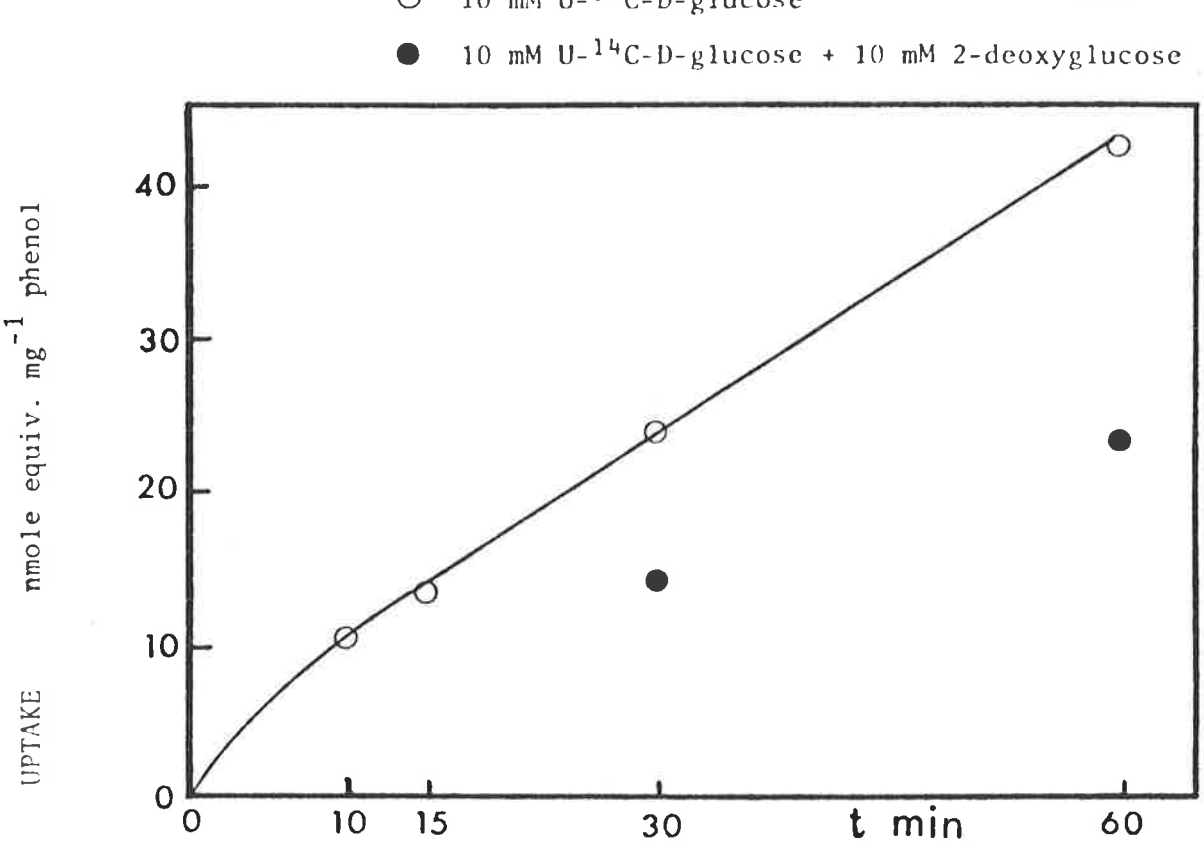


Fig. 11.5 Effect of 2-deoxyglucose upon uptake of ^{14}C from D-glucose by protoplasts from the skin of grape berries cv. Doradillo (5.1°Brix).
Points are means of duplicates. 10^6 protoplast ≡ 910 µg phenol
Medium included 10 mM cysteamine, 25 mM K^+

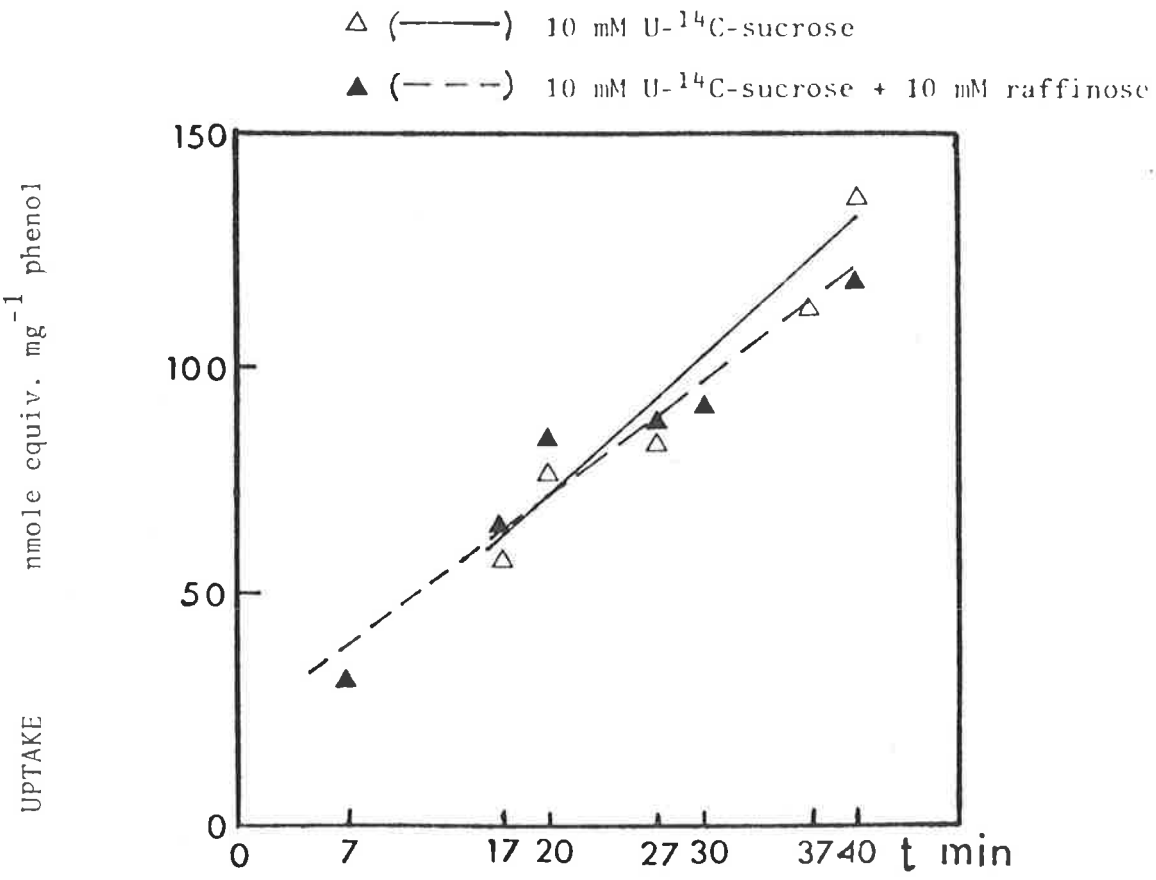


Fig. 11.6 Effect of 10 mM raffinose upon uptake of ^{14}C from 10 mM U-^{14}C-sucrose by protoplasts from skin of berries cv. Doradillo (7.5°Brix).
Single determinations. 10^6 protoplasts ≡ 775 µg phenol
Medium included 10 mM cysteamine HCl, 25 mM K^+

Table 11.1: Effect of 10 mM 2-deoxyglucose upon uptake of ^{14}C from 10 mM D-glucose by protoplasts from the skin of grape berries cv. Doradillo (7.5° Brix)

Incubation time (min)	7	10	20	27	30	37	40
^{14}C uptake ratio*	1.84	0.62	0.84	0.78	0.59	0.48	0.82

* $\frac{\text{Uptake in (D-glucose + 2-deoxyglucose)}}{\text{Uptake in (D-glucose alone)}}$

Upon addition of 10 mM raffinose, uptake by protoplasts of ^{14}C from 10 mM U-^{14}C-sucrose was unchanged (Fig. 11.6). An active transport of sucrose identified in beet vacuoles was competitively inhibited by raffinose (Willenbrink & Doll, 1979).

Competition by deoxyglucose indicates a component of D-glucose uptake to be mediated. Equimolar raffinose did not compete with sucrose uptake by protoplasts.

11.3.3 Alternative glucose radionuclides with protoplasts

Protoplasts were incubated in media separately containing the glucose radionuclides indicated in Fig. 11.7. Accumulation as nmoles equivalents (per incubation) is indexed against uptake of ^{14}C from U-^{14}C-D-glucose at 40 min.

Accumulation of radioactivity was less from 3-^{3}H-D-glucose than from U-^{14}C-D-glucose, and from I-^{3}H-D-glucose was much less still. With the assumption that glucose uptake was the same in each incubation it was possible that in each case hydrogen (tritium) was being removed from glucose. But when the two tritiated glucose stocks were checked by paper chromatography (Methods, Chapter 12) the 3-^{3}H-D-glucose was shown to be stable while the 1-^{3}H-D-glucose had exchanged to become cold glucose in tritiated water. Nevertheless, the fourth treatment in Fig. 11.7 does not amount to an estimate of the $^{3}H_2O$ volume of protoplasts

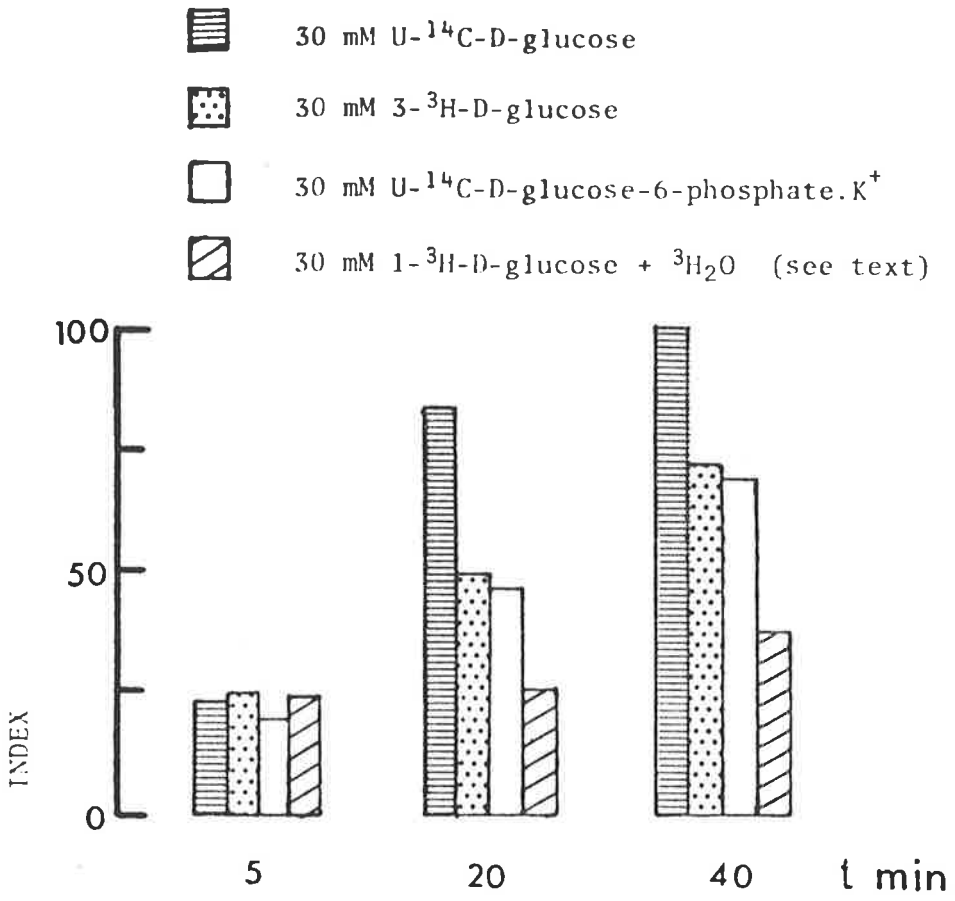


Fig. 11.7 Comparison of glucose radionuclides as substrates for accumulation of radioactivity by protoplasts from grape berries cv. Muscat (5.4°Brix).
(Refer chromatography, Table 12.8)

Incubation times 5, 20, 40 min.
All values indexed against (U-^{14}C-D-glucose at 40 min) = 100

as most $^{3}H_2O$ would exchange with the aqueous medium during centrifugation.

Uptake from G6P was at each time less than that from glucose.

These experiments revealed a tracer effect (^{14}C-glucose relative to 3-^{3}H-glucose) which had to be considered when using double-labelling techniques. The radiochemical products were later analyzed (Chapter 13).

11.3.4 Interaction of glucose and fructose

The relative rates of uptake by protoplasts of glucose and fructose in the presence or absence of the other are indicated in Fig. 11.8. This experiment was conducted prior to identification of the difference in uptake between U-^{14}C-D-glucose and 3-^{3}H-D-glucose (Fig. 11.7 and Table 12.8). Nevertheless accumulation of radiochemical from glucose was stimulated by inclusion of fructose in the medium (values above the index 1.00), while accumulation from fructose was unaffected by inclusion of D-glucose and in general was less than uptake from D-glucose alone. Subsequent analysis of labelling from 3-^{3}H-glucose discounted the "stimulation" arising simply by greater retention of tritium on the glucose molecule; it was evidently a real stimulation of glucose accumulation.

11.3.5 Effect of pH

The pH values of the media, PM and VM, had been selected to optimize yield and viability of protoplasts and vacuoles, rather than uptake of glucose and sucrose by protoplasts. To appreciate the contribution of pH to the differences between uptake in protoplasts (PM pH 5.5) and vacuoles (pH 7.3), the effect of pH upon uptake was investigated.

Moreover, H^{+} concentration in a medium may affect transport: via the proton gradient at a limiting membrane; or by protonation of a carrier (Komor & Tanner 1974 with Chlorella); by the pH-dependency of an associated enzyme; or by effects upon membrane structure (p. 130, 276 in Lüttge and Higinbotham 1979).

Protoplasts were prepared from grape berries cv. Muscat (10^{o} Brix), the suspension divided into five equal aliquots, sedimented at 1000 g for 1 min, the supernatant discarded and protoplasts resuspended to 4 ml

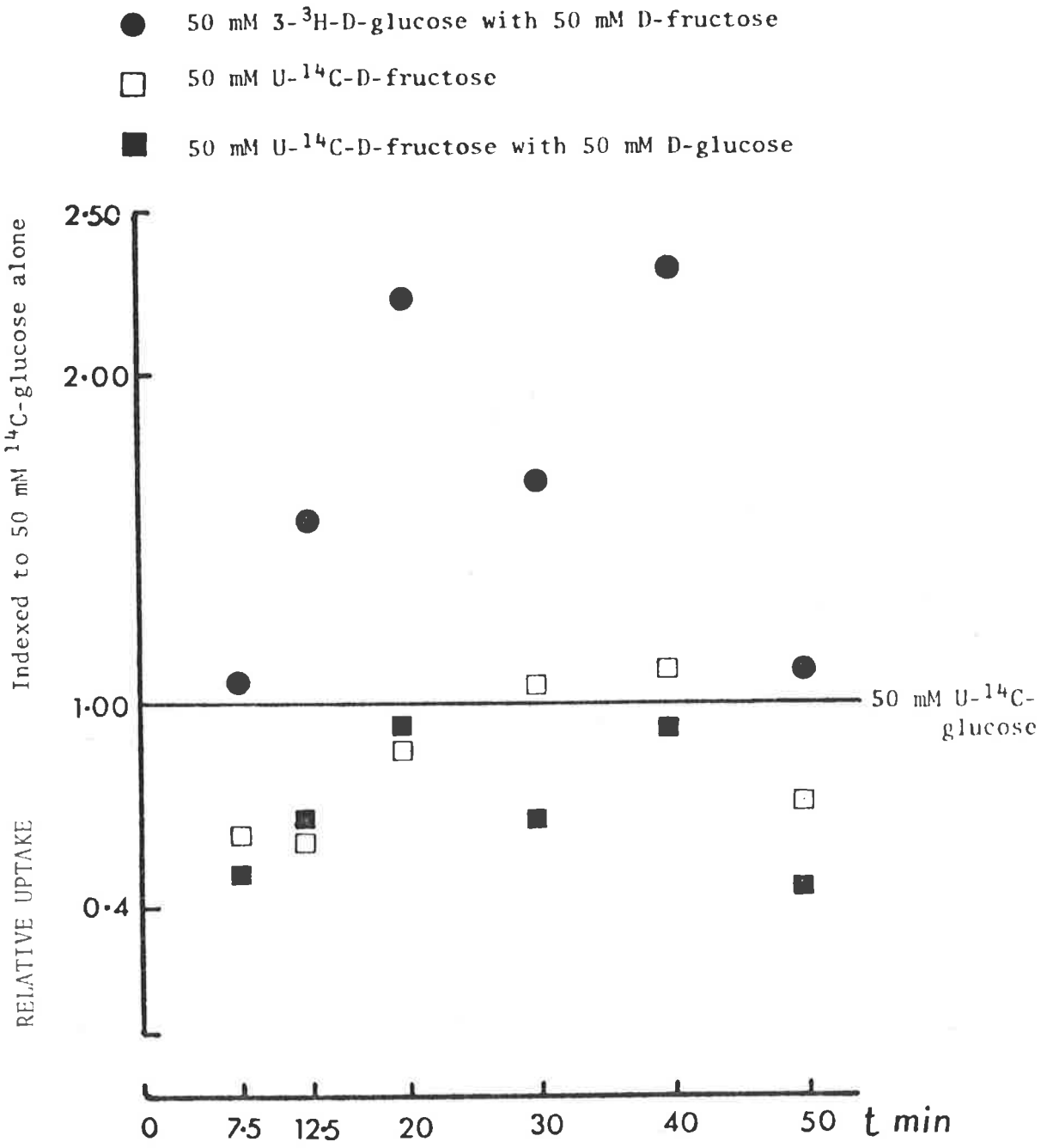


Fig. 11.8 Uptake by protoplasts of glucose and fructose in the presence or absence of the other. Data derived as nmoles mg^{-1} phenol, then indexed to 50 mM U-^{14}C-glucose = 1.00. Berries cv. Muscat. 5.6°Brix, 27 g l^{-1} glucose, 16 g l^{-1} fructose

with media at pH 4, 5, 6, 7 and 8. Aliquots of 950 µl were pipetted into modified Eppendorf reaction tubes and at time zero 50 µl differently labelled sugar stocks added, namely 400 mM U-^{14}C-sucrose and 400 mM 3-^{3}H-D-glucose containing respectively 1 µCi and 2 µCi activity. Incubation times were 20 and 60 min with duplication.

A pH series of PM was prepared by titrating MES acid with MES sodium salt. Use of one buffer avoided the uncertain side-effects in a pH series based upon several buffers: however, the pKa of MES being 6.15, poor buffering capacity was evident at the extremities. Nevertheless, the pH's indicated in Fig. 11.9 were those measured upon completion of the incubation described.

Excess protoplast suspension in each pH medium was assessed by haemocytometry before, during and after the incubation to ascertain decline of protoplast numbers.

After 1 h at pH 4.45 the recovery of phenols in the receiving cups was less than half that in the other incubations (results not shown), just as breakdown of protoplasts had been observed under the microscope. However, per mg phenol recovered, uptake of both glucose and sucrose was clearly greatest at pH 4.45 (Fig. 11.9). Otherwise, in the stable protoplasts, there was no clear dependency upon pH. At all pH's uptake from glucose exceeded that from sucrose at least four-fold.

Several interpretations could be placed upon the high apparent uptake in labile protoplasts. Firstly, that the membrane was increasingly semipermeable to sugars. Yet in this treatment perm-selectivity of glucose over sucrose was retained. Secondly, that uptake at the tonoplast was stimulated. For instance, the cytoplasm may become perturbed in a manner that stimulated transport at the tonoplast without perm-selectivity of the tonoplast being affected.

Since it was desirable to avoid changes to the pH of a medium containing protoplasts, it was concluded that uptake could be routinely studied at pH 5.5.

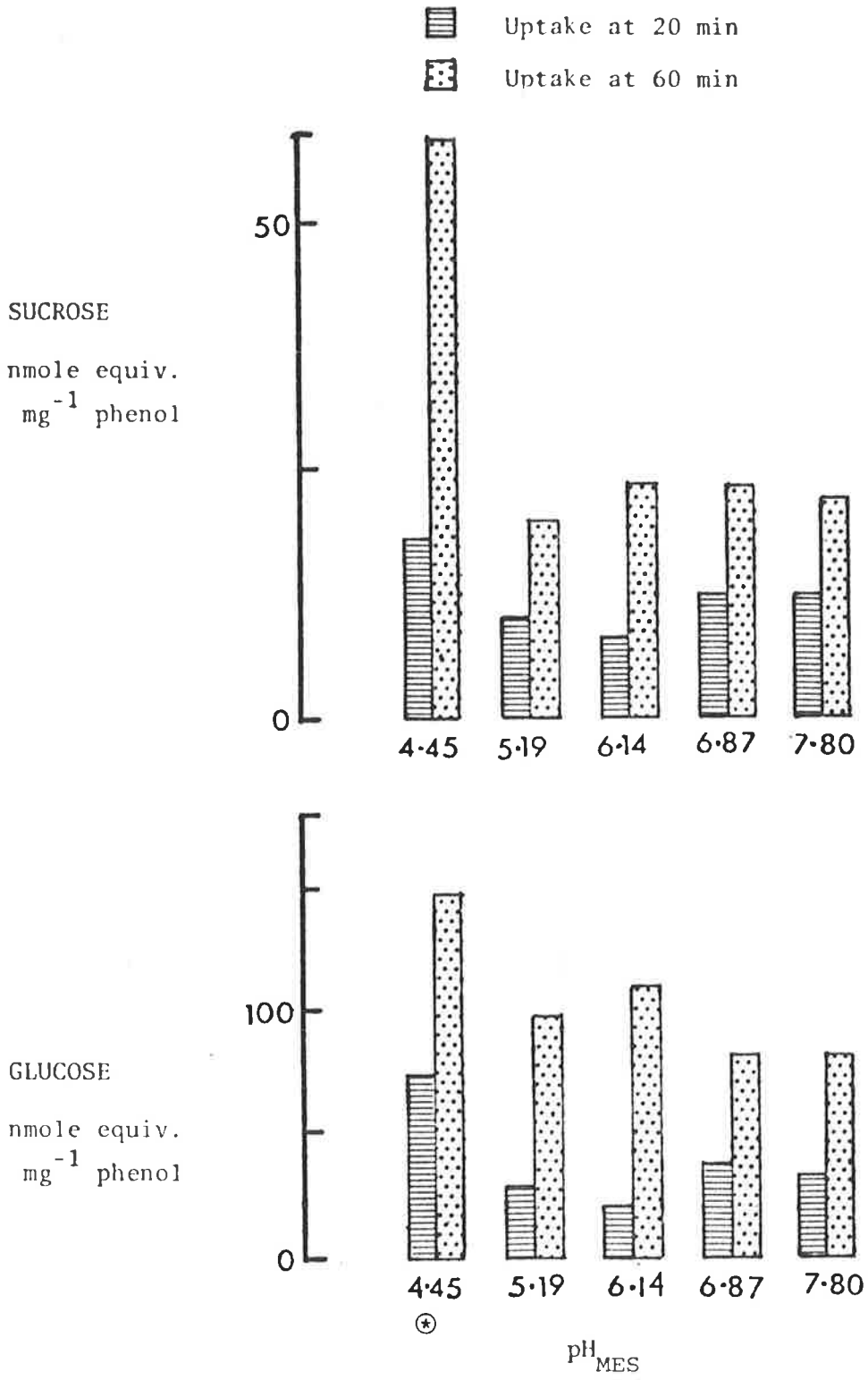


Fig. 11.9 The effect of pH upon accumulation of radioactivity from [20 mM U-^{14}C-sucrose + 20 mM 3-^{3}H-D-glucose] by protoplasts from grape berries cv. Muscat (10^{o} Brix)

Points are means of duplicates, and are corrected for differences in specific activity.
Note different scales. ⊛ labile protoplasts

11.3.6 Effect of pressure upon uptake by protoplasts

In Valonia hydrostatic pressure modulated K^+ transport (Gutknecht 1968; Zimmermann & Steudle 1978). The pressures applied were far below those significantly affecting the thermodynamic activity of ions and must be interpreted as physiological responses. Recently it has been suggested that phloem loading of sucrose is sensitive to pressure (Smith and Milburn 1980c).

Hence the effect of pressure upon uptake of ^{14}C by protoplasts was investigated using a Scholander Bomb as pressure chamber. Protoplasts were prepared from stored berries cv. Muscat (5.4° Brix) and pipetted into modified Eppendorf reaction tubes. Uptake of ^{14}C from 30 mM U-^{14}C-D-glucose was studied with duplicates at four pressures, 1, 2, 5, 10 Atm. As a control, protoplasts were incubated with L-glucose at 1 Atm.

90 sec were required to attain full pressue in the chamber, and 150 sec to reduce this pressure to 1 Atm. Therefore the incubation time of 20 min included 4 min of pressure below the nominal treatment. Randomisation was used in preparing incubation tubes and to execute the treatments: as only one chamber was available, duplicates were incubated simultaneously.

From Table 11.2 it is evident that protoplasts incubated at 5 and 10 Atm contained significantly more ^{14}C than those at 1 and 2 Atm. L-glucose uptake was high relative to many incubations of protoplasts (e.g. 11.3.1 above) and was indicative of berries stored for several months. This is interpreted as transport by passive diffusion, whereas uptake of D-glucose is by diffusion plus a mediated process (Chapter 12). The mean increase between the two significance groupings was 24%. After deducting the putative diffusion from each class (significance groups a and b) on the assumption that diffusion of D- and L-glucose was the same, that component attributed to mediated uptake was 320 cpm at 1 and 2 Atm and 574 cpm at 5 and 10 Atm, a mean increase of 80%.

The phenomenon warrants further investigation, both as a physiological process and for its implications regarding the hydrostatic pressures generated during ultracentrifugation.

Table 11.2 : Effect of pressure upon uptake of ^{14}C from 30 mM U-^{14}C-glucose by protoplasts from grape berries cv. Muscat (5.4°Brix)

Medium included 5 mM cysteamine HCl; 0.5 mM K^+
Protoplasts were incubated in a pressure chamber for 20 min of which 4 min were required to attain and release pressure. Values are means. F sig. at 5%; LSD (5%)=167 cpm. Values without common letters are significantly different at p = 0.05

Pressure (Atm)	Uptake (cpm per aliquot) Total
1	1090 a
2	1035 a
5	1340 b
10	1295 b

Uptake from 30mM U-^{14}C-L-glucose was 70% of D-glucose at 1 Atmosphere.

Smith and Milburn (1980 a, b, c) postulated that low hydrostatic pressure in the phloem stimulated phloem loading. Low turgidity in accumulatory tissue might arise where phloem unloading (and transpiration) raised the apoplastic osmotic potential to such a level that net water efflux occurred across the cell membranes. Conversely, pressure is generated in a turgid cell. At the plasmalemma, in such cases, there is obviously a thrust against the cell wall which may affect membrane properties by a number of mechanisms including micro-environment changes (charge, pH) or stretching of the membrane. At the tonoplast turgor is reflected as hydrostatic pressure: but this compression upon the membrane will be only slight. Nevertheless, as Zimmermann and Steudle (1980) point out, the membrane is an electro-mechanical entity and such compression will alter the transmembrane potential.

Wattiaux-De Coninck et al. (1980) observed increased permeability of the mitochondrial membrane to sucrose induced by hydrostatic pressure. Very high hydrostatic pressures may be generated in an ultracentrifuge (Rickwood 1978). As preparative ultracentrifucation is often used with protoplasts and isolated vacuoles it is appropriate to characterise any relationship between transport and hydrostatic pressure.

Due to unavailability of fresh plant material, the phenomenon was not investigated with isolated vacuoles, although the tonoplast is precisely the membrane at which a hydrostatic pressure effect is likely to act.

11.3.7 Vacuoles: Uptake of glucose and sucrose

From the skin of grape berries cv. Muscat (6 - 11° Brix) protoplasts were prepared and lysed to vacuoles. These were then diluted and sedimented at 1 g and contained some fine cytoplasmic debris but no unlysed protoplasts.

The vacuoles were incubated with replication for t = 10, 20, 30, 40, 50, 60 min in 50 mM U-^{14}C-D-glucose or 50 mM U-^{14}C-sucrose. Two aliquots of the source protoplasts were incubated in PM at pH 6.5 for 60 min. Two further aliquots of vacuoles were sonicated in ice for 30 sec using a Branson probe, and incubated for 10 min in either sugar.

At all sampling times accumulation of ^{14}C by vacuoles was greater from sucrose than glucose (Fig. 11.10) despite the considerable variability of data. Uptake from each sugar was diphasic as with protoplasts (e.g. 11.3.1); the rapid initial uptake was not characterised by the data but is apparent from the Y-intercept (or zero-time uptake).

Per mg phenol, accumulation from glucose by vacuoles was double that by protoplasts at 60 min. Sonicated vacuoles at 10 min contained only 5% and 25% of the radioactivity recovered from intact vacuoles in sucrose and glucose respectively; but expressed per mg phenol (and little phenol was pelleted) these comparisons were 27% and 100%. Surprisingly there were always "vacuoles" that survived vigorous sonication. These are considered to be composed of a resilient matrix as aqueous membrane vesicles certainly would not withstand this ultrasonication.

Uptake by sonicated vacuoles was comparable to zero-time uptake in ^{14}C-glucose. Furthermore, chromatographic analysis (Chapter 12) revealed the radioactivity of sonicated vacuoles in U-^{14}C-sucrose to be radioactive glucose. This suggests that zero-time uptake is only in part attributable to physico-chemical binding. It occurs in some preparations but not all, and occurred in this instance despite relatively high homogeneity of the vacuole suspension.

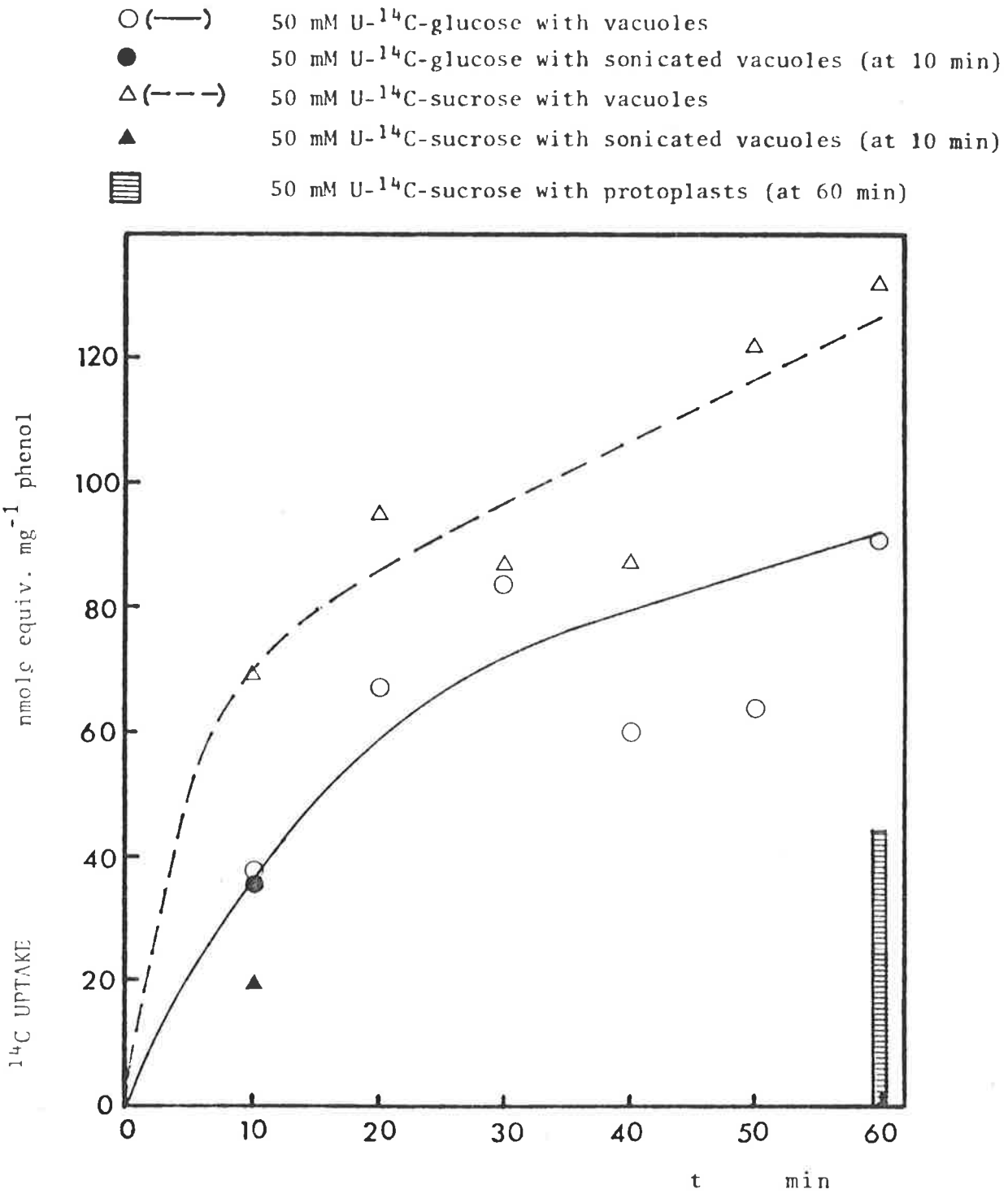


Fig. 11.10 Uptake of ^{14}C from U-^{14}C-glucose and U-^{14}C-sucrose by vacuoles isolated from the skin of grape berries cv. Muscat (6-10^{o}Brix)

(Refer chromatography, Table 12.9 and Fig. 12.12)

Vacuole medium contained 3 mM DTT; pH 7.2
Protoplast medium contained 10 mM DTT; pH 6.5

Values are means of duplicates.
10^6 vacuoles ≡ 25 µg phenol
Protoplast suspension included 27% vacuoles:
thus (0.73 protoplast + 0.27 vacuoles)10^6 ≡ 19.7 µg phenol

11.3.8 Variability between experiments

Considerable variability was encountered in many experiments with vacuoles, even when there was no decline in numbers during an incubation. Sometimes high accumulation would be observed in the short incubations while from longer incubations vacuoles were recovered with less radioactivity. Many results were therefore rejected on the basis of poor replication or reproducibility.

Notwithstanding such problems, it was evident that different preparations of protoplasts and vacuoles varied in their ability to sequester radioactive sugars. This has already been indicated (11.3.1) and is evident between Figs. 11.10 - 12 and Table 11.3.

Table 11.3 : Uptake of ^{14}C from 50mM U-^{14}C-sucrose and 50mM U-^{14}C-D-glucose by vacuoles isolated from the skin of grape berries cv. Muscat (5.1^{o}Brix).

Values ± S.D.

Substrate	Time (min)	Uptake (nmole equiv. mg^{-1} phenol)
U-^{14}C-sucrose	30	693 ± 71
	60	1211 ± 110
U-^{14}C-D-glucose	30	26 ± 12
	60	66 ± 20

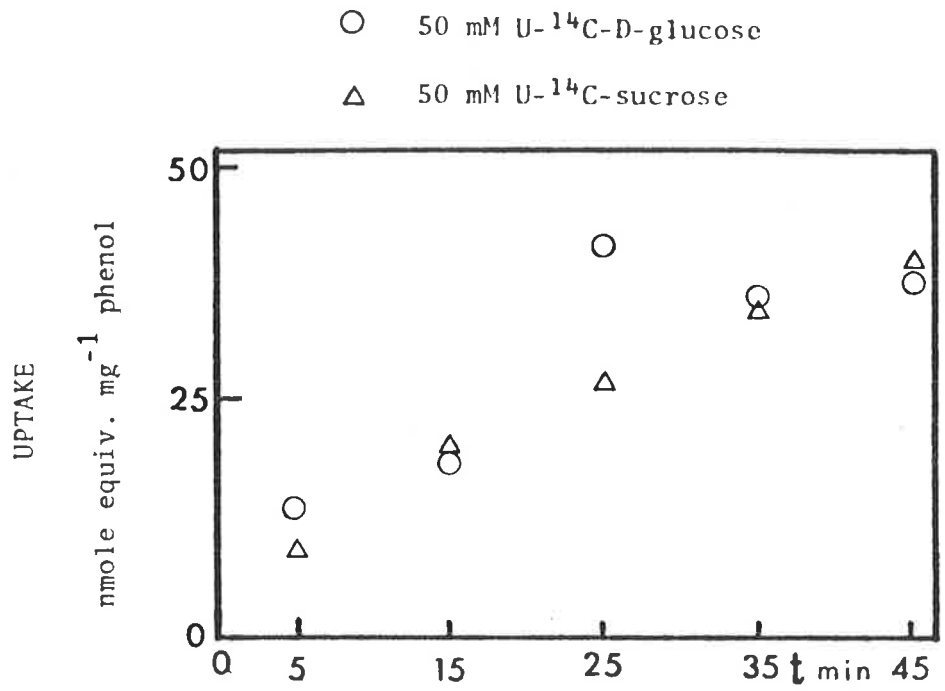


Fig. 11.11 Uptake of ^{14}C from U-^{14}C-glucose and U-^{14}C-sucrose by vacuoles isolated from skin of grape berries cv. Muscat (5.8° Brix).

To contrast with Figs. 11.10 & 11.12.

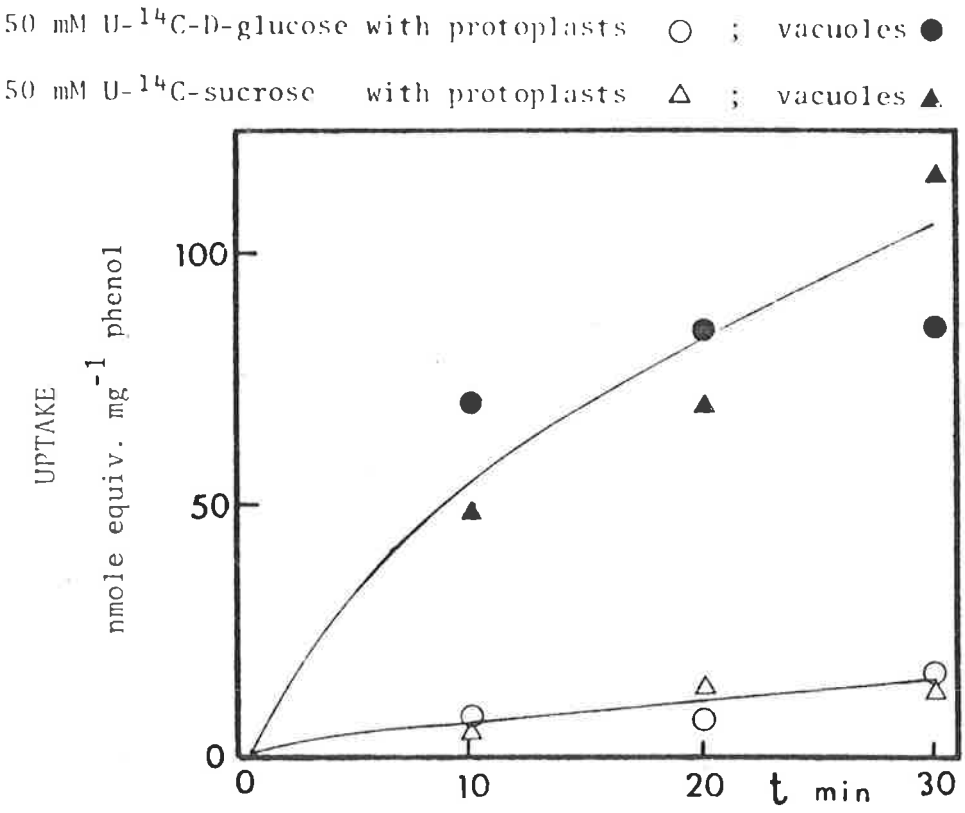


Fig. 11.12 Uptake of ^{14}C from U-^{14}C-D-glucose and U-^{14}C-sucrose by protoplasts and vacuoles isolated therefrom.

Source material: berries cv. Muscat (c. 5.0°Brix).

While in each of two preparations isolated vacuoles accumulated radioactivity from U-^{14}C-sucrose and U-^{14}C-D-glucose at similar rates, in one preparation this rate (per mg phenol) was three times greater than the other (c.f. Figs. 11.11 and 11.12). Uptake by protoplasts was one seventh.

In a previous season, vacuoles isolated from grape berries cv. Muscat (5.1^{o} Brix) accumulated ^{14}C from sucrose 20 times faster than from D-glucose (Table 11.3). This was regarded to be an important result, indicating both selective and time-dependent (linear) uptake. At the same time protoplasts were found to take up ^{14}C from sucrose more rapidly than previously. When such results could not be repeated, it was concluded that a sucrose transport had been lost during isolation. Hence composition of the media was altered, but it was not possible to reproduce the highly selective uptake of sucrose by vacuoles.

Many compounds were screened for an effect upon uptake of U-^{14}C-sucrose by vacuoles isolated from berries cv. Muscat (5 - 8^{o} Brix). The object was to inhibit and to stimulate selective uptake, such that a pulse-chase experiment could be conducted.

Aliquots of isolated vacuoles were preincubated for 10 min in VM containing the compound to be screened, then sucrose was added to 10 mM (including 1 µCi U-^{14}C-sucrose). Incubation times were 15 and 30 min without replication; or in one experiment: 15, 30, 45 min. Some compounds obviously interacted with the medium, such as $AgNO_3$ and $(NH_4)_6Mo_7O_{24}$ which formed precipitates or reduced. In Table 11.4 certain of the compounds are listed. Uptake has been indexed against controls (10 mM U-^{14}C-sucrose alone) that were run with each preparation of vacuoles.

The SH-reagent cysteamine.HCl stimulated uptake: the index relative to sucrose alone was, in four determinations, 395, 282, 424 and 338 - a mean stimulation of 260%. NaF (an inhibitor of phosphatase activity), $MnCl_2$ (inhibits the cleavage reaction of sucrose synthase) and CEPA (an ethylene producer) stimulated uptake by c.50%.

Table 11.4 : Effect of certain compounds upon U-^{14}C-sucrose uptake by vacuoles isolated from the skin of grape berries cv. Muscat (5-8°Brix)

As a screening operation, only 2 or 3 replicates were used. Incubation medium included HEPES pH 7.3, 0.7M mannitol, 10mM U-^{14}C-sucrose and the compounds indicated. All vacuole preparations included some cytoplasmic debris.

Compound	Index of uptake (10mM sucrose alone = 100)
5 mM glucose	59
5 mM fructose	59
10 mM stachyose	126
10 mM raffinose) (sucrose $\underline{\underline{30}}$mM)	123
5 mM UDPGlucose)	80
1 mM ADPGlucose	83
3 mM ATP.Mg^{2+} (sucrose 50mM)	80
0.1 mM $NADP^+$	83
0.1 mM NAD^+	89
60 mM KCl_2	64
60 mM $MgCl_2$	72
20 mM $MnCl_2$	156
2 mM $CaCl_2$	91
10 mM NaF	147
1 mM DTT	89
5 mM cysteamine	360
10 mM CEPA	145
10 µM ABA	79
10 µM Fusicoccin	67
30 µM 2,4-DNP	73
5 mM sodium diethyldithiocarbamate	67

Mistakenly, no vacuoles were incubated with L-glucose which would have provided an estimate of non-selective diffusion in these preparations. Other experiments using the same source of berries exhibited high uptake of L-glucose relative to the metabolizable sugar: 70% (Table 11.2) to 88% (Table 11.4).

In this case the stimulation of mediated uptake in particular may have been more than is immediately evident in the index. Similarly, other compounds in Table 11.4 warrant further study as inhibitors of mediated transport, and in particular the interaction of glucose and fructose with sucrose uptake. (Conversely, addition of unlabelled 5 mM sucrose to vacuoles in 10 mM U-^{14}C-glucose stimulated uptake by 46%).

11.3.9 Uptake by vacuoles from stored berries?

Vacuoles were isolated from the skin of grape berries cv. Muscat (5.4^{o} Brix) which had been stored for three months at 4^{o}C in sealed plastic bags. Protoplasts and vacuoles at this stage were incubated with a number of sugars as indicated in Table 11.5 with a view to stimulating a group transport (such as proposed in Fig. 7.1).

The rate of sucrose accumulation was similar to that of L-glucose and could thus be attributed to passive diffusion. Uptake from D-glucose and D-fructose was significantly greater than from L-glucose. Uptake of the phosphorylated sugars was less than the simple sugars, though G6P.K^{+} was greater than F6P.Na^{+}. Certain additives were included in two media as indicated in Table 11.5, the proposition being that *in situ* synthesis of sucrose or sucrose phosphate predisposes to transport (uptake). Unlabelled UDPGlucose with U-^{14}C-D-fructose are substrates to sucrose synthase, but this additive did not modify accumulation of ^{14}C. Thus there was no implication of sucrose synthase in uptake.

Unlabelled UDPGlucose and F6P are substrates to sucrose phosphate synthase; G6P is competitive for phosphatase (Boyer et al. Vol. IV, 1971) without affecting sucrose phosphate synthase (Salerno & Pontis 1978); NaF inhibits acid phosphatase (Torriani 1960) while EDTA, chelating Mg^{2+} inhibits sucrose phosphate phosphatase activity (Hawker 1969a).

Table 11.5: Uptake of ^{14}C from various sugar substrates by vacuoles isolated from the skin of grape berries cv. Muscat (5.4°Brix)

Berries had been stored for 3 months at 4°C.
Medium contained 5 mM cysteamine HCl
Values without common letters are significantly different at p = 0.05

Substrate (30mM)	Additives	Uptake in 20 min (Indexed; sucrose = 100)	
sucrose	-	(100)	ab
D-glucose	-	115	a
D-fructose	-	116	a
D-fructose	+ 10mM UDPGlucose	110	a
F6P.Na^+	-	47	d
F6P.Na^+	+ 10mM UDPGlucose, 10mM G6P, 20mM NaF, 10 mM EDTA	40	d
G6P.K^+	-	65	c
L-glucose	-	88	b

This "pot pourri" of reagents had no effect upon accumulation of ^{14}C from the substrate U-^{14}C-F6P.Na^+, so there was no implication for sucrose phosphate synthase in uptake.

These data reflect the loss of selective uptake in these vacuoles isolated from stored berries; such an investigation of group transport, with analysis of labelled products, should be conducted upon vacuoles isolated from fresh berries.

11.4 General Discussion

Protoplasts isolated from the skin of grape berries may be stored at 4°C for days or even weeks and remain viable as evidenced by fluorescein di-acetate (9.4.2), exclusion of Evans Blue, and maintenance of sufficient pH gradients at the tonoplast for Neutral Red to accumulate. Isolated vacuoles are less stable, but have been observed to keep at 4°C for 7 h without loss; accumulation of Neutral Red and a highly acid pH in the cell saphave been demonstrated (Chapter 9).

Such protoplasts and vacuoles will take up glucose, fructose and sucrose as indicated by tracer techniques. Is this merely passive diffusion across the relevant membranes?

In protoplasts, uptake from L-glucose was only 10% of that from D-glucose after 1 h. Molecules which were expected to have similar passive reflection coefficients at a membrane - namely D-glucose with sucrose - showed quite different uptake rates which were linear or diphasic with time, and each greater than L-glucose. 2-Deoxy-D-glucose reduced the uptake of ^{14}C-D-glucose at 30 or 60 min by 42%, while moderate hydrostatic pressure stimulated uptake. Fructose stimulated glucose uptake, but glucose did not affect fructose uptake. These pieces of evidence show that D-glucose transport into protoplasts is mediated; and sucrose uptake also is more rapid than expected of diffusion though the contribution of sucrose metabolism (e.g. inversion) on the outside or inside of the plasmalemma has yet to be considered.

In isolated vacuoles, uptake in some preparations may be substantially by diffusion: in Table 11.5, sucrose uptake was not significantly different from that of L-glucose, uptake from the charged sugar phosphates was less (as predicted for passive diffusion across a membrane). Yet in other preparations (and the difference is attributed to procedure rather than material) accumulation bears the characteristics of a mediated process: uptake from U-^{14}C-sucrose was in one instance 20 times that from ^{14}C-D-glucose; uptake of glucose and sucrose was six times greater in vacuoles than in protoplasts (compared per unit phenol); sucrose uptake was stimulated 260% with the sulfhydryl reagent cysteamine, stimulated also by Mn^{2+} and partially inhibited by a number of other compounds including glucose and fructose. So in many experiments vacuolar uptake had properties of a mediated process.

The enzymes sucrose synthase and sucrose phosphate synthase both contain essential SH-groups, and interact with Mn^{2+}, glucose and fructose. That exposure of the tonoplast to these compounds affects uptake of sucrose strengthens the thesis that these enzymes are involved in group transport of sucrose at this membrane.

Willenbrink and Doll (1979) found sucrose uptake in isolated beet vacuoles to be inhibited by raffinose and stimulated by ATP, while raffinose was slightly stimulatory in these vacuoles from grape pericarp and ATP was not. Metrizamide was without effect (data not presented), again in contrast to the 75% inhibition reported in beet vacuoles.

Despite the variability of the data, isolated protoplasts and vacuoles displayed patterns of difference in uptake. Generally radioactivity was accumulated more from glucose than sucrose in protoplasts, while the converse held for vacuoles. Uptake curves were diphasic with time. The first phase was greater and more rapid for glucose with protoplasts, and for sucrose with vacuoles (c.f. Figs. 11.3 and 11.10). Raffinose had no effect on sucrose uptake by protoplasts, but was perhaps stimulatory with vacuoles (c.f. Fig. 11.6 and Table 11.4). Finally, these experiments showed that uptake by vacuoles exceeded that by protoplasts (Figs. 11.11 and 11.12).

With respect to the hypothesis stated in the introduction there are several pertinent questions which unfortunately were not investigated due in part to the limited availability of plant material. For instance, does 2-deoxy-D-glucose compete with ^{14}C uptake by protoplasts from U-^{14}C-sucrose? What are the respective accumulation rates of ^{14}C-glucosyl- versus ^{14}C-fructosyl-sucrose, asymmetrically labelled? What are the relative rates of 3-O-Methyl-D-glucose and D-glucose uptake by protoplasts, and do these show similar pH-dependency (indicative of a pH effect upon transport rather than metabolism)? What efflux is observed from pre-loaded protoplasts and vacuoles? (With a companion pulse-chase analysis). What is the nature of uptake from sucrose phosphate, using both ^{14}C and ^{32}P labelling? Is vacuolar incorporation from γ-^{32}P-UTP and ^{14}C-uptake stoichiometric?

Nevertheless the results obtained are useful and are discussed later (Chapter 12) after the analysis of the identity of the radioactive compounds from some of these incubations.

CHAPTER 12: SUBSTRATE DEPENDENCY OF SUGAR UPTAKE BY PROTOPLASTS AND ISOLATED VACUOLES

SUBSTRATE DEPENDENCY OF SUGAR UPTAKE BY PROTOPLASTS AND ISOLATED VACUOLES

12.1 Introduction

The relationship between substrate concentration and rate of uptake of a radionuclide sugar by protoplasts and isolated vacuoles was examined in a series of experiments. Isotherms so derived may be described with certain constants such as k_D, J_{max} and K_T which facilitate design and interpretation of subsequent experiments to elucidate mechanisms responsible for substrate flux. Although the kinetics of a process cannot give conclusive evidence about the underlying mechanisms (Cram 1973) they do have indicative value in this respect.

Ideally, such studies are based upon initial rates, such as during 5 seconds (Kessler & Toggenburger 1979). This presents problems both of manipulation, and of analysis where the quantity to be assayed is extremely small. Hence, linearity of uptake with time is usually demonstrated to allow rate determination over a longer period which in turn is interpreted as an initial rate.

In previous experiments reasonable linearity of uptake was observed for up to one hour but substrate influx into different preparations of protoplasts and vacuoles varied. The chosen experimental design follows from the difficulties of preparing and handling large numbers of protoplasts or vacuoles.

12.2 Materials and Methods

In each experiment there were duplicate incubations of 30 min at 8 concentrations of sugar ($[S^o]$ = 1, 5, 20, 30, 30, 50, 100, 150 mM), plus at one concentration a time curve to assess linearity of flux during 30 min.

Berries were taken from late bunches of grapes cv. Muscat (5.4 - 5.8° Brix) except in expt. V where these berries had been stored at 4°C for 3 months.

Protoplasts were prepared as indicated in Fig. 9.3 and purified by repeated settling.

In expt. I, II, III the incubation (final volume 1 ml) contained respectively glucose, fructose or sucrose from 1 M stocks complemented with 1 M mannitol and water to 600 mOsmol; protoplast medium and about 2.5×10^5 protoplasts free of macerated cells but including up to 5 vacuoles per 100 protoplasts and some fine cellular debris. The relevant U-^{14}C-sugar was included at 1 µCi per incubation for the lower concentrations, and 2 µCi for for the 100 and 150 mM incubations. In each experiment at least 2 incubations of an alternative sugar were included as cross-references between the four protoplast preparations.

In expt. IV a large stock of protoplasts - about 30×10^6 - was prepared. An aliquot was kept at 4°C whilst the bulk was lysed to vacuoles (as indicated in the protocol: Fig. 9.4). This lysed suspension was used as a "vacuole preparation" without purification, though the lysing chemicals were diluted, and without inclusion of cysteamine·HCl. After 3 h, at completion of the experiment, the vacuole count was unchanged.

The incubation (final volume 1 ml) contained glucose, fructose or sucrose from 1 M stocks complemented with sufficient 1 M mannitol and water to give 700 mOsmol; 1 or 2 µCi of the relevant U-^{14}C-sugar; vacuole medium and about 2×10^5 vacuoles. Replicated incubations in 30 mM sugars of the conserved protoplasts allowed comparison of the two suspensions.

Expt. V was done three months later. Using berries stored for 3 months at 4°C, and with 5 mM cysteamine HCl in the medium, vacuoles were incubated in a sucrose concentration series without replication. Vacuoles were also incubated in 30 mM 3-^{3}H-glucose and 30 mM U-^{14}C-fructose, singly and in combination.

In all experiments protoplasts and vacuoles were quantified by haemacytrometry or by assaying phenolics (Method: Fig. 11.2).

As in other experiments (Chapter 11), incubation at 23°C in a modified 1.5 ml Eppendorf reaction tube was commenced by addition of protoplasts or vacuoles to the medium, and terminated by a 10 min centrifugation through a cold gradient of 7%/10% Ficoll in medium. Radioactivity of the entire pellet (50 µl) of the receiving cup was measured in 10 ml of ACS:toluene (2:1). The receiving cup was rinsed with 250 µl water which was then added to the counting vial.

12.3 Results and Discussion: Protoplasts

12.3.1 Data from protoplasts

Data for uptake against time are presented in Fig. 12.1: plotted time does not include any adjustment for the delay in attaining the effective centrifugation speed. For each preparation the phenol equivalence is shown.

With each sugar there was a rapid initial (<5 min) influx followed by a steady influx over the interval 5 - 30 min; the size of the initial influx is shown by extrapolation of the later steady rate to time zero. In suspension I, with 10 mM glucose, the Y-intercept was relatively low such that uptake was essentially linear from 0 - 30 min. In suspension II, with 30 mM fructose, uptake during 0 - 5 min was at least 1.7 times higher than during 5 - 10 min. In suspension III, with 30 mM sucrose, 50% of total uptake at 30 min was attributable to the period 0 - 5 min.

Radioactivity compartmented in 30 min from various substrate concentrations is presented in Fig. 12.2. These relationships are analysed in Figs. 12.2a and 12.3 as detailed later (12.3.4)

The rate of uptake of each sugar by protoplasts increased with increasing substrate concentration. From glucose and fructose the rate increased more at the lower concentrations (to 50 mM glucose and 30 mM fructose), then was linear with concentration. From sucrose, the relationship between accumulation at 30 min and substrate concentration was virtually linear. But the Eadie-Hofstee plot (Fig. 12.3) reveals systematic deviation from linearity at low molarities, suggesting that uptake at low molarities exceeded that attributable to the dominant linear process.

12.3.2 Efflux

In the analysis that follows no allowance is made for efflux as it was not measured.

Nor could one simply determine the specific activity of a pool from which efflux occurred, due to the dual compartmentation within protoplasts.

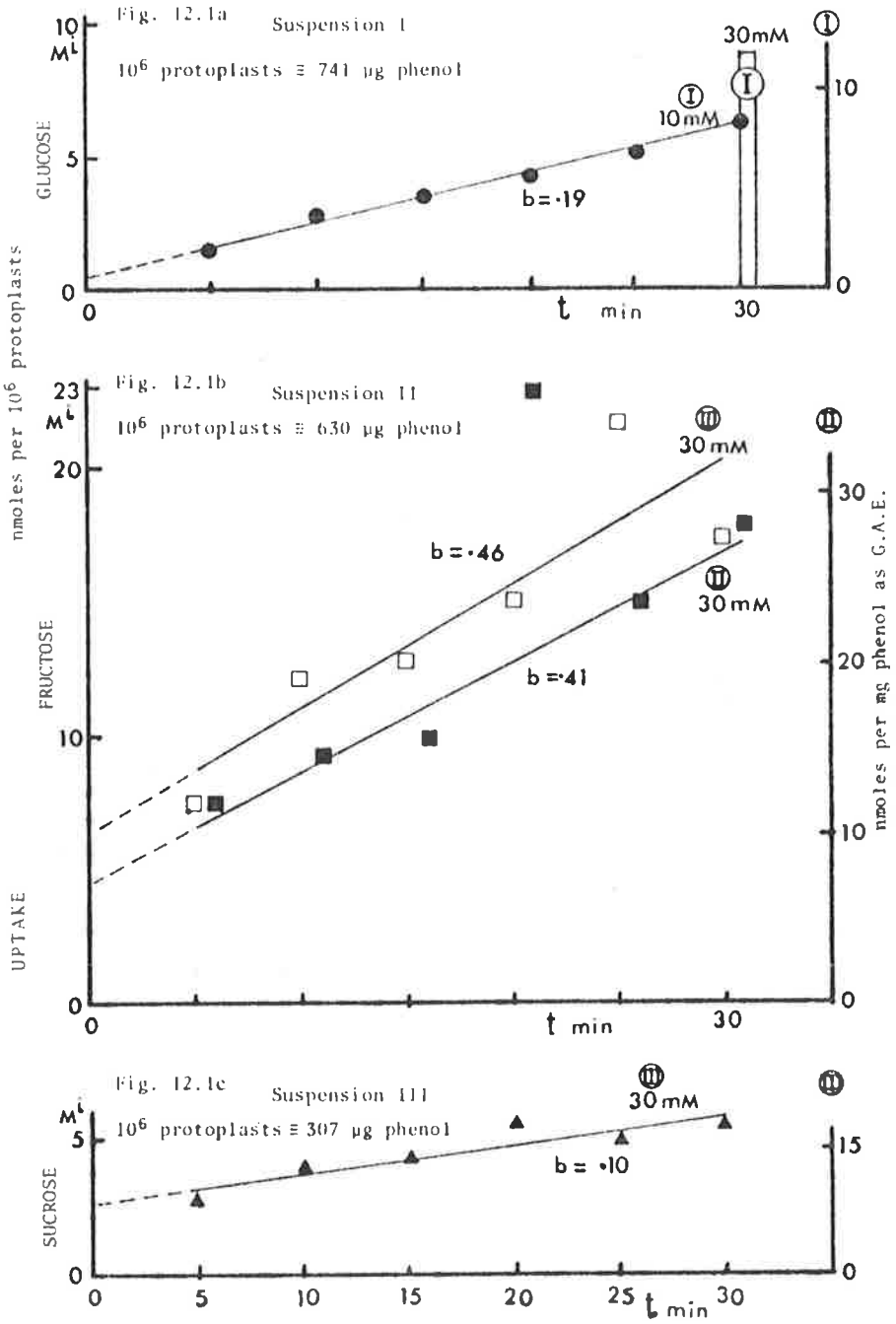


Fig. 12.1 Uptake of ^{14}C from (1a) U-^{14}C-glucose 10 mM; (1b) U-^{14}C-fructose 30 mM; (1c) U-^{14}C-sucrose 30 mM by 3 protoplast suspensions (I , II , III) at intervals to 31 min. Phenol content and slopes (b) are indicated.

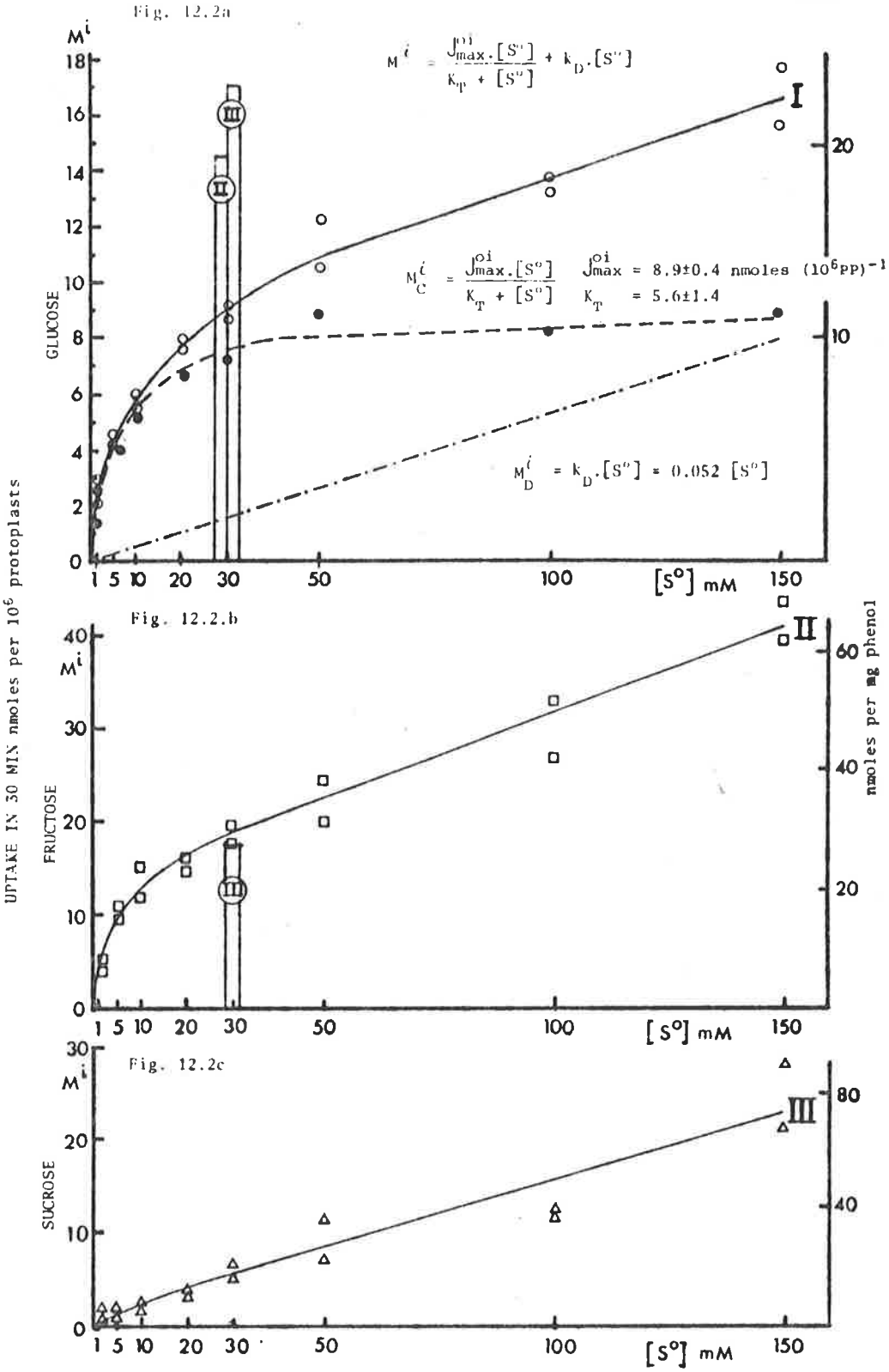


Fig. 12.2 Uptake in 30 min of ^{14}C from (2a) U-^{14}C-glucose (2b) U-^{14}C-fructose (2c) U-^{14}C-sucrose at different substrate concentrations by 3 protoplast suspensions (I, II, III). Histograms superimposed at 30 mM indicate uptake by alternative suspensions expressed as nmoles per 10^6 protoplasts (not per mg phenol). In 2a: the curve (—) fitted to the data (○) has been analysed to a linear component (–·–) and the mean residuals (●) fitt with a rectangular hyperbola (– –).

The concentrations of "compartmented" sugars in hypodermal cells of the berry, as used here, were c. 40 mM glucose and 10 mM fructose with negligible sucrose (Chapter 8). The fate of compartmented sugars during protoplast preparation is not known - though glucose has been measured in protoplast suspensions.

12.3.3 The nature of "zero-time uptake"

The major problem of interpretation is that the sampling period, 30 min, embraced two phases of uptake. Such diphasic curves have been reported in protoplasts uptake studies (Guy et al. 1978). The data are inadequate in this respect. While accumulation may be described by certain parameters, which phase determines which parameter?

Extrapolation of the slow phase to the Y-axis (termed "zero-time uptake") gave intercepts of 8, 30 and 47% of observed uptake at 30 min for glucose (10 mM), fructose and sucrose (30 mM) respectively (Fig. 12.1). What is the nature of zero-time uptake?

It is not attributed to ineffective separation of the incubation medium and protoplasts, being constant amongst the replicates. Nor did isotope sediment when medium alone was centrifuged over the Ficoll gradient. The lag period during which the centrifuge reaches an effective speed would account for only some of this. Boller et al. (1975) determined the lag as 0.3 min in a similar system.

There are three possible mechanisms:

(i) the incubation medium rapidly established equilibrium with a freely permeable volume external to the site of slower accumulation, across a semi-permeable membrane such that the process was non-selective amongst sugars and showed linear dependency upon concentration. In this volume diffusion was sufficiently restricted that the substrate was not removed in the Ficoll gradient, and was to some extent trapped in the pellet. Evidence is presented elsewhere (Paragraph 11.7) for recovery of a highly diffusive species, ^3H_2O, from such a centrifugation; (this molecule has a diffusion coefficient 6 times higher than, for example, sucrose: Arnold 1968).

(ii) Substrate binds to material by a mechanism not involving transport, and is pelleted by the methods used.

The following evidence, from other experiments, supports this and the first postulate:

- (Fig. 11.10) radioactivity was pelleted from sonicated vacuoles
- (Fig. 11.3) zero-time uptake from L-glucose and sucrose was similar; the phenomenon is not selective between these two sugars
- (Fig. 11.5) whereas 2-deoxy-glucose inhibited uptake from D-glucose it did not change the Y-intercept
- (Fig. 11.3) uptake of L-glucose was unchanged between 12 and 62 min. This indicates rapid equilibration with a certain volume or binding capacity.

On the other hand, as radioactivity was recovered from protoplasts principally as free sugars (Chapter 13) covalent binding was not a major contribution. Moreover, low affinity binding by low MW compounds have $t_{\frac{1}{2}}$ of less than a second (Carafoli and Semenza 1979) so any substrate on external binding sites of protoplasts would be removed during the washing procedure.

Also, most of the radioactivity was not recovered as ^{14}C-sucrose from incubation of protoplasts in that medium, which would have been expected from the first mechanism.

(iii) A third possible mechanism is a potentiated uptake system at a perm-selective membrane whereby rapid initial influx occurs with dissipation of an energy potential, or isotope influx occurs by exchange diffusion. Whilst the rate of a mediated process may be concentration dependent, the end-point may be independent of concentration - determined rather by the initial "potential".

The evidence for this, in Fig. 11.3, is the low value for sucrose relative to glucose after a 2 min incubation, indicative of a selective rapid initial uptake. Moreover the zero-time uptake from glucose is double that from sucrose.

Though non-specific uptake into a semipermeable volume (i) and non-specific binding (ii) may account for some sedimentation of radioactivity with protoplasts, it is concluded that a rapid phase of potentiated uptake (iii) exists. This capacity is rapidly dissipated upon addition of sugar while a slower process persists.

12.3.4 Algebraic description of accumulation

Given the inadequacies mentioned above, uptake at 30 min is analysed as a "black box", a net uptake with ill-defined contributors yet concentration dependent, for each sugar, with curvilinear and linear phases (Fig. 12.2). The analysis of this relationship for glucose is given as typical (Fig. 12.2a). A rectangular hyperbola was fitted to the unweighted data using an iterative computer program for non-linear least squares regression, to describe the relationship between uptake after 30 min (m^i, units of amount) and glucose concentration outside ($[S^o]$) in the form of a Michaelis-Menten equation:

$$m^i = \frac{k_1.[S^o]}{k_2 + [S^o]} \qquad \text{Eq. 12.1}$$

Superscripts i and o indicate inside and outside. The fit was poor, with systematic deviation at low $[S^o]$.

Transport data has commonly been resolved into two components, linear and hyperbolic (Neame and Richards 1972; Blackman and McDaniel 1978; Willenbrink and Doll 1979a). The linear phase is interpreted as describing a process of diffusion, assumed to remain linear with $[S^o]$ over the entire range of the analysis, and passing through the origin. Substracting this component from observed transport gives residual values attributed to a second process. This applies to parallel uptake (each linear with time) across a single membrane. (Systems described by the Torii-Laties hypothesis, with two transport processes in series being rate-limiting over different concentration ranges, are not simply additive in this manner. Refer Chapter 6, Lüttge and Higinbotham 1979.) To emphasize the limitation imposed by the present data, accumulation rather than rate of flux is the factor analysed. Despite this shortcoming, the conventional symbols are retained for the exercise: namely, J^{oi}_{max}, the maximal influx of a saturable component; K_T, a constant related to the affinity of the same; and k_D, a constant of non-saturating (diffusive) influx.

In the present experiment uptake at higher $[S^o]$ (50 to 150 mM glucose) appeared linear, such that the component of uptake attributable to passive diffusion, m^i_D, could be described $m^i_D = k_D.[S^o]$ with slope k_D = 0.052 µl (10^6 protoplasts)$^{-1}$. As shall be seen, the Y-intercept of that linear phase is an estimate of the saturation uptake by a second (hyperbolic) process.

Subtracting the linear process from observed values establishes a residual uptake, m_C^i which fits a rectangular hyperbola and may be described in a form common with mediated transport:

$$m_C^i = \frac{J_{max}^{oi}.[S^o]}{K_T + [S^o]} \qquad \text{(at t = 30 min)} \qquad \text{Eq. 12.2}$$

where J_{max}^{oi} = 8.9 ± 0.4 nmoles glucose $(10^6$ protoplasts$)^{-1}$ in 30 min and K_T = 5.6 ± 1.4 mM for glucose.

The observed uptake is described in a unified equation:

$$m^i = m_C^i + m_D^i = \frac{J_{max}^{oi}.[S^o]}{K_T + [S^o]} + k_D.[S^o] \qquad \text{(at t = 30 min)} \qquad \text{Eq. 12.3}$$

The determined values are indicated in Table 12.1 for glucose and fructose.

Table 12.1 The relationship between ^{14}C uptake by 10^6 protoplasts and $[S^o]$ analysed into two concentration-dependent components - linear and hyperbolic:

	K_T mM	J_{max}^{oi} (1) during 30 min nmoles $(10^6$ protoplast$)^{-1}$	k_D	Y-intercept of linear phase nmoles $(10^6$ protoplast)	$\frac{m_D^i}{m_C^i}$ (2) (fitted values) at 30 mM
GLUCOSE	5.6 ± 1.4	8.9 ± 0.4	0.052	8.5	0.21
FRUCTOSE	1.7 ± 0.4	12.8 ± 0.4	0.188	12.4	0.47
SUCROSE	(1.3 ± 0.4)	(0.8 ± 0.05)	0.142	1.3	5.4

(1) the exercise is such that J is only valid for these data

(2) ratio of uptake by diffusion to carrier-mediated uptake

Both diffusive and mediated efflux will occur during incubation and cleaning. The uptake equation is more precisely:

$$m^i = J_{max} \frac{[S^o]}{K_T + [S^o]} - \frac{[S^i]}{K_T + [S^i]} + k_D\,([S^o] - [S^i]) \qquad \text{(at t = 30 min)} \qquad \text{Eq. 12.4}$$

Furthermore, Neame and Richards (1972) showed that a transport process has a higher K_m when there are similar substrate concentrations on each side of the membrane than in an asymmetrical system. Ignoring efflux particularly causes underestimation of J_{max}.

Nevertheless, these are good indications that mediated transport exists for glucose and fructose into protoplasts prepared from grape skin with respective K_T of 5.6 and 1.7 mM; further, fructose transport has a higher maximal rate (it was shown in Chapter 8 that skin is a fructose accumulator in vivo).

The relationship $m^{\dot{i}}$ against $[S^o]$ was almost linear for sucrose with systematic deviation at low $[S^o]$ readily apparent in an Eadie-Hofstee plot, $m^{\dot{i}}$ against $m^{\dot{i}}/[S^o]$ (Fig. 12.3).

Dowd and Riggs (1965) demonstrated the weakness of different linear transformations of the Michaelis-Menten equation, a matter treated also by Neame and Richards (1972). The Eadie-Hofstee plot is one of the better transformations, less prone to deceptive fit and emphasizing errors in measurement of v (in this case, $m^{\dot{i}}$). Incubations at low $[S^o]$ were of high specific activity, yielding high counts and particularly good replication. In the insert of Fig. 12.3 $m^{\dot{i}}_C$ is analysed at [S] = 1, 5, 10, 20 mM sucrose, yielding the constants which of course agree with those from the iterative computer program in Table 12.1. These constants, K_T = 1.3 and J^{oi}_{max} = 0.8, may describe uptake of sucrose itself or of hexoses present in trace amounts.

Various K_T have been published for active sucrose uptake (Table 5.2): 8 mM by sugarbeet vacuoles (Cholodova 1973 using elution rate analysis); 22 mM by isolated red beet vacuoles (Willenbrink and Doll 1979); 30 mM by isolated mesophyll cells of N. tabacum (Cataldo 1974).

From the hyperbolae describing mediated uptake of hexoses (Table 12.1) one obtains rates very similar to those evident in the slope of the time curves (Fig. 12.1): respectively 5.7 and 5.6 nmoles at 10 mM glucose, 12.1 and 12.3 nmoles at 30 mM fructose.

12.3.5 And the physical basis?

Equation 12.3 (in the previous section) describes the uptake in two components which is to be observed where diffusion and mediated transport occur in parallel at a limiting membrane. Such was the interpretation of similar kinetics by Willenbrink and Doll (1979). But the unified equation 12.3 cannot be converted to a rate equation (substituting v for $m^{\dot{i}}$ and generalizing the time interval of J_{max}) without first demonstrating the simple time dependence of each component. Willenbrink and Doll have not adequately done so. Their data and these results using ^{14}C might be explained without reference to any mediated transport as follows.

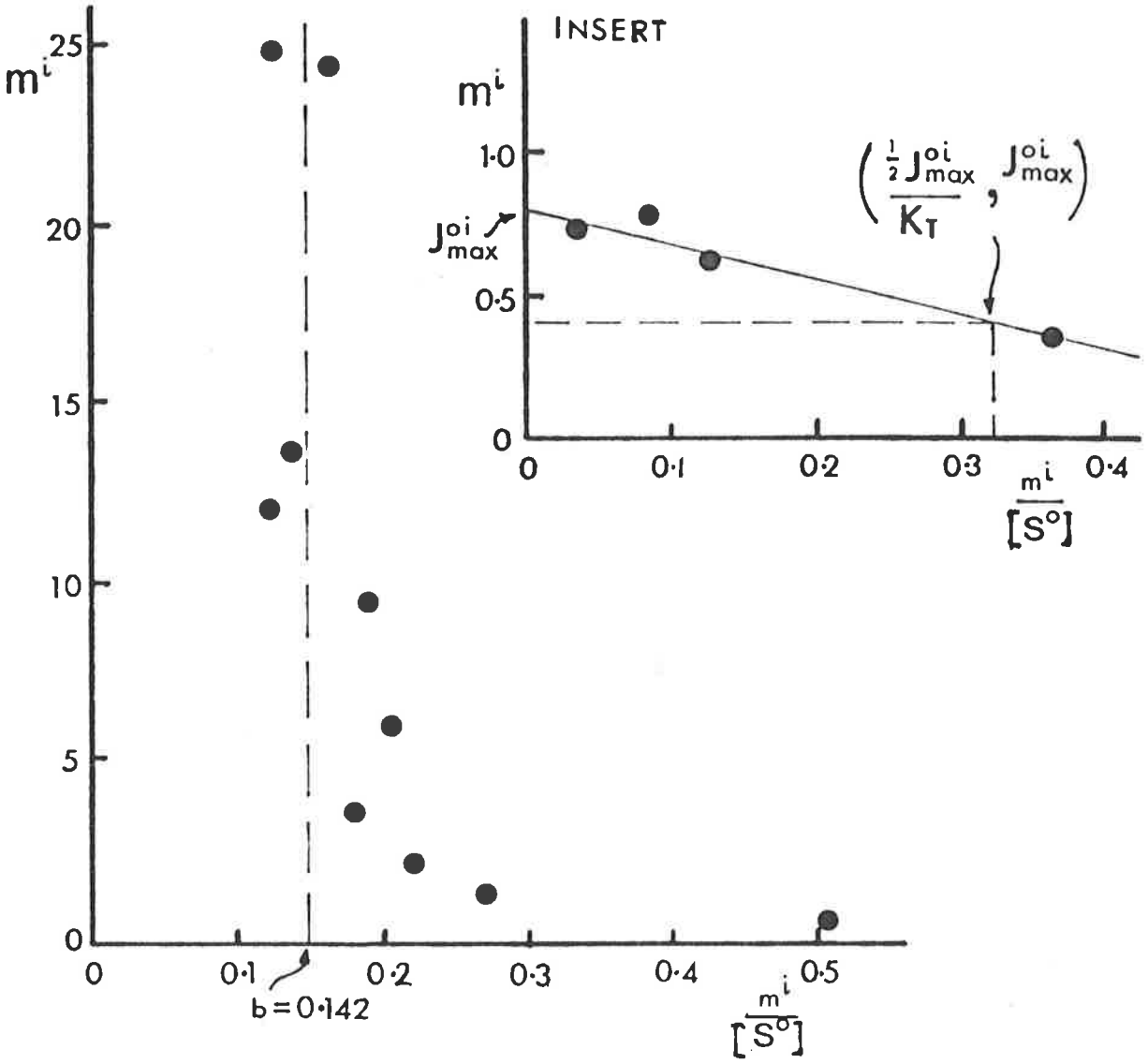


Fig. 12.3 Data from Fig. 12.2c: protoplasts in U-^{14}C-sucrose, transformed as an Eadie-Hofstee plot to demonstrate the multiphasic uptake.

Insert: Subtracting the linear phase (slope = 0.142) yields residuals which are analysed for constants, J^{oi}_{max} and K_T.
Each point is the mean of duplicates.

Consider a compartment whose limiting membrane is highly permeable to a substrate such that equilibrium is reached rapidly. Within the compartment exists an irreversible system demonstrating saturation kinetics capable of sequestering the substrate (as metabolic trapping). As the internalised substrate is sequestered the activity gradient at the membrane causes influx by diffusion.

If accumulation is assessed at a time interval longer than that required for diffusive equilibrium, apparent uptake of ^{14}C will be determined not by the transport rate but the internal volume and the sequestering process Thus from such analysis as Fig. 12.2a (or Willenbrink's Fig. 2) erroneous conclusions concerning transport may be drawn unless rates are clearly determined to be initial rates. In assaying internalised S chemically the transport process will not be confounded with subsequent substrate modification though the possibility of a vectorial group-translocator has then to be considered separately. Willenbrink has not been explicit as to which techniques - radiochemical or direct assays - served in his various experiments, and has not convincingly distinguished rates and uptake.

However, uptake by diffusion with metabolic trapping is rejected upon analysis of the radiochemical products (Chapter 13). The rate of metabolism of these sugars is much less than that necessary for such a process (e.g. Table 12.1, reference (2)).

Two propositions to consider with respect to hexose uptake in protoplasts are:

A. In a protoplast suspension every protoplast presents a permeable volume (cytoplasm) and an inner permselective volume into which mediated transport occurs.

B. In a protoplast suspension two subpopulations are distinguished - those semipermeable*, and those permselective* in which mediated transport may be observed.

How might one test these propositions?

In the relationship $m^i = k_D.[S^o]$ the constant k_D is directly related to the permeable volume per 10^6 protoplasts: if this volume is an inherent feature of protoplasts, it will show little variation between preparations. If on the other hand k_D varies appreciably between preparations, the permeable volume is varying and this should be treated as an artefact of isolation.

*defined in 4.3

Furthermore, for A the permeable volume and the number of transport sites per 10^6 protoplasts will tend to be constant. Conversely, for B the subpopulations are complementary and preparations with greater permeable volume will exhibit lesser mediated uptake.

Using different protoplast preparations, the relevant indices derived from identical incubations will vary as shown in Fig. 12.4.

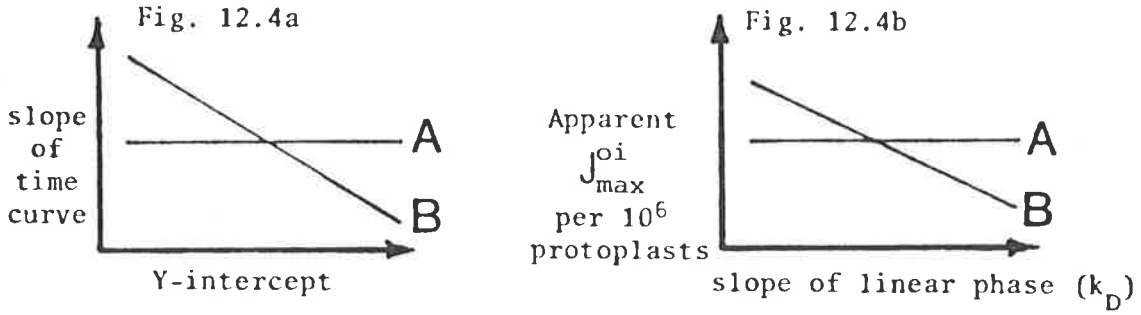


Fig. 12.4 Is the rapidly permeable volume an attribute of each protoplast (A) or of a subpopulation (B)? Indices derive from time curves (4a) or concentration series (4b).

Such data are not available, but there is modest evidence for proposition B - that a subpopulation of semipermeable protoplasts are present. The permeable volume varied considerably between preparations: from the zero-time uptake of 10 mM glucose and 30 mM fructose permeable volumes are calculated as 0.06, 0.15 and 0.22 µl (10^6 protoplasts $)^{-1}$ for preparations I, II and III; similarly the slopes k_D were 0.052, 0.188 and 0.142.

This permeable volume represents little of the predicted volume of protoplasts.

12.4 Results and Discussion: Vacuoles

12.4.1 Data from vacuoles

A protoplast stock of 35.4 x 10^6 was lysed to produce 15 x 10^6 vacuoles, with 1.4 x 10^6 protoplasts unlysed. The vacuole incubations reported here contained less than 10% protoplasts by count, and much subcellular material from protoplast lysis.

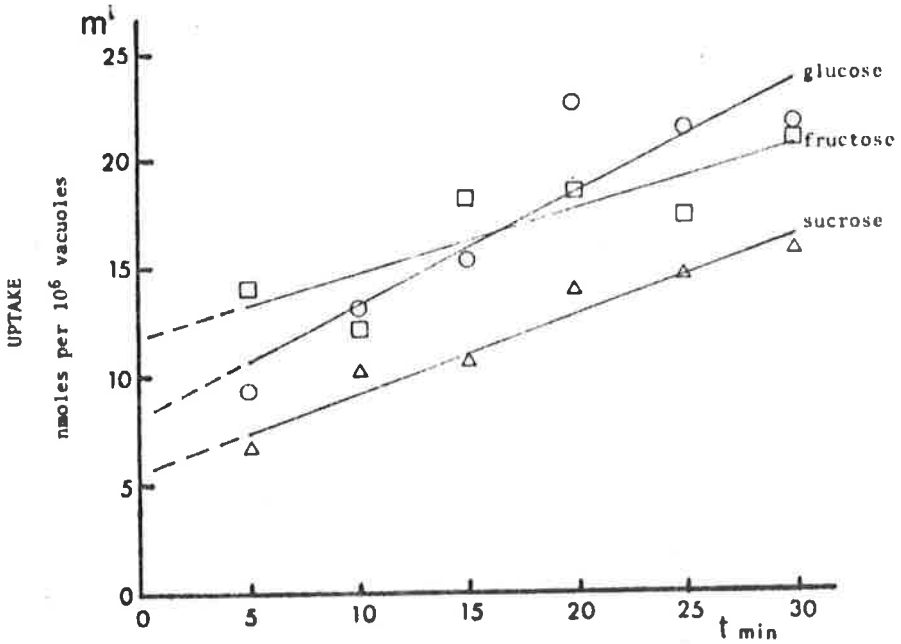


Fig. 12.5 Uptake of ^{14}C from U-^{14}C-glucose 30 mM (○), U-^{14}C-fructose 30 mM (□), U-^{14}C-sucrose 30 mM (△) by a vacuole suspension (IV) at intervals to 30 min. 10^6 vacuoles contain 940 µg phenols.

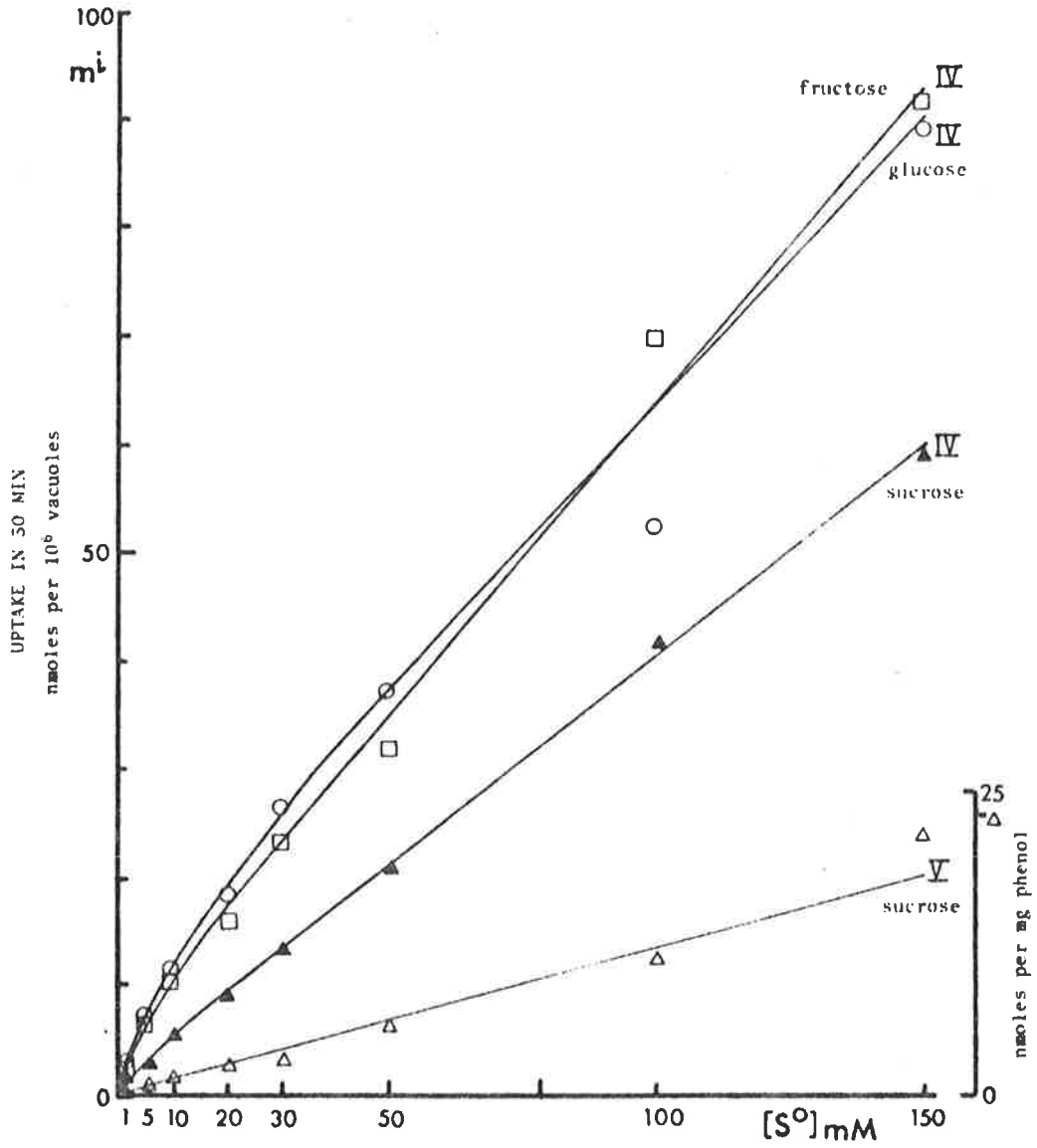


Fig. 12.6 Uptake in 30 min of ^{14}C from U-^{14}C-glucose (○), U-^{14}C-fructose (□), U-^{14}C-sucrose (▲) (△) at different substrate concentrations by 2 vacuole suspensions (IV, V).

Uptake by suspension IV: each point is mean of duplicate incubation.

Uptake by suspension V alone relates to the ordinate "nmoles per mg phenol" as numbers were not assessed: points unreplicated.

The analysis of uptake by vacuoles bears many similarities to the preceding analysis for protoplasts. ^{14}C uptake by vacuoles from the 3 sugars increased during the interval 5 to 30 min (Fig. 12.5) with the hexose incubations showing particular variability. Uptake (nmoles) from hexoses exceeded uptake from sucrose. Uptake versus time would more correctly be represented by parabolae passing through the origin, but extrapolated lines give an estimate of the rapid initial uptake (as in Fig. 12.1).

Clearly the rate of uptake was not constant from 0 to 30 min. Thus the relationship between uptake and substrate concentration (Fig. 12.6) compounds changes in the rapid initial rate and in the slower phase. The overall relationship appears linear with notable deviation from linearity at low $[S^o]$ which is more evident in the transformed data of an Hanes plot (Fig. 12.7). As in the previous section, accumulation is described by two components, one diffusive (linear) uptake and one mediated uptake showing saturation kinetics.

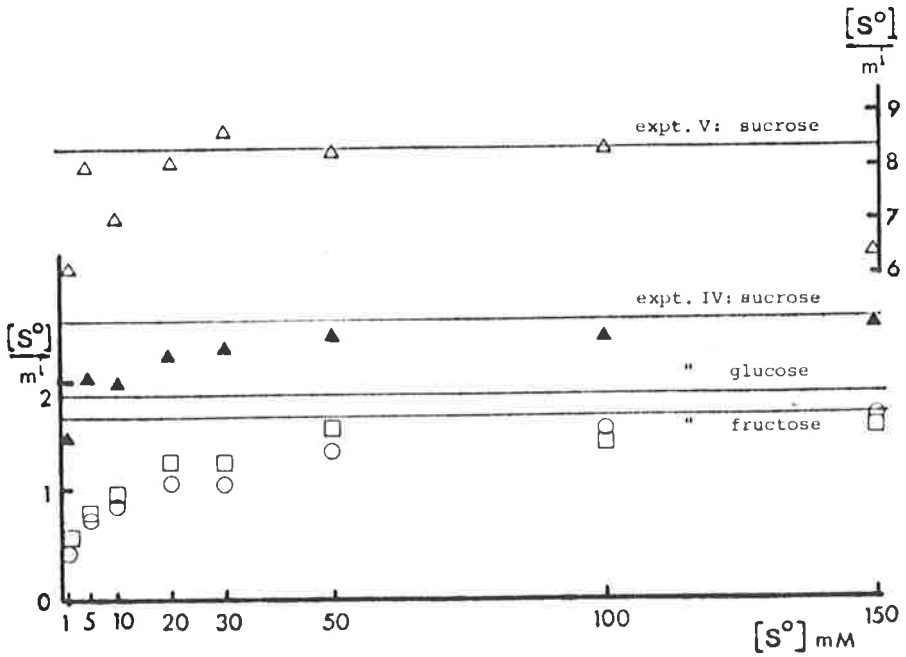


Fig. 12.7 Data from Fig. 12.5 transformed as an Hanes plot to demonstrate the multiphasic uptake by vacuoles of ^{14}C from U-^{14}C-glucose (O), U-^{14}C-fructose (□), U-^{14}C-sucrose (expt. IV: ▲; expt. V: Δ).

Diffusive uptake is represented by lines, parallel to the abscissa, of intercept $1/k_D$.

Plotted data is uptake per 10^6 vacuoles for expt. IV; per mg phenol for expt. V.

The curvilinear relationship could not be precisely described, despite the good replication at low $[S^o]$, because the linear process predominated and insufficient molarities were assessed. Hyperbolae fitting the residual values ($m^{\dot{i}} - k_D.[S^o]$) for $[S^o]$ = 1, 5, 10, 20, 30 mM indicated first approximations of K_T and J_{max} as in Table 12.2.

Table 12.2 Constants of the hyperbolic component of ^{14}C uptake by 10^6 vacuoles against substrate concentration.

Substrate	K_T	J_{max}^{oi} during 30 min
	mM	nmoles(10^6 vacuoles)$^{-1}$
GLUCOSE	10.3 ± 1.9	12.6 ± 0.8
FRUCTOSE	2.8 ± 1.3	5.5 ± 0.6
SUCROSE*	(8.0 ± 3.9)	(1.8 ± 0.3)

*values for sucrose are particularly tentative

12.4.2 The relative rates of accumulation

Accumulation by protoplasts and vacuoles is compared in Fig. 12.8. The hyperbolae defined by the constants J_{max}^{oi} and K_T are indicated in Fig. 12.9. Though these values are tentative, and would vary between preparations, their relationship to one another is instructive.

The accumulation of glucose and fructose increased with $[S^o]$ at a greater rate than that of sucrose. Moreover this sucrose rate varied between expt. IV using vacuoles from fresh berries and expt. V using vacuoles, from stored berries, in a medium with cysteamine.HCl. The relative rates of the sugars did not change between preparations IV and V (Fig. 12.8). Protoplasts from preparation IV accumulated ^{14}C more rapidly from 30 mM glucose, contrary to the previous experiments.

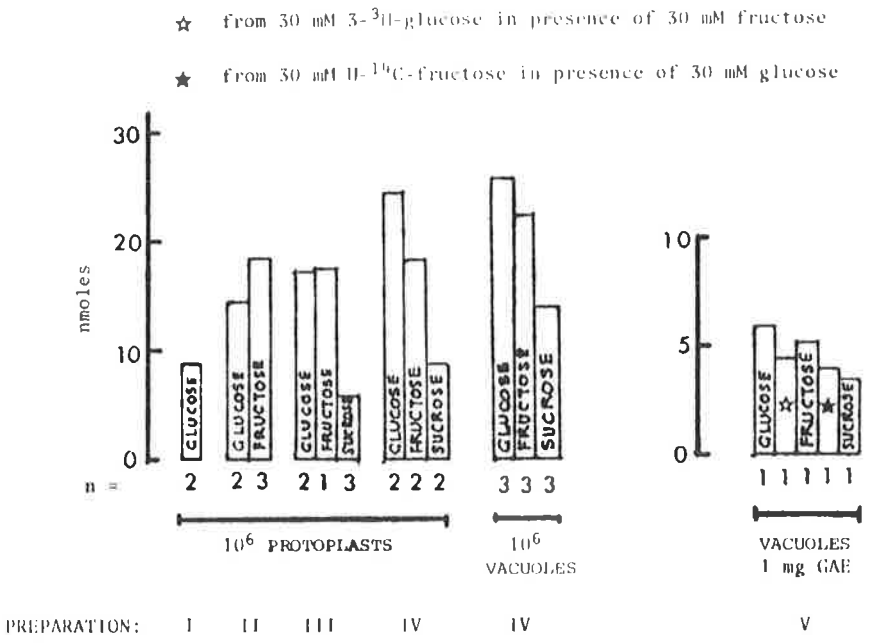


Fig. 12.8 Comparative uptake in 30 min of ^{14}C from U-^{14}C-sugars as indicated, each at 30 mM, by protoplasts or vacuoles of five preparations.

n = No. of incubations contributing to the mean shown. 5 blocks (left) related to count; 1 block (right) related to phenols.

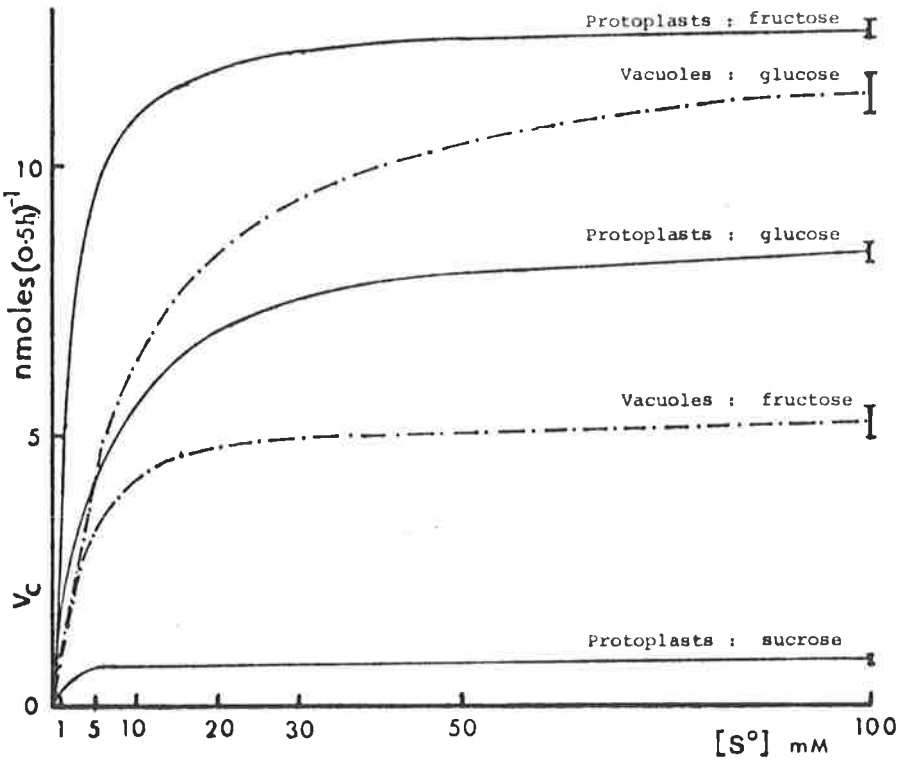


Fig. 12.9 Hyperbolae defined by the constants J_{max}^{oi} and K_T describing a saturable component of ^{14}C uptake from glucose, fructose and sucrose by 10^6 protoplasts (—) or 10^6 isolated vacuoles (· — ·). Standard deviation indicated (I)
1 x 10^6 protoplasts lysed to 2.3 x 10^6 vacuoles.

12.4.3 Zero-time uptake may have a different source in vacuoles

Zero-time uptake by vacuoles (Fig. 12.5) did not account for all the linear component ($k_D.[S^o]$) of the accumulation equation. Furthermore the slopes of uptake against time for each sugar were much more than the rates expected of the mediated process (Table 12.3).

Table 12.3 Sugar uptake by vacuoles

Substrate:	Rate from slope of time curve at 30 min	Rate of mediated process alone, from hyperbolae at 30 min
	$m\dot{\ell}\,(min)^{-1}$	
30 mM glucose	0.52	0.31
30 mM fructose	0.29	0.17
30 mM sucrose	0.36	0.05

Unlike the protoplast result, the linear and hyperbolic processes must be coincident in the interval beyond 5 min.

Steudle and Zimmermann (1974) showed the reflection coefficients of glucose and sucrose to be very similar in Nitella flexilis. The slower ^{14}C uptake by vacuoles from sucrose reflects not only physical properties of that molecule but selectivity of the tonoplast.

Uptake of each hexose was similar for protoplasts and vacuoles in their respective media while sucrose uptake by vacuoles was 1.7 times that of the source protoplasts (expt. IV, Fig. 12.7). Assessed as C-6 units, accumulation by protoplasts was more rapid from the hexoses; by vacuoles it was most rapid from sucrose.

As observed with protoplasts, it is difficult to explain the mechanism by which accumulated substrate was retained during the washing procedure, given the apparent permeability of the membranes. The recovered counts might indeed have been a small part of compartmented counts with a major portion lost during cleaning - in which case a better procedure is required. Yet one would have expected unmanageable variability of data in this case.

Similarly tissue which accumulates hexoses *in vivo* to more than 1 M (Coombe 1976a) is expected to show compartmentation without significant "leakage". Thus, the rapid initial uptake is either an isotope effect, or is essentially unidirectional, or is a consequence of the *in vitro* procedures.

If sugars passed across the tonoplast by an exchange mechanism, subject to a trans effect, one could explain the uptake of isotope and its subsequent retention (in a cleaning medium). Such processes have been reported (Ussing 1947; Heinz 1978). Saturation kinetics would be expected.

Bange (1979) proposed a model with negative cooperativity arising from movement of substrate particles both in the bound and the free state through a membrane channel with oscillating binding sites. Multiphasic kinetics are predicted. Aspects of this "common-site" model are relevant: direct interaction of the diffusive and mediated processes is possible, and control of the latter may affect the former.

12.5 Conclusions

In both protoplasts and vacuoles isolated from grape pericarp, accumulation of ^{14}C from uniformly labelled glucose, fructose and sucrose was concentration dependent over the range 1 to 150 mM. During a 30 min incubation the rate of uptake changed from a rapid to a slower phase. The following relationship between uptake at 30 min ($m^{\dot{i}}$) and substrate concentration ($[S^o]$ held:

$$m^{\dot{i}} = \frac{J^{oi}_{max}.[S^o]}{K_T + [S^o]} + k_D.[S^o] \qquad \text{Eq. 12.3}$$

Where J^{oi}_{max} is a constant characteristic of saturable (non-linear) flux(es), K_T is a constant related to the affinity of the same, and k_D is a constant of a non-saturating influx.

Although this equation describes the data from these experiments, the constants, especially J, may be quite different in repeated experimentation, because changing rates of flux contribute to uptake at 30 min i.e. the constants will be affected by sampling time. Nevertheless the kinetic constants of the non-linear process were determined for protoplasts (Table 12.1) and vacuoles (Table 12.2). The two K_T for fructose, into protoplasts and into vacuoles, were not significantly different (1.7 ± 0.4 and 2.8 ± 1.3 mM respectively) and could have arisen from the same interaction

at the tonoplast with transport across the plasmalemma being by passive diffusion alone.

In vacuoles accumulation was dominated by a non-saturating uptake determined by substrate concentration.

For each sugar tested, protoplasts and vacuoles accumulated radioactivity at similar rates at 30 mM but, at higher [S], uptake by vacuoles was more than by protoplasts.

The kinetics of uptake by protoplasts and vacuoles were consistent with two vectorial processes, one carrier-mediated and one not saturable. The latter varied between preparations and could be an *in vitro* phenomenon. On the other hand, given the diffusion of sugars from excised skin (Chapter 8) this non-saturating process may also occur *in vivo* but with some manner of regulation (12.4.3).

In these experiments with vacuoles molar sucrose uptake was less than that of the hexoses. Results presented in Fig. 11.10 show molar sucrose uptake greater than that of the hexoses, while in Figs. 11.11 and 12 the rates are similar to one another. Together these demonstrate the "susceptible" nature of sucrose uptake (see 11.3.8).

It is evident that:

(i) sugar uptake by protoplasts and vacuoles from grape pericarp should be examined in the concentration range 0.1 - 20 mM

(ii) the rapid initial uptake requires to be characterised by samplings at <5 min over a range of [S], and with identification of the radiochemical products

(iii) the slope of the slower phase, 5 - 30 min, against [S] should be defined with at least 6 points (5, 10, 15, 20, 25, 30 min) and analysed separately from the rapid initial uptake.

CHAPTER 13: RADIOCHEMICAL LABELLING FROM THE INCUBATION OF SKIN SEGMENTS, PROTOPLASTS AND VACUOLES OF THE GRAPE BERRY

RADIOCHEMICAL LABELLING FROM THE INCUBATION OF SKIN SEGMENTS, PROTOPLASTS AND VACUOLES OF THE GRAPE BERRY

13.1 Introduction

The grape berry accumulates free glucose and fructose. In an initial hypothesis (Chapter 11) it was proposed that this accumulation within the vacuole involved both sucrose hydrolysis in the apoplasm and resynthesis of sucrose or sucrose phosphate in the cytoplasm.

From ^{14}C studies upon grape berries (reviewed in 2.2) it was evident that during the first growth phase diverse metabolism of glucose occurs (such as to organic acids) whilst in the second growth phase accumulation as hexoses was dominant.

Hardy (1967, 1968) administered labelled sugars via the pedicel of excised berries in both growth cycles (cv. Sultana) to find that sucrose was rapidly converted to hexoses; fructose mostly remained as such whereas from glucose he recovered equal amounts of labelled glucose and fructose. Small amounts of sucrose were synthesized by the berries during both growth stages.

Thus it was expected that from uptake studies upon the ripening grape berry *in vitro* labelled compounds would be recovered which were indicative of the accumulation process itself rather than of the various pathways of carbohydrate catabolism (reviewed in Chapter 3).

Accumulation of unlabelled glucose by excised skin of grape berries was approximately linear for 44 h (Coombe & Matile 1980). In contrast uptake of radiochemical sugars by isolated protoplasts and vacuoles was sometimes linear during 1 h but more often diphasic (e.g. Fig. 11.4).

An indication was sought as to the compartmentation and the sites of metabolism of radiochemical sugars by an efflux analysis developed from that described in Chapter 8. Preliminary evidence indicated that rapid hydrolysis of labelled sucrose occurred during incubation of skin segments, and resultant hexoses were recoverable in a fast efflux fraction.

In view of the stated hypothesis and the possibility of distinguishing diffusible and residual fractions, excised skin incubated in U-^{14}C-glucose and U-^{14}C-fructose was analysed. The following questions were posed: Is the asymmetry of labelling in sucrose consistent with the ratio of labelling in compartmented hexoses as predicted where the latter derive from the former? Does interconversion of hexoses occur and is this associated with compartmentation? What other labelled compounds are evident? Are these apparent intermediates or do they accumulate?

Similarly, the distribution of label was assessed in protoplasts and vacuoles incubated in radioactive sugars (reported in Chapter 11). With protoplasts, extracellular material was either absent or did not sediment through the Ficoll cleaning gradient. With vacuoles, residual cytoplasmic material did not sediment through the gradient. Additional questions were posed with respect to these preparations: What is the nature of the rapid initial uptake commonly observed (Chapter 12)? Are there intermediates common to uptake from glucose, fructose and sucrose, and how do these relate to rate of ^{14}C accumulation? What sugar phosphates are labelled? Is there evidence of perturbed metabolism contrasting with that in skin segments?

Results and Discussion are presented in three sections pertaining to excised skins, to analysis by electrophoresis, and to protoplasts and vacuoles.

13.2 Materials and Methods

Paper chromatography: Aliquots were streaked onto Whatman 3MM paper as 40 mm zones, separated by 40 mm, and developed for 16 h by descending ethyl acetate:pyridine:water, 10:4:3. Samples from the incubation of protoplasts or vacuoles were applied without prior partitioning. To each side of the paper a marker was applied, containing 5 µg sucrose, 1 µg glucose and 1 µg fructose. Mobilities were expressed relative to fructose i.e. $R_{FRUCTOSE}$.

Electrophoresis: Whatman 3MM paper was cut 150 x 570 mm, retaining the machine direction of the paper. An origin was indicated at the sides 150 mm from one end without marking across the surface. Samples were applied on 10 mm fronts with 10 mm spacing and 20 mm at the edges. A combination marker (1 µl) was spotted 10 mm from the sides. This contained 2-deoxyadenosine, ATP, P_i, PP_i, sodium phytate, fructose, xylene-cyanol blue and orange G.

Papers were run in a glazed ceramic tank with baffles as described by Tate (1968), using perclene as insulator and water coils to cool. The spotted paper was immersed in buffer from one end up to within 20 mm of the origin and blotted; this was repeated from the other end, taking care not to blot or blur the origin. Once the buffer had absorbed up to the origin, the paper was mounted upon a glass A-frame, inverted, and immersed in the perclene to abutt at each end against the ceramic baffles.

A wick of 3 MM paper linked the electrophoretogramme with 125 ml of buffer in reservoirs behind each baffle. Electrodes were inserted here and 2000 V applied, or sometimes less, keeping V.I below 3000 units. Running time was 30 - 60 min. Upon completion the paper was dried before a fan and inspected under U.V.

The following buffers were used:

- 0.05 M sodium borate pH 9.2 : 19.07 g l^{-1} sodium tetraborate in d.d. water. Attention is given to remove any borate buffer from both the unit and the sample before using alternative buffers in which artefacts will occur if residual borate complexes with a compound.
- formic/acetic acid pH 1.9 : 28.4 ml 98% formic acid with 59.2 ml acetic acid to 1 l with d.d. water.
- ammonia/ammonium bicarbonate pH 9.4 : 0.1 M ammonium bicarbonate (i.e. 7.906 g l^{-1}) titrated with conc. ammonia solution to pH 9.4
- 0.1 N NaOH : freshly prepared to avoid the complications caused by CO_2 absorption.

The relative mobilities were derived from the mobile orange G ($R_{ORANGE\ G}$ = 1.00) and the U.V. absorbing 2-deoxyadenosine (0.00), the movement of which accounts for any shift of the origin by diffusion or bulk (buffer) flow. With 0.1 N NaOH the origin was indicated by glycerol (staining with Ag^{2+}). With formic/acetic, fructose was the indicator of the origin.

Stains: Markers were stained for sugars using Ag^{2+} after Anet and Reynolds (1954); and for phosphates using a modification (Tate, pers. comm.) to procedure of Harrap (1960). A stock phosphate revelator was prepared: 50 g $Na_2MoO_4.2H_2O$ in 250 ml water, add 500 ml 1N HCl, 210 ml 72% $HClO_4$, this then diluted 1:1 with water. The reagent was 40 ml stock added to 160 ml acetone, and kept for 1 h only.

Scintillation counting and elution: Chromatogrammes and electrophoretogrammes to be counted were cut at 10 or 15 mm intervals and the papers inserted into counting pots containing 10 ml toluene/PPO/POPOP scintillant. Where strips were sufficiently narrow, papers were placed in disposable plastic 2 ml inserts for counting.

If the material was to be recovered the scintillant was dried by storage on $CaCl_2$. In this manner any toluene-insoluble material could be recovered after counting by one of two methods: (i) elution through a capillary of two appressed microscope slides (Dent 1947) (ii) repeated elution into a tube by centrifugation of the moistened papers suspended in an open plastic cone within the tube.

Invertase: Sucrose was eluted from samples as indicated and hydrolysed with yeast invertase in 50 mM sodium acetate pH 4.6 at 30°C for 20 h (Downton & Hawker 1973). The products were similarly chromatographed and counted.

Alkaline phosphatase: A sample was made 200 µl with 50 mM glycine-NaOH buffer pH 10.6 (Gomori 1955) and 5 mM $MgCl_2$. 5 µl calf intestine phosphatase (Calbiochem B Grade), reconstituted as 0.065 U μl^{-1} in the same buffer pH 8.6, was added to incubate for 15 h at 30°C. Alternatively phosphatase ex E. coli (Sigma type III) was used. Prior to analysis the incubate was made 5 mM EDTA to chelate Mg^{2+} which tends to affect the mobility of compounds.

Incubation of skin segments: The skin of grape berries cv. Muscat (7° Brix : hypodermal juice sample 17.8 g l^{-1} fructose) was peeled into 8 segments and placed for 1 h in 20 ml washing medium containing 200 mM mannitol, 50 mM MES pH 6.5, 2 mM PEG 4000, 10 mM DTT, 0.5 mM $K^+.P_i$ buffer pH 5.5, 2 mM $MgSO_4$, 0.2 mM $Ca(NO_3)_2$. Segments were then blotted and together placed into 0.5 ml of the above medium modified to contain 100 mM mannitol and 100 mM D-glucose or D-fructose. In a parallel series of incubations 100 mM unlabelled fructose was included in the ^{14}C-glucose medium, and vice versa.

Upon completion of an incubation the radioactive material was partitioned into three fractions according to rates of efflux as follows: at 0.5 and 4 h two skin segments were removed, blotted, weighed and placed sequentially, with blotting at each transfer, into (i) 1 ml washing medium for 10 min, (ii) another 1 ml washing medium for 30 min, and, finally (iii) 1 ml water which was then three times frozen and thawed in liquid nitrogen. 50 µl aliquots of the media were counted for radioactivity in 5 ml xylene/triton/PPO/POPOP scintillant.

Incubation of protoplasts and isolated vacuoles: These were prepared as indicated in Chapter 9, purified as indicated herein (with details in Chapter 10), incubated in the relevant medium (PM or VM: Chapter 9), and in all cases removed from the incubation medium by centrifugation through a 7%/10% Ficoll gradient (Fig. 11.1).

13.3 Labelling patterns in skin segments

13.3.1 Results

For simplicity the following terminology is used: for "the ^{14}C-glucose incubation" read "the incubation of tissue in medium containing 100 mM D-glucose and 5 μCi U-^{14}C-D-glucose". When considering incubations of glucose and fructose, "the other hexose" refers specifically to the complementary hexose of this pair. Radioactivity in zones corresponding to sucrose, glucose and fructose markers is assigned to these compounds without chemical confirmation other than hydrolysis of sucrose.

As indicated in Coombe and Matile (1980), and as shall be demonstrated here, the material in each fraction could be characterised as

(i) First Wash, 0 - 10 min: the rapidly effluxed, "diffusible" material including medium from surface adhesion and from damaged cells;

(ii) Second Wash, 10 - 40 min: a slower efflux dominated by "diffusible" material;

(iii) Residual, at 40 min: released by destruction of the washed tissue and hence predominantly compartmented material.

Table 13.1 shows the distribution of radioactivity between the first wash, second wash and residual fractions, and between the zones of the chromatogrammes corresponding to various compounds at 0.5 and 4 h. (In addition to these nominal incubation times there was a 40 min efflux period to be considered.) Values are the means of two incubations, 100 mM labelled hexose with or without 100 mM of the other hexose unlabelled.

A representative series of chromatogrammes is presented in Fig. 13.1, being the distribution of label within the three efflux fractions.

The first wash was dominated in each case by the hexose substrate supplied in the incubation; for example in Fig. 13.1a the predominant activity of glucose is evident. In each case less than 20% of radioactivity was recovered in other metabolites, principally the other hexose

Table 13.1 Uptake and metabolism of ^{14}C-hexoses by excised skin of the grape berry

Radioactive hexose in the incubation	t (h)	Paper chromatography peaks of radioactivity corresponding to compounds as cpm mg^{-1} FWt ; percentile distribution also indicated ( ) X	Y	Z	sucrose	glucose	fructose	Σ
FIRST WASH								
Glucose	0.5	0 (0)	115 (0.5)	0 (0)	2,680 (12)	17,400 (78)	2,100 (9)	22,295 (100)
	4	310 (1)	60 (0.2)	165 (0.6)	4,235 (15)	21,485 (79)	1,005 (4)	27,260 (100)
Fructose	0.5	0 (0)	50 (0.2)	140 (0.5)	2,080 (8)	2,390 (9)	22,510 (83)	27,170 (100)
	4	240 (1)	330 (1.3)	120 (0.5)	1,610 (6)	2,800 (11)	20,350 (80)	25,450 (100)
SECOND WASH								
Glucose	0.5	20 (0.3)	45 (0.7)	25 (0.4)	1,485 (25)	3,725 (62)	725 (12)	6,025 (100)
	4	40 (0.4)	55 (0.6)	30 (0.3)	2,735 (28)	6,640 (67)	430 (4)	9,930 (100)
Fructose	0.5	0 (0)	45 (0.5)	20 (0.2)	870 (9)	540 (6)	7,885 (84)	9,360 (100)
	4	15 (0.1)	45 (0.4)	40 (0.3)	1,155 (9)	565 (5)	10,585 (85)	12,405 (100)
RESIDUAL								
Glucose	0.5	260 (5)	50 (1)	30 (0.6)	60 (1.2)	2,870 (59)	1,625 (33)	4,895 (100)
	4	2,555 (10)	95 (0.4)	260 (1)	2,575 (10)	12,660 (49)	7,640 (30)	25,785 (100)
Fructose	0.5	60 (1.8)	10 (0.3)	20 (0.6)	50 (1.5)	195 (6)	2,930 (90)	3,265 (100)
	4	390 (5.6)	50 (0.7)	60 (0.9)	255 (3.7)	550 (8)	5,670 (81)	6,975 (100)

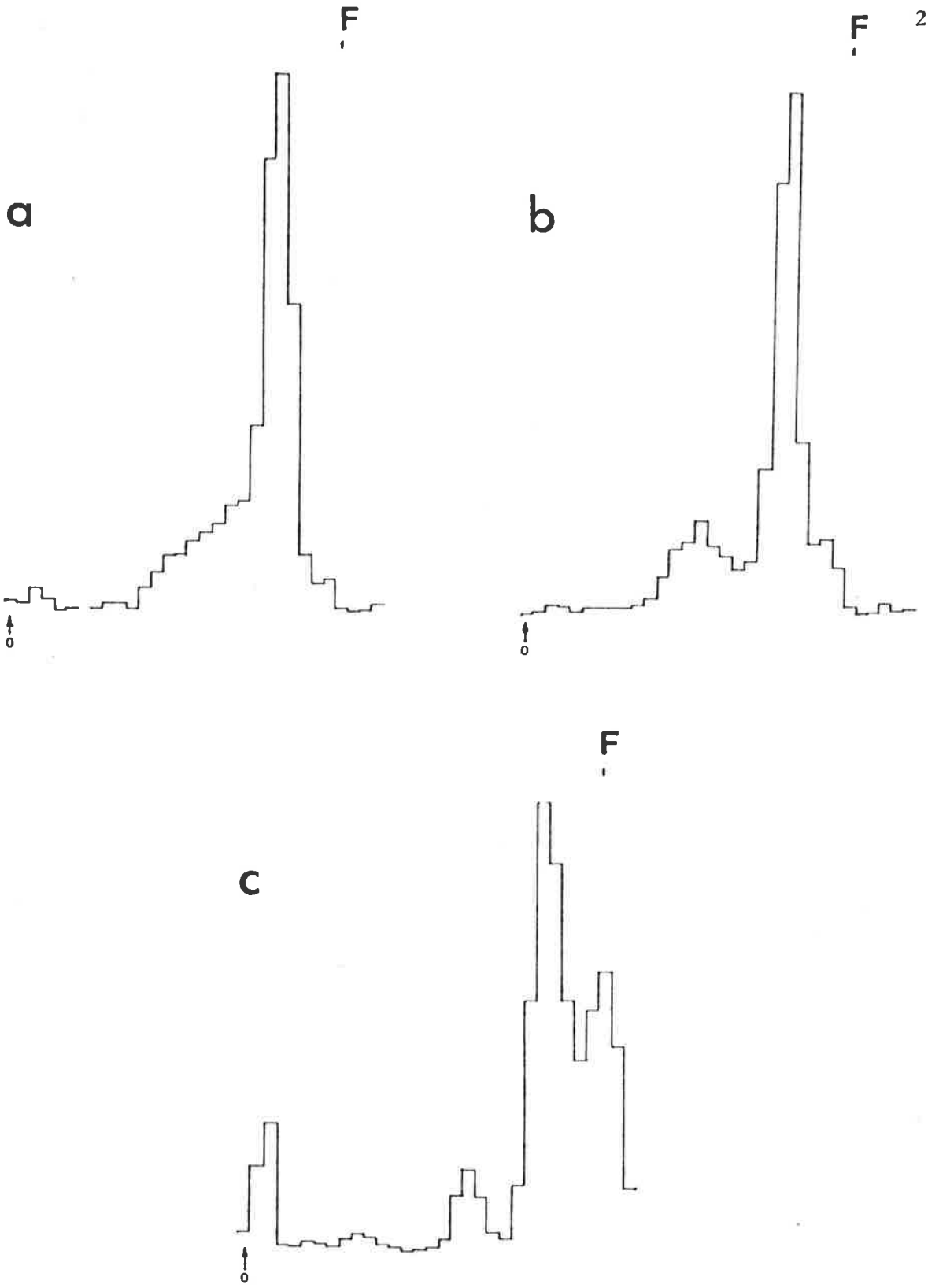


Fig. 13.1 Paper chromatography of radioactivity in excised skin of the grape berry incubated for 4 h in 100 mM U-^{14}C-glucose.

(a) First wash: material effluxing in 0-10 min

(b) Second wash: material effluxing in 10-40 min

(c) Residual

SCALES OF THE Y-AXES ARE DIFFERENT IN EACH REPRESENTATION, AND THE SAME APPLIES FOR EACH FIGURE OF CHAPTER THIRTEEN!

F = fructose marker

and in sucrose: in this wash twice as much label appeared in sucrose from a ^{14}C-glucose incubation as from a ^{14}C-fructose incubation. Three zones of activity were often evident in the region between the origin and the position of sucrose (Fig. 13.1a). These were designated X, Y and Z with approximate $R_{FRUCTOSE}$ of 0.04, 0.17 and 0.31 and are shown as such in Table 12.1. Several compounds might have contributed to each zone, though it is possible they would be of a similar class (such as sugar phosphates at the origin, and di- or oligo-saccharides between the origin and sucrose).

The second wash (Fig. 13.1b) bore similarities to the previous material, but contained a higher proportion of sucrose. In skin segments from a ^{14}C-glucose incubation 25% of the label in this wash was as sucrose after 30 min. Again more label appeared in sucrose for a ^{14}C-glucose incubation than from a ^{14}C-fructose incubation. In each case the other hexose was labelled and traces at X, Y, and Z were apparent, but less than in the first wash.

The residual material also contained principally the hexose provided in the medium. From a 30 min ^{14}C-glucose incubation 59% of the label was still as glucose whereas 33% was converted to fructose and 1% to sucrose; 5% was at the origin in zone X. The absolute amount of label in sucrose was particularly high after ^{14}C-glucose incubation for 4 h, as was compound X. In contrast, conversion of ^{14}C-fructose as substrate to glucose and other compounds was less in both relative and absolute terms. 80 - 90% of label in the residual from ^{14}C-fructose incubation was recovered as such, unaltered.

Total residual counts, at 4 h, of tissue incubated in ^{14}C-glucose were about four times those in tissue from incubation in ^{14}C-fructose. Between 0.5 and 4 h, residual counts increased 5-fold in the ^{14}C-glucose incubation and doubled in the ^{14}C-fructose incubation.

Table 13.2 presents part of the data pertaining to inclusion of the other hexose, unlabelled, in the radioactive medium. In this manner it was possible to compare strictly the movement of ^{14}C as supplied in different hexoses without varying the treatment. Surprisingly, inclusion of the other hexose did not alter uptake nor the labelling pattern. Consequently, as in Table 13.1, data is presented as the mean of these two incubations (with and without the other hexose unlabelled).

Table 13.2 : Effect of 100 mM fructose upon uptake and metabolism of 100 mM U-^{14}C-glucose, and the reciprocal comparison: incubation of skin segments

(Data from residual material only, at two times)

U-^{14}C- as: (100 mM)	Complementary sugar: (100 mM)	t (h)	cpm mg^{-1} corresponding to compounds					
			X	Y	Z	sucrose	glucose	fructose
glucose	mannitol	0.5	270	80	50	80	3,005	1,840
glucose	fructose	0.5	250	20	10	40	2,740	1,410
glucose	mannitol	4	2,540	120	320	2,190	14,240	8,390
glucose	fructose	4	2,570	70	200	2,960	11,080	6,890
fructose	mannitol	0.5	80	0	0	0	210	3,250
fructose	fructose	0.5	40	20	40	95	180	2,600
fructose	mannitol	4	430	60	70	300	650	7,230
fructose	fructose	4	350	40	50	210	450	4,110

The sucrose zone was eluted from material of the second wash of each 4 h incubation, treated with invertase, rechromatographed and counted. Counts were found only in the glucose and fructose positions (Table 13.3).

Table 13.3 : Distribution of ^{14}C in moieties of sucrose as assessed by inversion and chromatography

	% radioactivity recovered from the moiety:	
	glucosyl	fructosyl
From the second wash, tissue incubated for 4 h in:		
U-^{14}C-glucose	93	7
U-^{14}C-fructose	11	89

That from a ^{14}C-fructose incubation showed labelling of glucosyl:fructosyl of 11:89 resembling that of residual hexoses (namely 8:81 at 4 h). In contrast, from a ^{14}C-glucose incubation, labelling of hexose moieties in sucrose in the second wash at 4 h (93:7) did not resemble that of the free residual hexoses at 4 h (glucose:fructose was 49:30).

13.3.2 Discussion

(i) The distinctive nature of each fraction.

Variability due to experimental technique was anticipated in the first wash because of variable degree of carry over - despite blotting - of the highly active medium. Yet the recoveries per mg were quite constant.

There were qualitative differences between the first and second washes. Considering an 0.5 h ^{14}C-glucose incubation, the glucose:sucrose ratio changed from 6.5 to 2.5; conversely, the sucrose:fructose ratio was different in the washes.

Moreover, labelling in the residual differed markedly from that in the second wash. In a 30 min ^{14}C-glucose incubation, glucose:sucrose increased from 2.5 to 48 while the proportion isomerized to fructose was much higher in the residual than in the wash. Clearly the residual material was (i) distinctive from the washes in composition (ii) characteristic of the hexose substrate and (iii) dependent upon time. These residual counts are therefore attributed to cellular compartmentation as was done by Coombe and Matile (1980). Indeed these fractions - first wash, second wash and residual material - characterise substantially different compartments despite certain overlap.

(ii) X, Y and Z

Compound X was predominantly compartmented, was greater at 4 h than 0.5 h and was most active at 4 h in a ^{14}C-glucose incubation. Radioactivity due to compound Y was low but equally distributed between the three fractions and showed no trend with time. Compound Z, appearing in both the washes and residual fractions, increased with time.

The identification of these compounds is discussed below (13.4.ii).

(iii) On specific activities.

Interpretation of data such as Table 13.1 is complex as one has no measure of specific activities of the different compounds in various fractions. That specific activities will be quite different is evident since the concentration of endogenous glucose is five times that of fructose.

It is obvious that specific activities cannot develop to a higher level than that in the incubation medium. A corollary is that the increase in specific activity of a metabolite will follow that of the substrate. Assuming that metabolism is consistent throughout the incubation, information can be deduced as to the specific activities of the hexoses and the sites of metabolism.

The ratio of counts in glucose, fructose and sucrose for any single treatment, (that is, the ratio between a substrate and each product; Table 13.4) did not change between 0.5 and 4 h. Thus the specific activity of the supplied substrate, at the site of metabolism to the other hexose and to sucrose, was already equilibrated with the medium at 0.5 h and appeared not to change thereafter. For this to be the case, the large (unequal) endogenous pools of hexoses must be isolated from this metabolism.

Table 13.4 : Total counts (cpm mg^{-1}) recovered in each metabolite from incubation of skin segments in ^{14}C-hexose substrate

The ratio of counts in the substrate to those in its metabolite is indicated in brackets.

Substrate	Time (h)	Radioactivity corresponding to compounds: X	Y	Z	Sucrose	Glucose	Fructose
U-^{14}C-glucose*	0.5	280	210	55	4225	24000	4450
		(86)	(114)	(436)	(5)	*	(5)
	4	2905	210	455	9545	40785	9075
		(14)	(194)	(90)	(4)	*	(5)
U-^{14}C-fructose*	0.5	60	105	180	3000	3125	33325
		(555)	(317)	(185)	(11)	(11)	*
	4	645	425	220	3020	3915	36605
		(57)	(86)	(166)	(12)	(9)	*

Conversely the 10-fold increase in X between 0.5 and 4 h indicates that this compound was either accumulating or being synthesized from intermediates of slowly increasing specific activity. The appearance of X was unaffected by the presence of absence of the other hexose, unlabelled, in the incubation. Therefore, the substantial labelling of X at 4 h was not so much a manifestation of increasing specific activities but was due more to accumulation of X.

(iv) Sucrose.

That sucrose was recovered in these experiments requires comment since, in other experiments, extracellular invertase activity was evident in skin segments incubated in U-^{14}C-sucrose. It is possible that product inhibition of invertase occurs, as demonstrated in sugarcane leaf (Sanpietro et al. 1980). Sucrose was predominantly in the washes, an exception being the 4 h ^{14}C-glucose incubations in which 27% of sucrose was residual (and in which zone X was similarly active). Total labelled sucrose increased with time only in ^{14}C-glucose incubations (Table 13.4). This increase could arise from accumulation of sucrose or from higher specific activity in the precursors to sucrose.

The labelling pattern in sucrose will be determined by the specific activities, not the total counts, of the relevant glucosyl and fructosyl substrates (NDPGlucose, fructose and fructose-6-phosphate). The products of sucrose hydrolysis, if this occurs, will obviously be labelled in the same ratio as the sucrose moieties irrespective of the hexose pools into which this occurs.

Thus the labelling pattern in the residual fraction from ^{14}C-fructose incubations was consistent with transport via sucrose and subsequent hydrolysis in the compartment. However, for ^{14}C-glucose incubations there was a large disparity: labelling pattern in sucrose was glucosyl:fructosyl 13:1 but in the residual 1.6:1.

(v) Glucose, fructose and isomerization.

Glucose was the substrate from which more counts were compartmented and was the more metabolized of the two hexose substrates both in absolute and proportionate terms. It appears that glucose was being converted to fructose with little of the reverse process. Isomerization may occur by the pathway: (UDPG ⟵ GIP ⟶:)Glucose ⟷ G6P ⟷ F6P ⟷ Fructose. Bieleski and Redgwell (1977) pointed out that the ratio of F6P:G6P in many plants is c. 0.3, reflecting an equilibrium state of hexose phosphate pools under the control of phospho-glucose isomerase. Fig. 1.5 indicates this to be so in the grape berry also (from Ruffner et al. 1976).

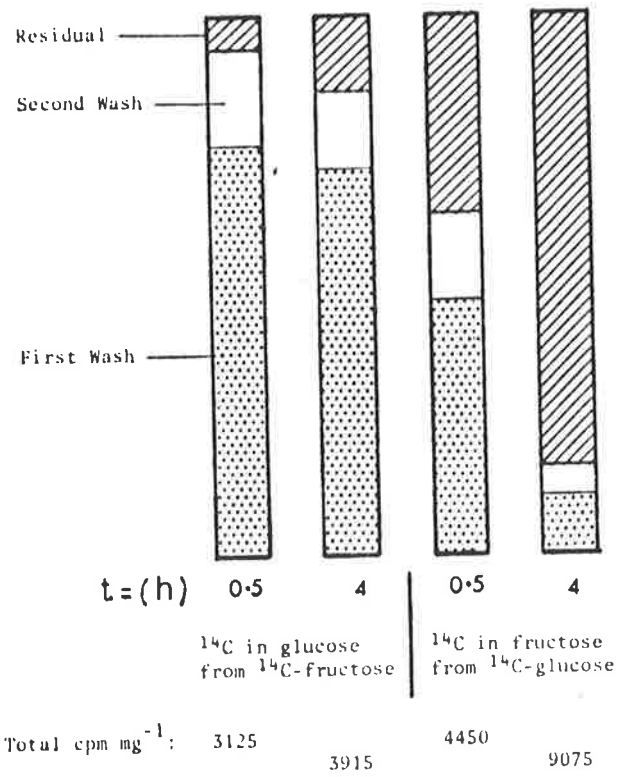


Fig. 13.2 Percentile distribution of ^{14}C, between two washes and residual material, in each hexose as derived by isomerization.

The isomerization of hexoses in the tissue showed a distinct spatial character, as depicted in Fig. 13.2. Labelled glucose derived from ^{14}C-fructose was mostly in the first wash; labelled fructose derived from ^{14}C-glucose was mostly in the residual, markedly at 4 h. It appears that conversion of glucose to fructose disposes to compartmentation, while conversion of fructose to glucose does not.

This could arise by several mechanisms.

(a) a specific activity effect: isomerization of glucose to fructose occurs in a pool low in fructose such that its uptake is from a pool of high specific activity. Yet inclusion of 100 mM unlabelled fructose in the ^{14}C-glucose incubation, which should have largely decreased the specific activity of intermediates, only reduced the compartmentation of isomerized hexose by 18%. Furthermore, high radioactivity of glucose in the washes predisposes against low specific activity here.

(b) due to intermediates: preferred transport of fructose-6-phosphate, for example, would favour isomerization from glucose with uptake. But the criticism raised above also applies here.

(c) a sucrose-mediated effect: in which fructosyl derived from glucose is incorporated into sucrose via fructose or its phosphate and compartmented. Studying the accumulation of ^{14}C from hexoses in bean pod tissue, Sacher (1966) concluded that uptake was via sucrose synthesis: the more rapid uptake from ^{14}C-glucose derived from glucose being a better source of glucose-1-phosphate and fructose than fructose was of glucose-1-phosphate. But the present data differs in two key aspects from Sacher's. Firstly, in the ^{14}C-glucose incubation, ^{14}C-asymmetry in sucrose is quite unlike the labelling pattern in residual hexoses. Secondly, uptake of fructose was not inhibited by inclusion of unlabelled glucose.

- a sucrose-mediated effect: in which labelled glucosyl is converted to fructose within the residual compartment without first entering the free glucose pool. Sucrose catabolism by sucrose synthase or sucrose phosphate synthase yields NDPGlucose. Isomerization would involve NDPGlucose, GIP, G6P, F6P to fructose. Given the equilibrium of phosphogluco-isomerase favouring G6P, and the high activity of phosphatases in storage compartments, this is an improbable process.

(d) a group transport: a glucosyl moiety is transported across a membrane with isomerization to fructosyl. This proposition, for which there is no precedent, would entail an association of hexokinase, phosphogluco-isomerase and a membrane with F6P being vectorially released and hydrolysed to fructose. Stoichiometric phosphate transport would be observed. Such a model fits the data although the evidence is circumstantial and has not been tested further.

To summarize this section,

- Labelled glucose is converted to labelled fructose within grape skin with little of the reverse reaction.
- This particular isomerization produces compartmentation.
- The isomerization is apparently unaffected by the metabolic pool size of fructose with which any scalar reaction would equilibrate.
- The labelling pattern of hexoses in the compartment is unlike that in diffusible sucrose.
- Isomerization within the storage compartment is improbable.

Moreover,

- Field data on the grape berry indicate that after veraison fructose accumulates more rapidly than glucose.
- The interaction of transport and phosphorylation has been indicated in sugarcane (Paragraph 5.4) in which transported ^{14}C-fructose was recovered as free hexoses.

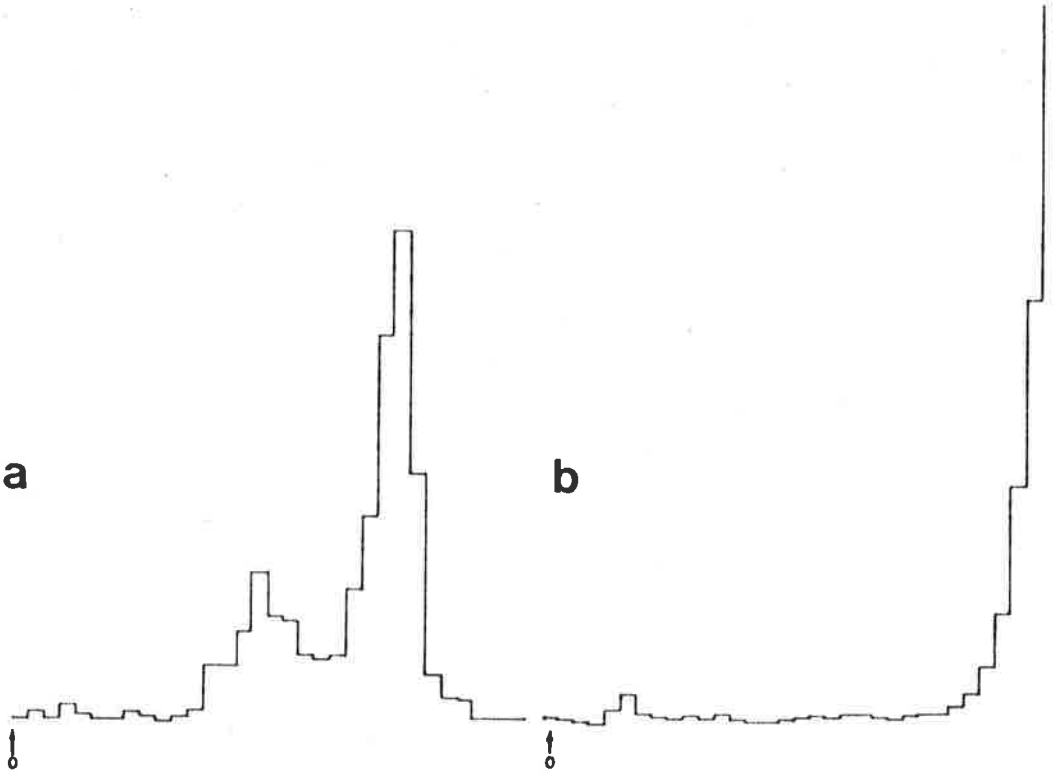


Fig. 13.3 Effect of storage at 4° for 3 months upon compounds labelled by incubation of skin segments in 100 mM U-^{14}C-glucose

Paper chromatography of an aliquot from the second wash
(a) before and (b) after storage

(vi) Changes upon storage of washings.

When duplicate samples which had been stored at 4°C for three months were chromatographed the pattern was quite different in the two washings (Fig. 13.3a, b). X and Z and sucrose were no longer labelled. Y had increased 300%. Most of the radioactivity was again at the hexose as supplied. The residual fraction was unchanged. It appears that enzymatic degradation had occurred in the samples from washings, while these enzymes had been inactivated in the residual material, possibly by endogenous phenols.

13.4 Electrophoresis of ^{14}C-compounds
Results and Discussion

(i) Mobility of standards in electrophoresis and chromatography

The mobilities of several sugars, nucleotides, nucleosides, and markers are indicated in Table 13.5. In some instances only a single determination was made. Sucrose phosphate mobility was determined using radiochemically labelled material subsequently verified by enzymatic degradation using phosphatase, invertase and α-glucosidase.

Table 13.5 Mobilities of some sugars, nucleotides and markers in chromatography and electrophoresis, with notation concerning reactivity with Ag^{2+}

	CHROMATOGRAPHY	ELECTROPHORESIS				Ag^{2+} STAINING*	
	pyridine:ethyl acetate:water	Na_2BO_3 pH 9.2	NH_3/NH_4HCO_3 pH 9.4	Formic/Acetic pH 1.9	0·1N NaOH	RESPONSE	INTENSITY
	$R_{FRUCTOSE}$	$R_{ORANGE\ G}$	$R_{ORANGE\ G}$	$R_{ORANGE\ G}$	$R_{ORANGE\ G}$	*Borate reduces sensitivity	
Cellobiose	0.47					rapid	++
Fructose	1.00	0.80	0.03	0	.63, .37	rapid	+++
Galactose	0.79	0.82	0.03	0	.55, .32	rapid	++++
Glucose	0.84	0.92	0.02	0	.60, .34	rapid	+++
Meso-Inositol	0.33					rapid	+++
Maltose	0.51	0.32				rapid	+
Mannitol	0.82					rapid	++
Mannose	1.04	0.67				rapid	++
Melibiose	0.33	0.70	0.04		.26, .19	rapid	+++
Raffinose	0.25	0.25	0.05	0	.17, .13	slow	+
Stachyose		0.31					
Sucrose	0.63	0.16	0.02	0	.19, .13	with heating	+
Xylose	1.21					rapid	++++
Xylene-cyanol blue		0.35	0.39		.33, .32		
Fructose-6-phosphate	0.04	1.19	1.14	0.55		rapid	+++
Fructose-1, 6-diphosphate	0.0					with heating	++
Glucose-1-phosphate	0.03	1.06				slow	++
Glucose-6-phosphate	0.02	1.17	1.12	0.52		rapid	+++
Glucose-1, 6-diphosphate		1.34					
Sucrose-phosphate	0.0	0.80	0.97	0.65			
ATP		0.89	1.06				
ADP		1.05					
UDP		1.17					
UDPGlucose	0.04	1.03	0.98	0.74		with heating	+
UDPGalactose		1.06	0.95	0.74			
UDPMannose		1.05					
ADPGlucose	0.02	0.95	0.81	0.37		with heating	+

The mobilities of other compounds have been tabulated by Foster (1962).

(ii) X^1, Y^1, Z^1

From the following analysis it appeared that the zone X contained a hexose phosphate while zones Y and Z were low order oligosaccharides, such as melibiose or raffinose. The data were derived from work with skin segments (previous section, 13.3) and with protoplasts and vacuoles (following section, 13.5).

Protoplasts were incubated in 50 mM U-^{14}C-glucose, -fructose and -sucrose for 30 and 60 min, cleaned through Ficoll, immediately frozen, and the resultant compartmented label was chromatographed.

Radioactivity on the chromatogrammes corresponding with certain compounds is shown in Table 13.6. ^{14}C-glucose was partly isomerized to fructose, and no labelled sucrose was recovered from incubation of protoplasts in U-^{14}C-sucrose. There were three distinct peaks (Fig. 13.4) analogous to X, Y, Z recovered when incubating excised skin of grape berries in ^{14}C-sugars. (To distinguish compounds derived from protoplasts or vacuoles, rather than skin tissue, the symbol is primed.) Z^1 contained up to 14% of total radioactivity taken up by the protoplasts.

Compound Z^1 was eluted from the chromatogrammes and electrophoresed in borate buffer. Borate will confer mobility on sugars at pH 9.2 by forming a complex. Z^1, whether derived from protoplasts incubated in glucose, fructose or sucrose, formed two peaks under electrophoresis. More than 90% of recovered counts were at $R_{ORANGE\ G} = 0.24$ (e.g. raffinose). In each, a trace of radioactivity was evident at about $R_{ORANGE\ G} = 0.57$ (e.g. Fig. 13.5).

Z^1 did not move in formic/acetic buffer and was re-eluted from the origin free of any residual borate. Thus it did not have strong acid groups such as phosphate or sulphate. In ammonia/ammonium bicarbonate buffer at pH 9.4 it was also immobile. Therefore it did not contain weak acids and mobility in borate was due to complex formation.

Table 13.6 : Chromatographic distribution at 30 and 60 min of ^{14}C taken up by protoplasts in 50 mM sugars as indicated. cpm are corrected for different specific activities in the incubations.

SUBSTRATE ($U-^{14}C$)	t (min)	cpm per aliquot of protoplasts					
		X^1	Y^1	Z^1	Sucrose	Glucose	Fructose
Glucose	30	1750	0	1150	0	7325	400
	60	tr	0	1450	0	7900	900
Fructose	30	tr	0	650	0	tr	5680
	60	205	tr	1030	0	tr	6030
Sucrose	30	tr	tr	864	0	4055	3920
	60	270	130	1390	0	4610	4190
$R_{FRUCTOSE}$		0.04	0.17	0.31	0.63	0.83	1.00

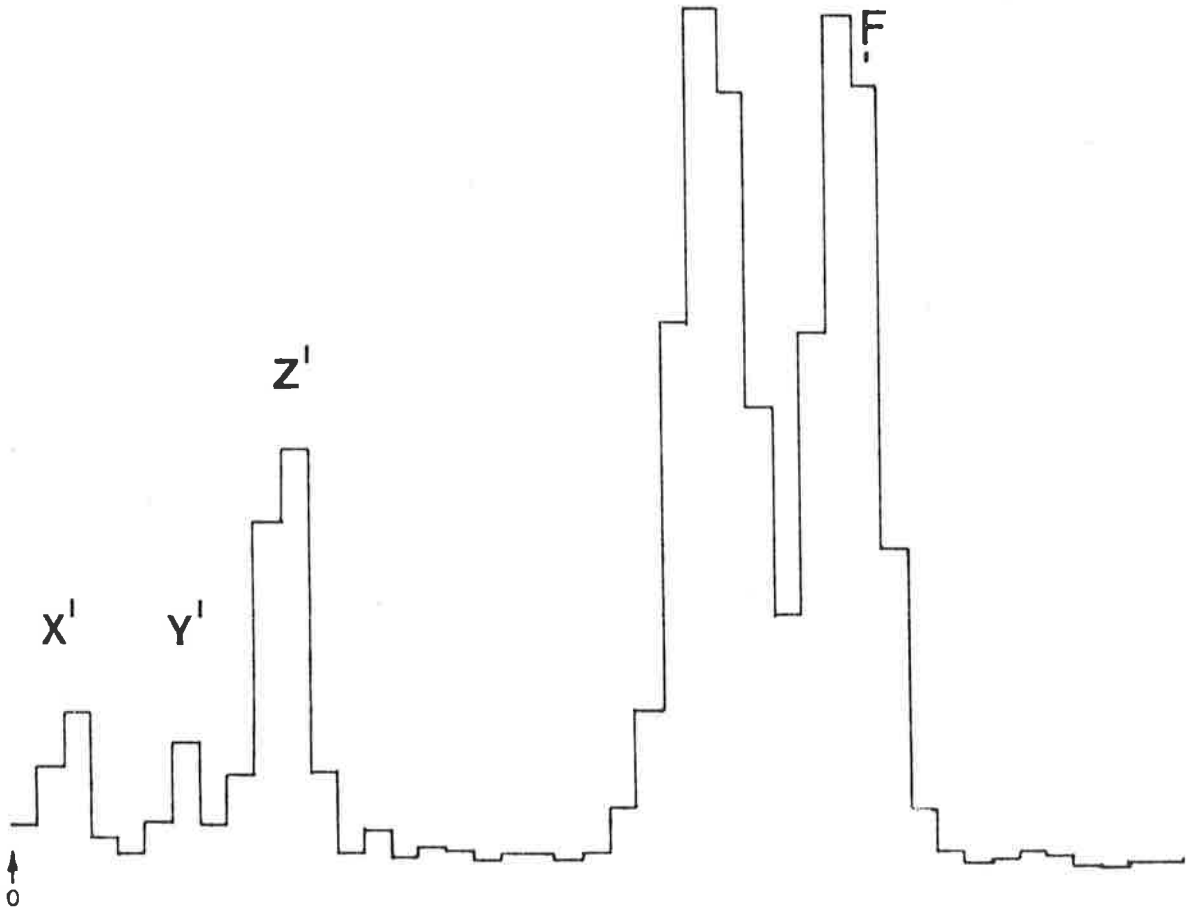


Fig. 13.4 Chromatograph of radioactivity in protoplasts after 60 min incubation in 50 mM $U-^{14}C$-sucrose

ethyl acetate: pyridine :water 10:4:3

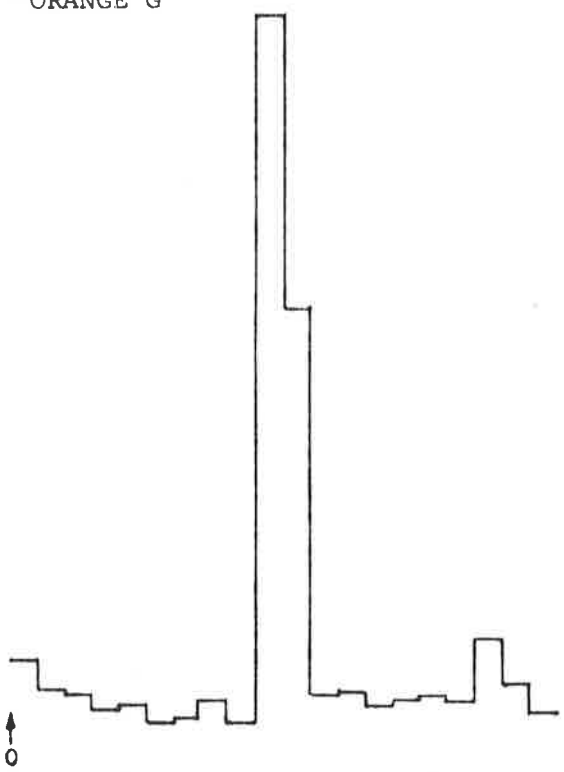


Fig. 13.5 Electrophoresis of Z^1 derived from protoplasts incubated in U-^{14}C-glucose

Borate buffer pH 9.2

From the residual material of incubation of excised skin in U-^{14}C-glucose (see 13.3.1), compounds X and Z were recovered. Borate electrophoresis of X produced two small zones of radioactivity (Fig. 13.6a), at $R_{ORANGE\ G}$ = 0.60 and 1.14 (c.f. F6P 1.19, G6P 1.17). This more mobile zone was labile to phosphatase, but the total radioactivity was too low for the products of phosphatase hydrolysis to be identified.

Fig. 13.6b is an electrophoretogramme of Z, from excised skin, developed in borate buffer. Three peaks are evident: two minor ones coincident with sucrose and with $R_{ORANGE\ G}$ = 0.24 as in Z^1, and major activity at $R_{ORANGE\ G}$ = 0.60 where protoplast material had indicated only a trace. This compound did not move in formic/acetic nor in ammonia/ammonium bicarbonate buffers.

Compounds Z and Z^1 were electrophoresed in 0.1 N NaOH (Fig. 13.7). Evidently the compounds were broken down by the alkali, indicative of reducing sugars. The analysis could not be continued as no intact material remained. Compound Z^1, deriving originally from ^{14}C-glucose, contained at least two labelled moieties (Fig. 13.7a) whereas Z^1 from ^{14}C-fructose contained one dominant moiety (Fig. 13.7b). Z, from

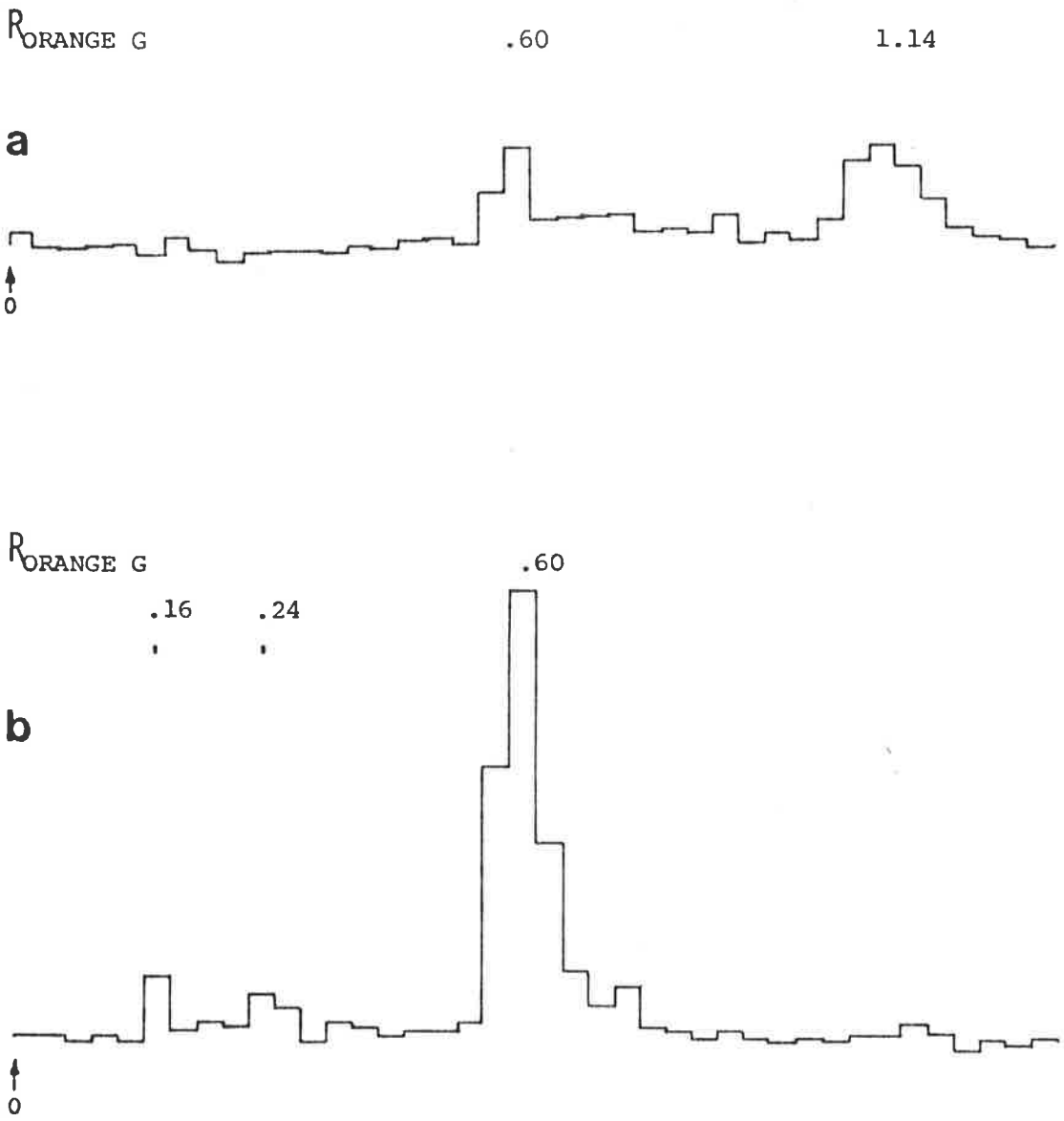


Fig. 13.6 Electrophoresis of radioactivity of X and Z derived from excised skin incubated in U-^{14}C-glucose

Borate buffer pH 9.2

13.6a Zone X

13.6b Zone Z

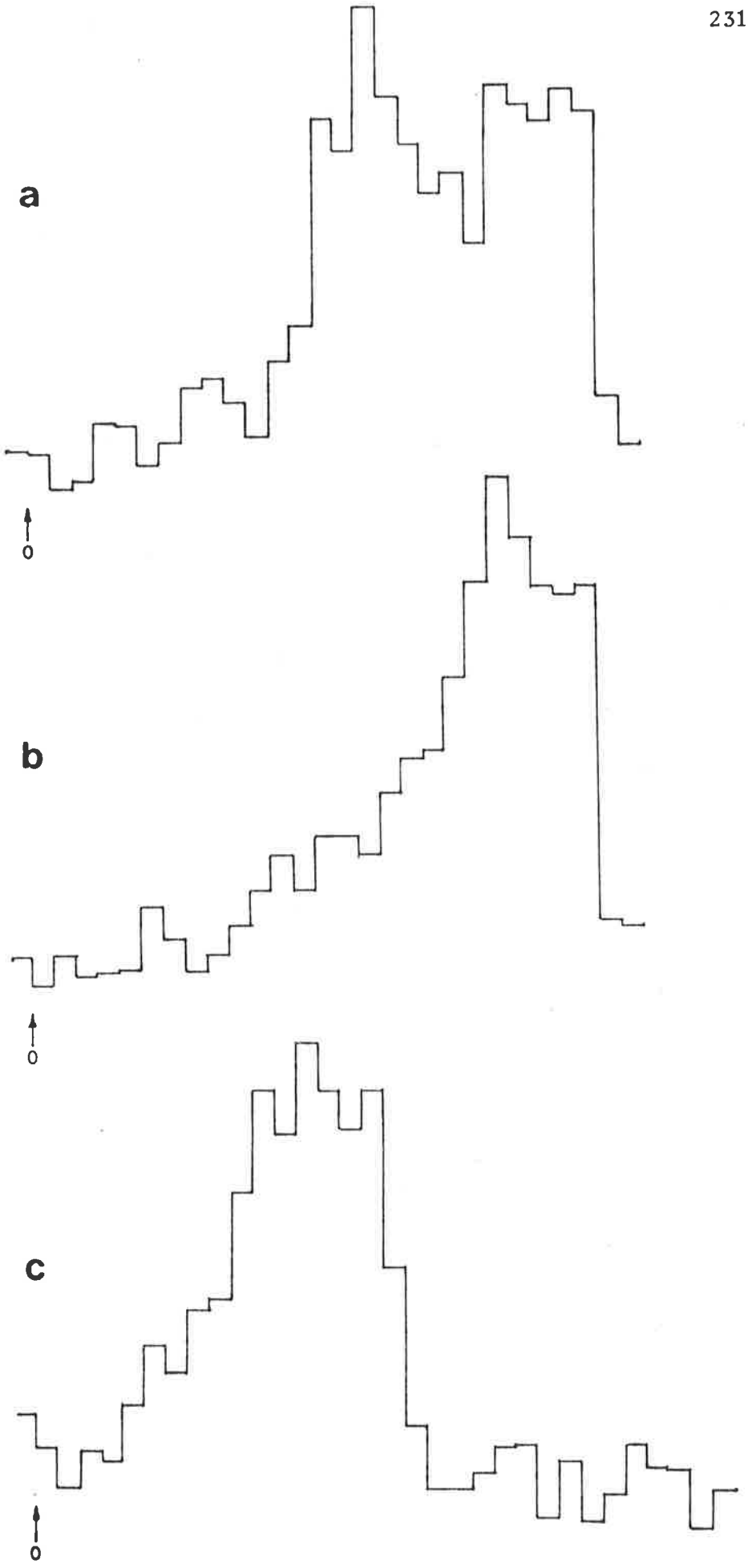


Fig. 13.7 Electrophoresis of radioactivity of Z and Z^1: O·IN NaOH electrolyte

13.7a Z^1 from protoplasts incubated in ^{14}C-glucose

13.7b Z^1 from protoplasts incubated in ^{14}C-fructose

13.7c Z from excised skin incubated in ^{14}C-glucose

^{14}C-glucose incubation of excised skin, ran as one broad peak slower than the two zones in Z[1] (Fig. 13.7c). The mobility of Z in each system used, and its reduction by alkali, is consistent with the properties of melibiose.

Hence from excised skin incubated in U-^{14}C-glucose a sugar phosphate was recovered, and low order oligosaccharides (or di-saccharides). From protoplasts similar products are recovered, but the degree of labelling was changed (in borate buffer, more at $R_{ORANGE\cdot G} = 0.24$ and less at $R_{ORANGE\ G} = 0.60$).

(iii) Labelling of galactose.

Electrophoresis of the residual material in excised skin of berries after incubation in ^{14}C-glucose (as in 13.3) merely substantiated what was apparent from chromatography: glucose, fructose and sucrose were each labelled (Fig. 13.8a). The zones corresponding to the hexoses were eluted and redeveloped both in electrophoresis and chromatography. The putative glucose developed as expected on the chromatogramme, but the fructose split into two zones (though still running as a single peak in electrophoresis), the lesser of which was $R_{FRUCTOSE} = 0.82$ (Fig. 13.8b, c).

Galactose has similar mobility to glucose in the chromatography system employed, but similar mobility to fructose in borate electrophoresis. Moreover, recovery of oligosaccharides probably of the raffinose family suggests epimerization of glucose to galactose. Free galactose is not normally recovered from plant tissue; its presence in ripe prunes was attributed to in vitro hydrolysis of galactose-containing compounds (Dey 1980).

Evidently, investigations of carbohydrate accumulation in fruit should take account of galactose, even if only to assess its contribution to assays for the principal hexoses.

The term activated hexoses is used in reference to phosphorylated and nucleotide hexoses which are disposed to transfer reactions (glycosylation (see also 13.5.iv) or to epimerization. The high levels of activated hexoses lead to epimerization and transfer reactions, and these products (of the raffinose family, for instance) may act in a feedback regulation of uptake at the plasmalemma. In this context, the grape berry cells in vitro appear to be perturbed by high levels of cytoplasmic sugar incommensurate with the capacity for export or compartmentation.

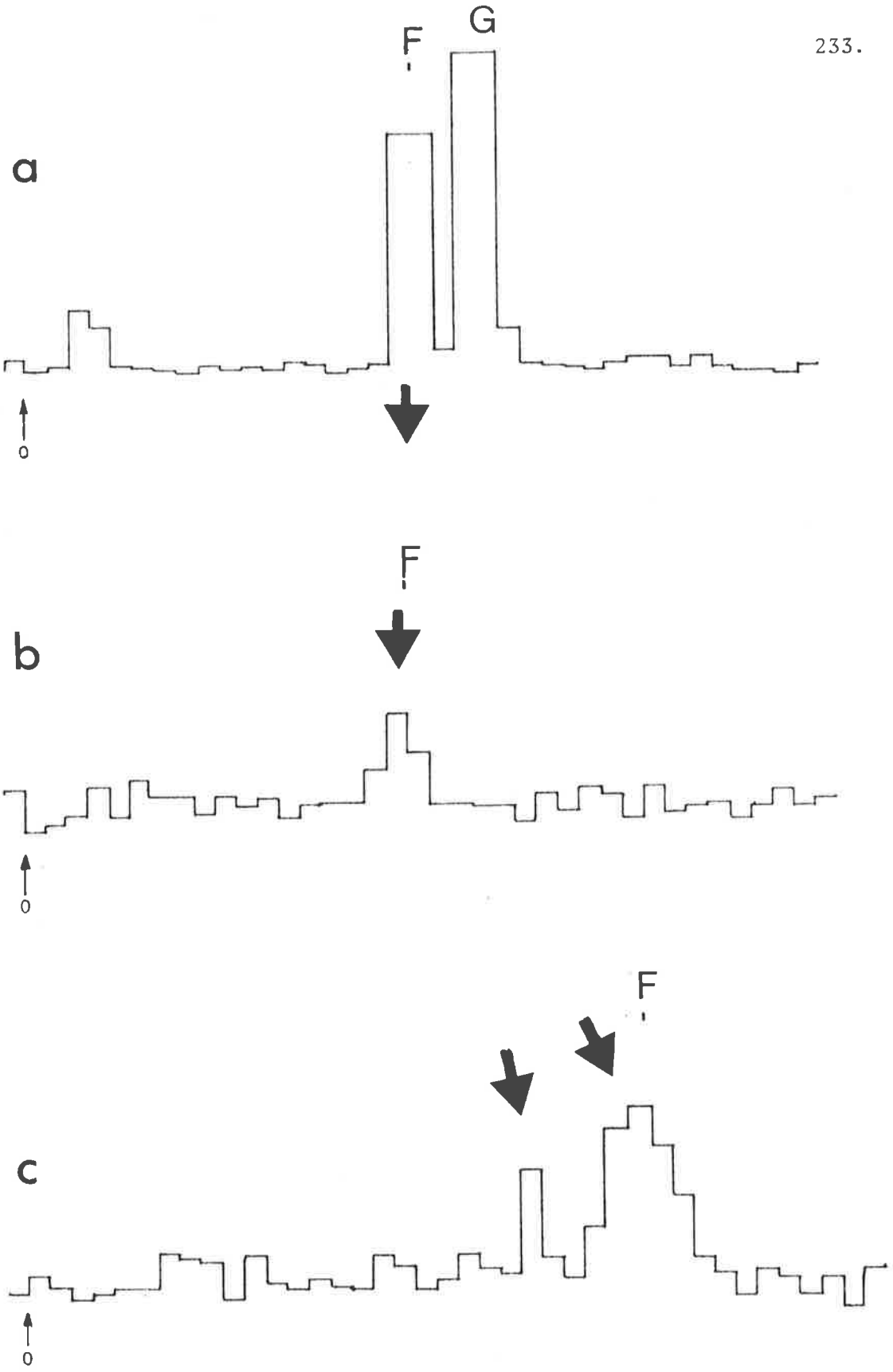


Fig. 13.8 Electrophoresis of radioactivity in the residual compartment of excised skin of the grape berry incubated in U-^{14}C-glucose G

The zone presumed to be fructose (in 13.8a) again electrophoresed as a single peak (13.8b) but two compounds were evident by chromatography (13.8c).

13.5 ^{14}C labelling in protoplasts and vacuoles

Results and Discussion

(i) Metabolism of sucrose and isomerization of glucose in protoplasts

Protoplasts from berries of grape cv. Muscat c. 10^{o} Brix were cleaned by ultracentrifugation in Percoll such that the suspension appeared free of cell-wall material. These protoplasts accumulated ^{14}C from uniformly labelled glucose and sucrose, each 20 mM. At 62 min protoplasts in glucose had taken up four times as much label as those in sucrose. As an indication of passive uptake, accumulation from L-glucose was only 20% of that from D-glucose (refer Fig. 11.3).

Sampling times were 2, 7, 12, 62 min of incubation; however, subsequent recovery of protoplasts from the medium took about 15 min during which time some metabolism of accumulated label could have occurred.

The percentile distribution of ^{14}C between sucrose, glucose and fructose is indicated in Fig. 13.9, and chromatogrammes of the 62 min sampling are shown (Fig. 13.10).

From ^{14}C-sucrose the label was already 76% as glucose and fructose after 2 min. At each time more label was in glucose than fructose; at 62 min fructose contained only half as much label as glucose. Though the relative proportion changed little, the level of sucrose increased four-fold from 2 to 62 min.

Thus the rapid initial uptake from U-^{14}C-sucrose by protoplasts was associated with hydrolysis and the slower phase with less hydrolysis. This is the converse of what is expected of uptake by diffusion. It appears that sucrose crosses the plasmalemma of protoplasts (by diffusion?) and is taken up at the tonoplast with rapid subsequent hydrolysis: this characterises the rapid uptake phase.

From ^{14}C-glucose predominantly glucose accumulated, while up to 10% of the label was recovered as sucrose. Fructose was labelled at the first sampling (2 min) and the proportion of label therein increased throughout to equal 29% of the activity at glucose after 62 min. In actual counts protoplasts incubated in ^{14}C-glucose synthesized as much sucrose as accumulated by protoplasts in ^{14}C-sucrose.

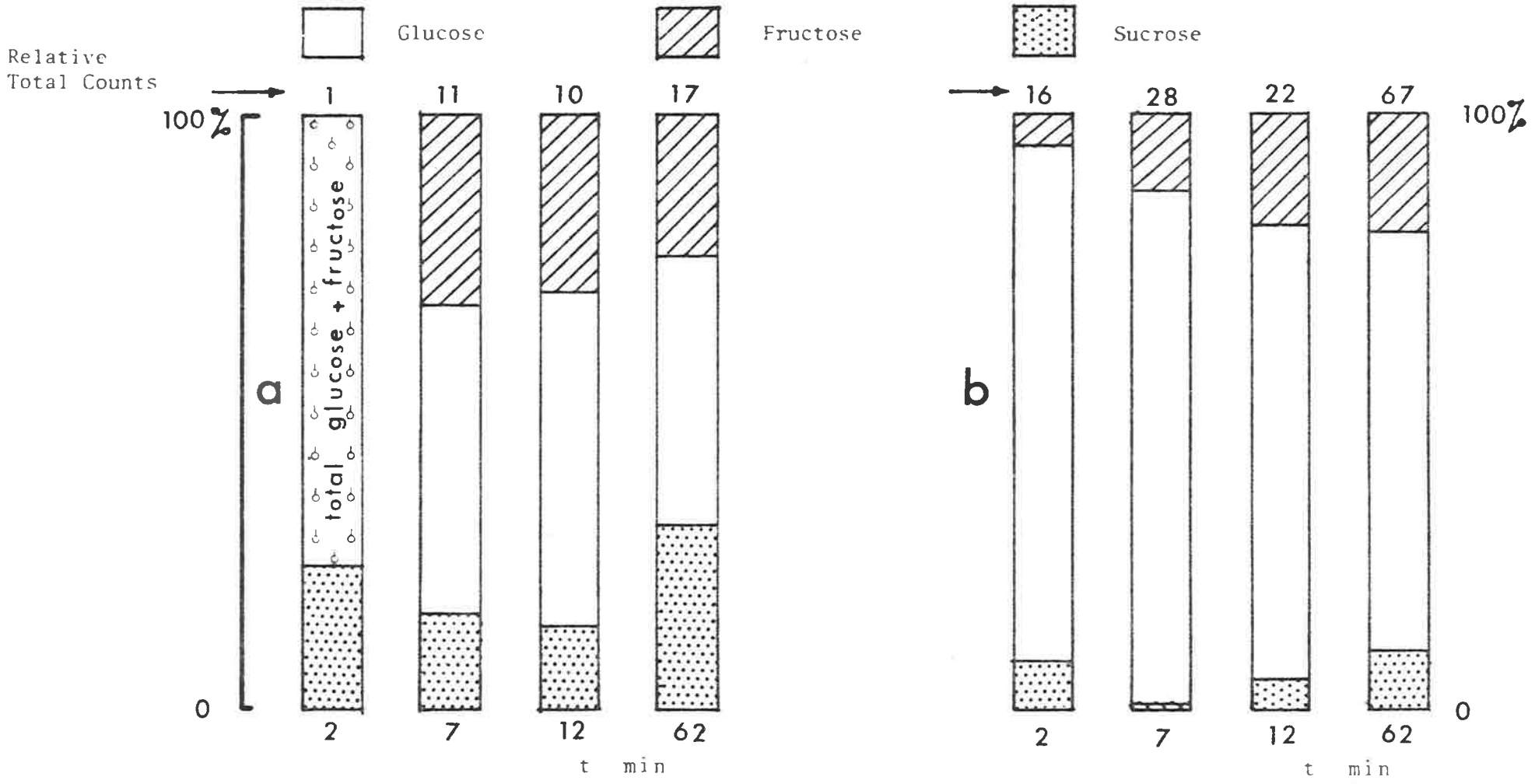


Fig. 13.9 Percentile distribution of ^{14}C between sucrose, glucose and fructose. Protoplasts incubated in (a) U-^{14}C-sucrose (b) U-^{14}C-glucose, each 20 mM, for 2, 7, 12, 62 min.

Also indicated, relative total ^{14}C uptake.
For ^{14}C-sucrose at 2 min, total of hexoses is shown. Refer for uptake: Fig. 11.3

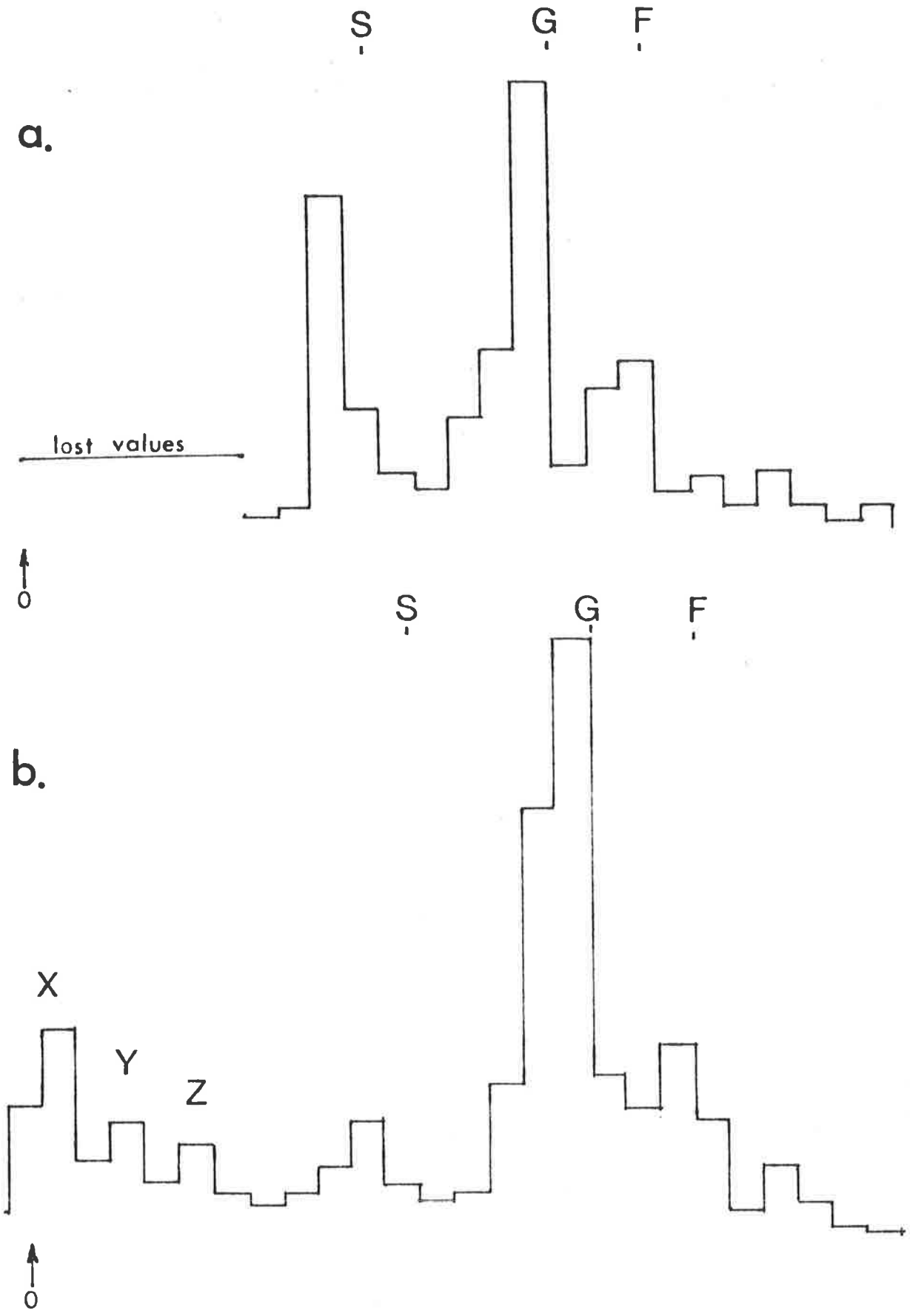


Fig. 13.10 Chromatography of radioactivity incorporated by protoplasts in 62 min, from (a) U-^{14}C-sucrose or (b) U-^{14}C-glucose, each 20 mM. The markers fructose (F), glucose (G) and sucrose (S) are indicated, as also the zones denoted X, Y and Z. Results are quantified in Fig. 13.9

L-glucose was recovered as such. Compounds in zones X^1, Y^1, Z^1 are evident in Fig. 13.10b as in many other samples. However, contamination about the origin of several papers precluded any further consideration of these three zones.

These results are enigmatic in two respects:

Firstly, sucrose hydrolysis up to 7 min (Fig. 13.9) was sufficient that at 62 min no sucrose should have remained, yet it was three times higher at 62 min. A fraction of the cellular sucrose appears "protected" for hydrolysis.

The appearance of sucrose in protoplasts incubated in ^{14}C-glucose substantiates this. As much label was incorporated into sucrose as was recovered in the ^{14}C-sucrose incubation: indeed, if one was asymmetrically labelled and the latter uniformly labelled (as supplied), molar sucrose synthesis was twice as much as the accumulation from U-^{14}C-sucrose. This sucrose is probably cytoplasmic.

Secondly, where is the fructosyl moiety from hydrolysis of uniformly labelled sucrose (Fig. 13.9a)? Either sucrose is inverted and the hexoses taken up at different rates; or fructose efflux is greater than that of glucose; or fructose is metabolised more than glucose.

In summary, the rapid phase of uptake from sucrose was characterised by sucrose transport with subsequent hydrolysis. The fate of a proportion of the fructose deriving from sucrose was not evident. The relatively faster uptake from glucose was also associated with sucrose synthesis and isomerization of glucose to fructose.

Evidently sucrose synthesis is cytoplasmic (or at the tonoplast) while hydrolysis is in another compartment (presumably the vacuole). The roughly equimolar ratio of glucose and fructose in the ripening grape berry is apparently not a consequence of their equivalence in translocated sucrose but rather is the product of intracellular metabolism and transport.

Invertase activity was evident in a protoplast suspension. Incubated in 50 mM U-^{14}C-sucrose, pH 5.5, the sum of label in hexoses free in the medium at 15 min, 4.1% exceeded the accumulation of label by these particular protoplasts: that is, inversion was sufficient to supply substrate (albeit at low concentration) for any hexose transporter.

(ii) Evidence for fructose-, glucose- and sucrose-phosphate from protoplasts.

From different chromatogrammes a zone near to the origin (-10 to +70 mm) was eluted. Aliquots of these eluants did not interfere with phosphatase action upon G6P, and from U-^{14}C-G6P treated with phosphatase 92% of the ^{14}C was recovered at the glucose position.

Two chromatogrammes of phosphatase-treated eluates are presented in Fig. 13.11. Compounds Y^1 and Z^1 were not labile to phosphatase. However, several compounds derived from protoplast incubation medium were labile to phosphatase. From U-^{14}C-sucrose medium a single compound appeared just ahead of a parallel fructose marker (Fig. 13.11a). Allowing for variability, especially in the presence of phosphatase medium, this was interpreted to be fructose and to indicate fructose phosphate. From ^{14}C-glucose medium (Fig. 13.11b) peaks appeared after phosphatase treatment at the positions corresponding to sucrose and glucose (and a trace of fructose).

The incorporation of both fructose and glucose into sucrose in skin segments (13.3 above), indicated sucrose synthase and/or sucrose phosphate synthase activity. The identification here of phosphates of fructose, glucose and sucrose indicates sucrose phosphate synthase to be active in protoplasts, as for the postulated mechanism of accumulation.

(iii) Accumulation of Z^1 is cytoplasmic.

The labelling pattern in aliquots from the incubation of protoplasts and of vacuoles is presented in Table 13.7. From protoplasts incubated in ^{14}C-glucose, X^1 and Y^1 appeared in the medium and X^1 and Z^1 pelleted with protoplasts. Indeed 24% of ^{14}C taken up was in Z^1. Of material in zone X^1 synthesized during incubation, 93% was recovered in the medium rather than in the protoplasts. From incubation of vacuoles (with associated cytoplasmic material), in ^{14}C-sucrose, less than 4% of compounds X^1, Y^1, Z^1 pelleted with the vacuoles.

It appears then that Z^1 is synthesized in the cytoplasm and neither crosses the plasmalemma nor the tonoplast.

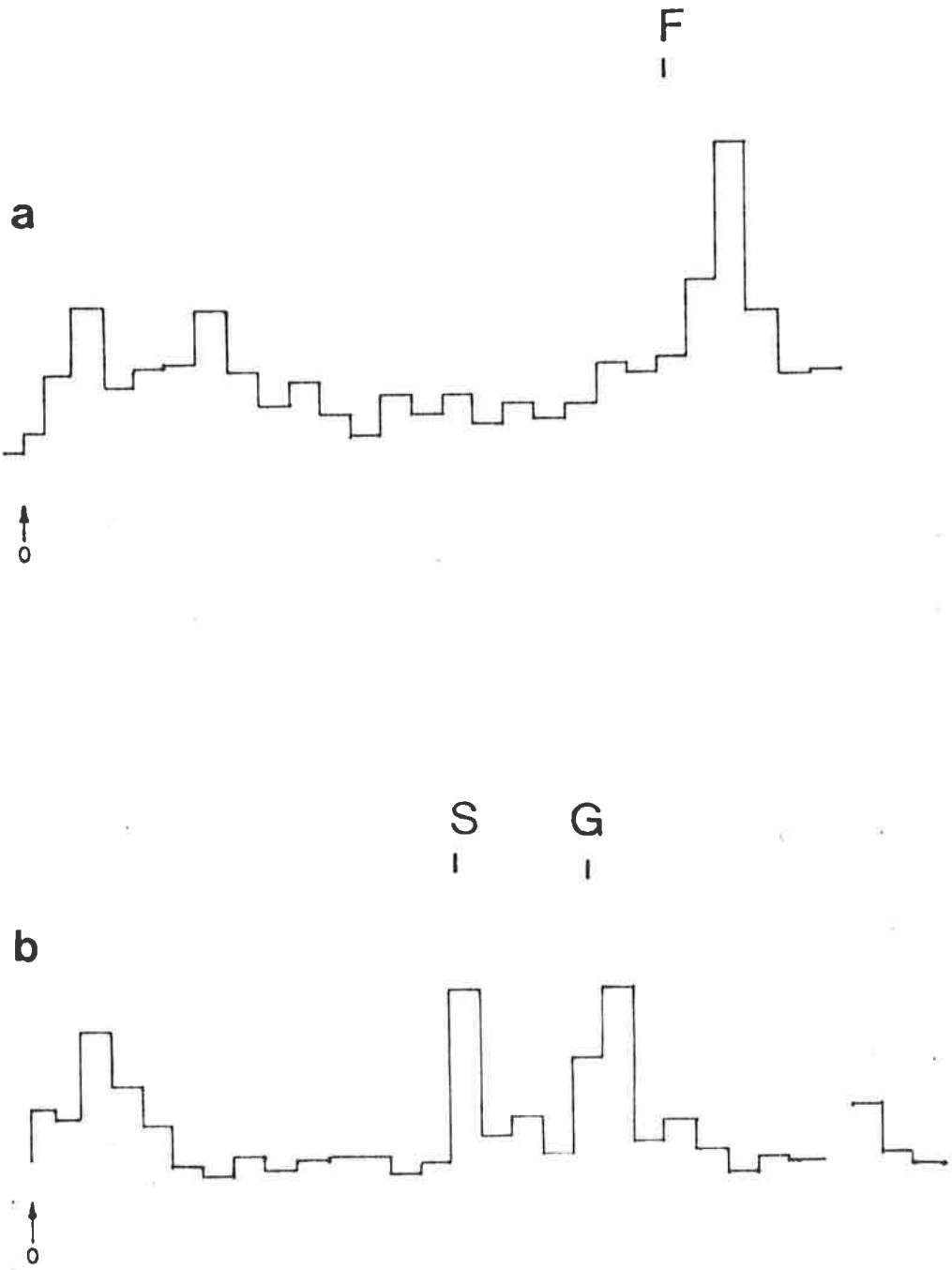


Fig. 13.11 Chromatography of radioactivity derived previously from the origin then incubated with phosphatase.

Material from (a) U-^{14}C-sucrose and (b) U-^{14}C-glucose incubation media after removal of protoplasts at 15 min.

Table 13.7 : Radioactivity of zones on chromatogrammes corresponding to X^1, Y^1 and Z^1 deriving from incubation of protoplasts and isolated vacuoles for 20 min

(a) From the incubation of protoplasts with 30 mM U-^{14}C-glucose
(b) From the incubation of vacuoles with 30 mM U-^{14}C-sucrose

			cpm per ml of protoplast or vacuole incubate			
			X^1	Y^1	Z^1	
a.	(i)	Incubation medium	29,200	14,000	0	
			(0.8)	(0.4)		(%)
	(ii)	Protoplasts	2,000	0	4,300	
			(11)		(24)	(%)
b.	(i)	Incubation medium	46,000	0	52,000	
			(2)		(2)	(%)
	(ii)	Vacuoles	3,100	0	650	
			(5)		(4)	(%)

In brackets, as percent of total ^{14}C chromatographed.

(iv) Z^1 was not labelled from 3-^{3}H-glucose, but rapidly from U-^{14}C-G6P.

Protoplasts from berries of grape cv. Muscat were cleaned by low speed centrifugation onto Ficoll and subsequently incubated in (a, b) 30 mM glucose and (c) 30 mM G6P.K^+ including respectively the following labels: (a) U-^{14}C-D-glucose (b) 3-^{3}H-D-glucose and (c) U-^{14}C-G6P.K^+. After 40 min incubation protoplasts were removed from the medium (refer Fig. 11.7). The pelleted protoplasts were made 0.3 N perchloric acid and frozen in N_2. The next day samples were neutralized and chromatographed. This delay was such that any sucrose would have been hydrolysed by the acid. The distribution of label is indicated in Table 13.8. U-^{14}C-G6P of course runs next to the origin in the zone X^1.

From U-^{14}C-glucose, as previously shown, fructose was labelled as also were compounds X^1 and Z^1. From 3-^{3}H-glucose the hexose labelling was similar but Z^1 was unlabelled.

(This surprising result has no ready explanation. Formation of heptulose, a sugar recovered in avocado fruit (Pigman & Horton Vol. IA, 1972) would involve loss of this C-3 hydrogen.)

Table 13.8 : Chromatography of radioactivity taken up by protoplasts from three radioisotopes of glucose in 40 min

30 mM substrates including:	cpm as % recovered in these compounds					
	X	Y	Z	*sucrose	glucose	fructose
U-^{14}C-D-glucose	16	0	12	(0)	60	12
3-^{3}H-D-glucose	22	0	0	(0)	69	9
U-^{14}C-G6P.K^{+}	47	4	21	(0)	22	6

*labile to perchloric acid

Half the label from G6P was still at the origin, as if G6P was taken up by protoplasts without hydrolysis of the phosphate ester. Fructose was labelled in addition to free glucose. It is most striking that label was rapidly incorporated into Z^1 from G6P and to a lesser extent into Y^1.

This is further evidence that hexose phosphates (in this case, G6P) are being metabolised rather than participating in an accumulatory process. It reinforces the interpretation placed upon recovery of galactose (13.4.iii above).

(v) Sucrose synthesis and hydrolysis by isolated vacuoles.

From the skin of grape berries cv. Muscat (6 - 11° Brix) vacuoles were prepared. These were free of any protoplasts but contained some cytoplasmic debris after dilution and sedimentation of the lysed protoplasts. In 1 h the pelleted vacuoles had accumulated 40% more radioactivity from 50 mM ^{14}C-sucrose than from 50 mM ^{14}C-glucose and from each sugar this uptake was double that observed in protoplasts (per phenol equivalent). Vacuoles sonicated for 30 sec with a Branson probe, prior to incubation in sucrose or glucose, accumulated respectively 5% and 10% of counts recovered from intact vacuoles (refer Fig. 11.10).

Radioactivity corresponding to sucrose, glucose and fructose is indicated in Table 13.9, and selected chromatogrammes are represented in Fig. 13.12.

Table 13.9 : Radioactivity from chromatogrammes corresponding to sucrose, glucose and fructose following incubation of vacuoles in ^{14}C-sucrose and ^{14}C-glucose

50 mM substrate including:	t (min)	cpm per ml of vacuole suspension		
		sucrose	glucose	fructose
U-^{14}C-sucrose	10	218	288	328
	20	58	717	596
	40	-	440	388
	50	63	405	430
	60	-	646	613
U-^{14}C-glucose	10	88	210	28
	20			
	40	tr	390	33
	50	20	295	33
	60	-	543	25

At 10 min vacuoles in U-^{14}C-sucrose (Fig. 13.12a, b) contained equal amounts of labelled glucose and fructose while 26% of ^{14}C was in sucrose. At 20 min 4% of recovered ^{14}C was at the sucrose position, while at 60 min there was no activity there, the hexoses were equally labelled, and 16% of the radioactivity was in a broad zone closer to the origin. That sucrose was recovered only initially is indicative of rapid sucrose uptake which was not maintained. This is consistent with a declining total ^{14}C uptake rate (Fig. 11.10) or with diphasic uptake (Chapter 12).

Moreover, if the counts in sucrose at 10 min were distributed into the hexose pools subsequently it is evident that the rate of hexose accumulation also declined. For instance, total free hexoses at 10 min and 60 min were 620 cpm and 1260 cpm respectively, of which 220 cpm could have derived from sucrose already present at 10 min. If this were so, net uptake of hexoses in the internal 20 to 60 min was 430 cpm, one third the initial rate.

From U-^{14}C-glucose (Fig. 13.12c, d) the substrate hexose was the principal form of label within the vacuoles, but sucrose and fructose synthesis was evident as also was activity in a broad zone near the origin. Fructose labelling did not increase during 1 h, sucrose disappeared, glucose labelling increased and at 1 h the unknowns (X^1, Y^1, Z^1) contained one third of the radioactivity.

Of the low radioactivity in sonicated vacuoles, that from a ^{14}C-glucose incubation was nondescript while that from U-^{14}C-sucrose occurred as a small glucose peak.

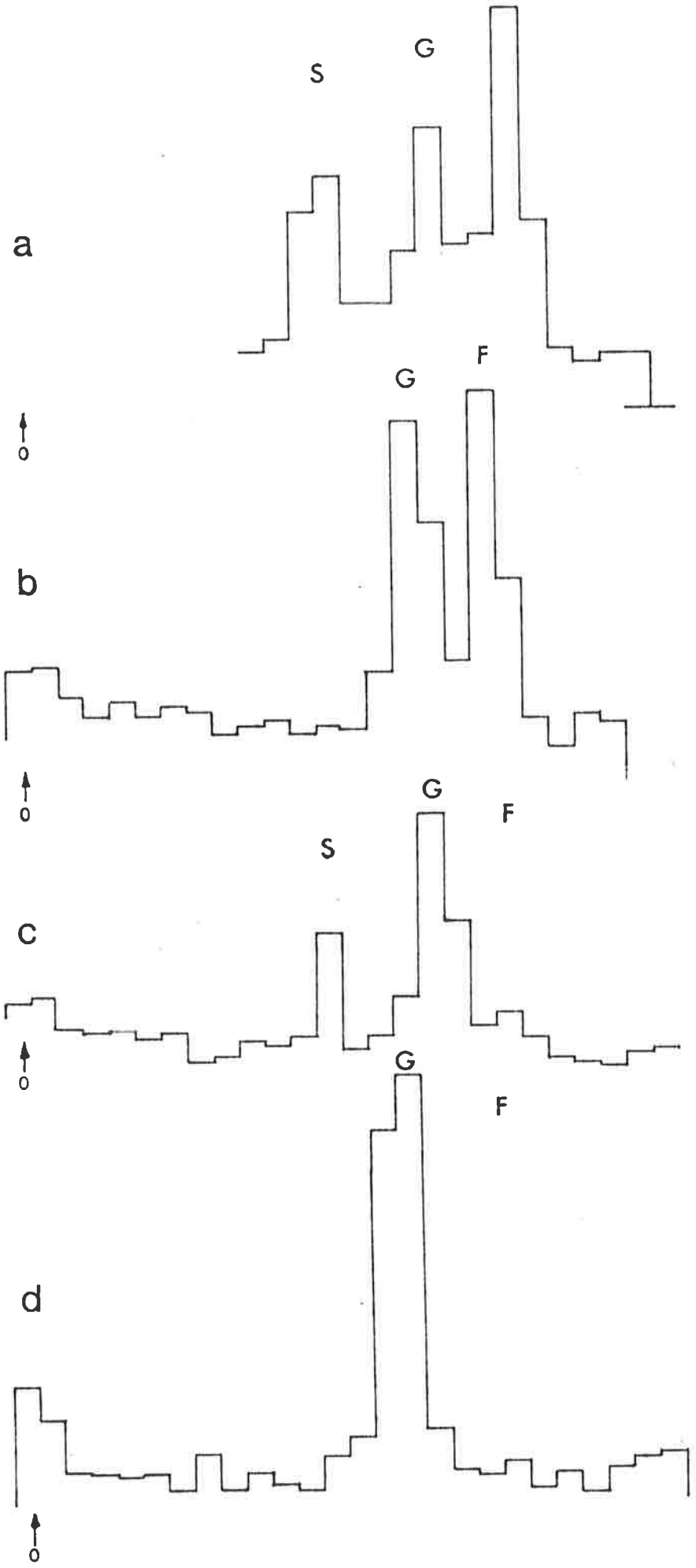


Fig. 13.12 Chromatography of radioactivity taken up by vacuoles from 50 mM U-^{14}C-sucrose (a, b) and U-^{14}C-glucose (c, d) at 10 and 60 min.

These results complement the data from protoplasts and the conclusions drawn thereby (13.5.i above). They suggest that vacuoles are taking up sucrose rapidly: 50% of uptake over 1 h was in fact during the first 10 min (Fig. 11.10). This uptake exceeded the rate of hydrolysis (i.e. sucrose was recovered in the vacuoles). Though sucrose was synthesized (e.g. from ^{14}C-glucose, Table 13.9), as observed also in protoplasts, it did not accumulate. Therefore sucrose accumulation observed in protoplasts is concluded to be cytoplasmic.

Labelling of fructose and sucrose from ^{14}C-glucose, with compartmentation, requires hexokinase, phosphoglucose-isomerase, phosphogluco-mutase, pyrophosphorylase and a synthase (see 3.3b). It is improbable that this occurs in the diluted and dispersed cytoplasm, let alone that the products be transported from such dilution. Thus the recovery of labelled fructose and sucrose is taken as evidence for enzyme complexes associated with the tonoplast, with vectorial synthesis occurring.

13.6 General Discussion

Analysis of the radiochemical products of incubations of skin segments, protoplasts and vacuoles of the grape berry indicated a distinct vectorial property of certain metabolism, as also a surprising degree of interconversion during the accumulation of hexoses.

Of the three sugars considered, glucose was the most rapidly accumulated by excised skin and protoplasts. Yet this accumulation proceeded with concurrent synthesis of sucrose to a greater level than that recovered from U-^{14}C-sucrose incubations (13.5. i). Isomerization to fructose in protoplasts was evident within 2 min and this isomerization appeared associated with group transport (13.3.2.v). A declining rate of glucose uptake by vacuoles was associated with cessation both of sucrose synthesis and of isomerization to fructose (Table 13.9).

In ^{14}C-fructose incubations, isomerization to glucose and incorporation to sucrose was much less. Uptake from ^{14}C-fructose by excised skin only increased marginally between 0.5 and 4 h; most of the sucrose synthesized was diffusible. That treatment which gave the highest accumulation, namely ^{14}C-glucose incubations (Table 13.1), was the exception and it is significant that high levels of compartmented sucrose accompanied this response.

So in each instance there appeared an association between compartmentation of sucrose and continued rapid uptake. In isolated protoplasts and vacuoles declining uptake was reflected in disappearance of compartmented sucrose. Indeed, the diphasic uptake of label by protoplasts and vacuoles is attributed to a rapid initial uptake of sucrose at the tonoplast (13.5. i) which was not maintained, for some reason, in most preparations.

Labelled compounds thought to be fructose phosphate, glucose phosphate and sucrose phosphate (13.5. ii) were recovered, providing supporting evidence for the activity of sucrose phosphate synthase.

The compounds X, which included phosphorylated sugars, increased with time (13.3.2.ii), especially in ^{14}C-glucose medium; they appeared mostly in the medium and the diffusible fraction (13.5. iii) and were unstable upon storage.

Compound(s) Y, probably an oligosaccharide, likewise appeared mostly in the medium or diffusible fraction (13.3.2.ii and 13.5. iii) but was stable upon storage.

The zone Z appeared labelled both in the residual and diffusible fraction from skin segments and contained most probably melibiose (13.4. ii). In protoplasts the labelling in this zone was different: mobility in four systems was indicative of raffinose (except that alkali apparently reduced the compound). Z accumulated in protoplasts, but not vacuoles, and is concluded to be cytoplasmic.

Compounds Y and Z may only be significant *in vitro*, or may also occur *in vivo* as regulators of influx into the cytoplasm and into the extracellular space (Y being diffusible); possibly they act as inhibitors of transport.

Though uptake of fructose was consistent with the postulate that accumulation is via synthesis of sucrose (or sucrose phosphate), the present results suggest that two modifications to this postulate are necessary:

(i) That vectorial synthesis of sucrose occurs at the tonoplast (ref. Fig. 7.1b) as an enzyme-complex not only including the synthases but also hexokinase and possibly phospho-glucose isomerase and phospho-glucomutase.

(ii) That another mechanism of glucose accumulation exists in parallel; this may be characteristic of the pre-veraison berry and not of later accumulation.

The epimerization of glucose to galactose (13.4. iii) and the incorporation of label into other saccharides (Y, Z), is indicative of high cytoplasmic levels of intermediates to glycosyl transfer reactions and epimerization. Similarly, the surprisingly high incorporation of ^{14}C into diffusible sucrose in skin - despite low endogenous levels - is attributed to the high levels of supplied hexoses. The labelling-pattern in this diffusible sucrose will not necessarily be that of the sucrose transported at the tonoplast. It is as if transport was determined not only by cytoplasmic sucrose but by an integrated process at the tonoplast.

The recovery of labelled fructose and sucrose from isolated vacuoles incubated in ^{14}C-glucose is good evidence for enzyme complexes associated with the tonoplast, with vectorial sucrose synthesis occurring (13.5.v).

CHAPTER 14 INTEGRATIVE DISCUSSION

CHAPTER 14 INTEGRATIVE DISCUSSION

14.1 Preamble

The initial hypothesis, derived from Coombe (1976a), was stated as follows (11.1):

> The pathway of carbohydrate movement during the accumulating stage of ripening in the grape berry is in the first place apoplasmic. Sucrose is unloaded from the phloem into the intercellular space where transfer through the tissue by diffusion, and inversion, occurs. Glucose and fructose traverse the plasmalemma; sucrose (or sucrose phosphate) is synthesized in the cytoplasm and transported at the tonoplast; glucose and fructose are produced in the vacuole by the action of an invertase.

In this thesis the sugar accumulation process in pericarp tissue of the developing grape berry was studied by incubation of excised skin, or protoplasts and vacuoles isolated therefrom, with sucrose, glucose or fructose radionuclides. Evidence which supports the operation of group transport at the tonoplast derives in part from the kinetics of uptake, but particularly from analysis of the products of uptake.

14.2 Mediated Transport

The evidence for mediated transport across the membrane of protoplasts, operating additionally to passive diffusion, is considerable: stereo-specific accumulation from D-glucose (11.3.1) with competition by 2-deoxy-D-glucose (11.3.2), selectivity for glucose over sucrose (11.3.1), and stimulation of glucose uptake by fructose (11.3.4). The latter implicates metabolism in this uptake process. The concentration dependency of uptake clearly showed a saturable component, as expected of a mediated process. The K_Ts for glucose and fructose were (mM): 5.6 ± 1.4 and 1.7 ± 0.4 respectively (Table 12.1).

Similarly, there was evidence for mediated sugar transport in isolated vacuoles despite high levels of diffusion across the tonoplast in these preparations (e.g. as shown by that of L-glucose in Table 11.5). Uptake was selective in that accumulation from L-glucose was less than from D-glucose or sucrose. Moreover, uptake of glucose and sucrose (compared per unit phenol) was appreciably greater in vacuoles than in protoplasts (11.3.1, 11.3.7, 11.3.8); sucrose uptake was stimulated 260% with cysteamine, stimulated also by Mn^{2+} and partially inhibited by a number of other

compounds (11.3.8) . This sucrose uptake was evidently sensitive to the vagaries of protoplast preparation and vacuole isolation (11.3.8). The concentration dependency of uptake by vacuoles showed a saturable component which was analysed for glucose and fructose to have K_Ts (mM) of: 10.3 ± 1.9 and 2.8 ± 1.3 respectively (Table 12.2).

The two K_Ts for fructose, into protoplasts and into vacuoles, were not significantly different and could have arisen from the same interaction at the tonoplast with transport across the plasmalemma being by passive diffusion alone. This, and the more rapid uptake by vacuoles, leads to consideration of "accumulation" as in fact a regulatory mechanism sensitive to cytoplasmic sugar levels and "functioning" to attenuate these (a notion reviewed in 1.3.b.ii).

14.3 Indeed, Group Transport ...

There is strong evidence for group transport of sugars at the tonoplast, a conclusion which is compatible with afore-mentioned characteristics of mediated uptake and, as well, accommodates the following results:

Isolated vacuoles incubated in U-^{14}C-glucose and cleaned through a Ficoll gradient contained not only labelled glucose but also labelled sucrose and fructose within 10 min (13.5.V). A declining rate of glucose uptake by vacuoles appeared to be associated with cessation both of isomerization to fructose and of sucrose synthesis (Table 13.9). This is taken as evidence for enzyme complexes associated with the tonoplast and catalysing vectorial modification of the ^{14}C-glucose moiety.

This conclusion is reinforced by evidence from protoplast incubations, in which isomerization and sucrose synthesis were also observed. Not all sucrose synthesized was vacuolar, as a fraction was not inverted (Fig. 13.9), in contrast to complete inversion in isolated vacuoles. But the rapid initial uptake of sugars by protoplasts was attributed to more rapid transport at the tonoplast (13.5.i) which was not maintained beyond 10 min in most preparations. It was as if a potentiated transport system existed, with evident distinction from any mechanism of diffusion. Such diphasic uptake has been observed in other incubation studies with protoplasts (e.g. Guy et al. 1977, 1980).

When excised skin was incubated in 100 mM ^{14}C-glucose or ^{14}C-fructose, uptake from glucose was greater and, in particular, increased in a linear manner from 0.5 h to 4 h. Analysis of the radiochemical products indicated synthesis of sucrose within the tissue and marked compartmentation of sucrose in that treatment which gave the highest accumulation (13.3.iv) in contrast to high levels of diffusible sucrose in other incubations where accumulation was low. An important observation in this respect was the apparent vectorial isomerization of glucose to fructose in skin segments (Fig. 13.2). Moreover, these observations were substantially unaffected by inclusion of 100 mM unlabelled fructose in the 100 mM U-^{14}C-glucose incubation (Table 13.2). This would have dramatically altered equilibria between G6P, F6P, fructose and glucose in the cytoplasm, yet the isomerization of glucose to fructose continued, indicating that it occurred across a membrane unaffected by pool equilibria (mechanism d, in 13.3.2.v). This conclusion needs to be incorporated into the original hypothesis (see Conclusion: 14.7).

14.4 Sucrose Synthesizing Apparatus

Many observations are consistent with the following statement:

In cells of the grape berry in vivo, sucrose synthesis occurs in a multienzyme complex at the tonoplast as a group transport mechanism. In vitro these same cells are perturbed, due in part to dissociation of the synthesizing complex from the tonoplast and in part to high hexose levels in the cytoplasm. Metabolism appears to be partially de-controlled. High levels of activated hexoses (or even sucrose-phosphate) appear in the cytoplasm and transglycosylation reactions proceed (including sucrose synthesis).

These observations are as follows:

- Substantial labelling of sucrose in vitro is in contrast with low sucrose concentrations in vivo (Table 13.1 versus Fig. 8.3). The in vitro labelled sucrose was mostly diffusible (Table 13.1).
- Uptake of sugars by protoplasts and vacuoles was often diphasic with time, and declining sucrose synthesis was associated with increased synthesis of cytoplasmic Z^1 which is considered as an alternative glycosyl transfer reaction (13.5.iii).
- Epimerization to galactose was evident in skin segments (13.4.iii).
- Labelled sucrose-phosphate was recovered from protoplasts which, considering the phosphatase activity attributed to the vacuolar contents, must have been cytoplasmic.
- Applied ^{14}C-G6P was incorporated into Z (13.5.iv) whereas with sucrose synthesis occurring, with uptake, it was expected to be accumulated as hexoses. Thus the spatial organisation of sucrose synthesis - at the tonoplast - appears to be important.

The labelling pattern of the hexose moieties within diffusible sucrose (Table 13.3), as the product of this non-vectorial synthesis, was typical of the labelled hexose as supplied in the incubation medium. In the case of ^{14}C-fructose incubations, labelling pattern of compartmented hexoses was consistent with this same synthesis, as predicted in the initial hypothesis. In the case of ^{14}C-glucose incubation, it was not. The apparent vectorial isomerization of glucose to fructose mentioned above (paragraph 14.3) is pertinent. Phospho-glucose-isomerase may form part of the multienzyme complex such that sucrose synthesis here may proceed without supply of fructose. This leads to more labelling of residual fructose than found in "cytoplasmic" (diffusible) sucrose synthesis from ^{14}C-glucose. The equilibrium of phospho-glucose-isomerase (F6P/G6P = 0.3: Table 3.1) is such that isomerization of fructose to glucose will be much less (as observed) than the opposite reaction: but nevertheless, why is not this isomerization equally vectorial if occurring at the same site (Fig. 13.2)? It is suggested that this arises from the pathway of incorporation to sucrose. F6P (from glucose isomerization, for example) is directly incorporated (and transported) while G6P (from the lesser fructose isomerization, for example) is not directly incorporated but must pass a circuitous route of GIP and NDPGlucose to sucrose.

As labelled glucose is incorporated to the fructosyl moiety of sucrose despite high levels of fructose (e.g. Table 13.2, incubations in ^{14}C-glucose containing 100 mM fructose) it must be synthesis from F6P. (To copy Sacher's terminology from 13.3.2.v.c: glucose may be a better source of F6P than fructose, but it cannot be a better source of fructose!) Thus, the group transport would appear to be sucrose phosphate synthase. In this case, the speculation presented in Fig. 7.1b should be modified to incorporate other enzymes of the complex (Fig. 14.1).

Hence the conclusion is drawn that the equimolar concentrations of hexoses in grape juice are not a consequence of their 1:1 ratio in the translocated sugar, but result from the characteristics of the sucrose transport mechanism at the final membrane, the tonoplast.

14.5 Hexose compartmentation in the developing grape berry

An efflux analysis of hexose compartmentation indicated (Chapter 8): At veraison half of the sugar in the skin was freely diffusible but two weeks later 80% (of an increased total) was diffusible. Hence it

appears that accumulation is primarily associated with unloading from the phloem into the apoplasm and not loading into the final compartment, the vacuole. This result raises several questions.

The first question concerns the meaning of efflux into a medium containing 100 mM mannitol, ions, buffer, SO_2 and antibiotic (refer 8.2). Similar efflux from radiochemically labelled material revealed the distinctive nature of two efflux fractions and the residual (13.3.2.i). But what is the consequence of ripening upon this phenomenon, especially when no increment is made to the osmoticum? This remains unanswered.

Secondly, what are the differences between flesh and skin cells with respect to sugar accumulation? In 8.4.1 it was argued that the higher total sugar measured in the flesh of grape berries must have been due to more compartmentation in the flesh. The distinctive ontogeny of skin and flesh cells, and histochemical differences, were evident from early in berry development (1.3). It is possible that the skin tissue acts as some sort of buffer tissue which can accumulate or re-export sugars (to other tissue of the same fruit, for example). This would explain the variable residual glucose levels indicated in Fig. 8.3. The "re-export" of hexoses may be significant not only as a source but also as a regulatory mechanism, impinging upon the neighbouring vascular tissue (for instance, affecting invertase activity).

One speculates that lesser compartmentation in skin cells results in higher cytoplasmic sugar levels with the consequential activation of shikimic acid and phenyl-ammonium lyase pathways (Pirie & Mullins 1976, 1977).

Thirdly, what is the difference between pre-veraison and post-veraison tissue? From the pre-veraison green berry, glucose is recovered. The fructose moiety of translocated sucrose is apparently metabolized (respired, etc.) more rapidly than glucose, or at least is not sequestered away from metabolism. In line with this, Kliewer's (1964) work with ^{14}C showed fructose to be the more metabolized hexose in the berry, while Hardy (1968) showed fructose uptake to be slower (reviewed in 2.2). So in the green berry the evidence is that the sucrose translocation at the tonoplast is not active, and only glucose is accumulating.

The post-veraison tissue assessed in Chapter 8 appears to be a net fructose accumulator. But studying Fig. 8.3, the increase in residual fructose was associated with a decreased variability of "residual" glucose. It is possible that sucrose compartmentation (with hydrolysis) is occurring, while the reserve of glucose evident in green berries is being

remobilized from within the same or different cells.

If efflux analysis can be taken as a useful indicator of compartmentation in vivo, the most important observation in Chapter 8 is the apparent concentration gradient between the diffusible and residual volumes, diffusible being high. In the skin tissue, there would appear to be a resistance to sugar movement into the storage compartment even up to 16° Brix. Despite this, accumulation in the whole berry proceeds. A primary change which disposes to the accumulation of sugars appears to be phloem unloading, rather than cellular compartmentation. This was not a tested conclusion, but certainly requires examination.

14.6 *Inter alia*

It is worth recalling certain points from earlier chapters pertaining either to physiology or to techniques.

(i) The concept of an "ambiquitous" enzyme (3.4.c) may be relevant to sucrose storage in plant cells, wherein a multienzyme complex associated with a membrane serves as a group translocator, or, solubilized, may synthesize sucrose for symplasmic re-export.

(ii) Compounds such as Y and Z arising from glycosyl transfer reactions (13.4.iii) may act in a feedback regulation of uptake at the plasmalemma. Coombe & Matile (1980) observed that washing of skin segments from the berry predisposed to subsequent glucose accumulation in vitro. It is possible that di- or oligo-saccharides are inhibiting accumulation in vivo and are diluted upon washing. Willenbrink and Doll (1980) found raffinose to inhibit sucrose uptake, though this was not the case here.

(iii) In the literature review (2.5) the water and osmotic relationships of fruit tissue were discussed. It was argued that extracellular fluid will attain a maximum of 10% hexoses before phloem loses turgidity. This calculation wrongly ignored solutes other than sucrose contributing to phloem turgor, for Smith and Milburn (1980c) indicate that in Ricinus phloem sap "other solutes" contribute about half the osmotic pressure. So a similar phloem sap in the grape berry would allow (theoretically) about 20% hexose accumulation from mere hydrolysis of unloaded sucrose without any requisite concentrative accumulation. (Above 20%, phloem plasmolysis would occur.)

Nevertheless, the point remains that the extracellular space may be considered, to some extent, as part of an osmometer in which respect measurement of resistance to water and sugar flow in the pedicel is of interest.

The effect of hydrostatic pressure upon transport in higher plants is just receiving attention (Smith and Milburn 1980a, b, c); the surprising result here that pressures of 5 and 10 Atm stimulated glucose uptake by gradients and hydrostatic pressure (in addition to osmotic pressure) during protoplast and vacuole isolation should also be considered.

(iv) It is suggested that further work with protoplasts and vacuoles isolated from grape tissue such as this should examine sugar uptake in a range 0.1 - 20 mM (refer 12.5), conducting the necessary experiments to identify the nature and contribution of any rapid initial uptake (Fig. 12.4). As a routine, L-glucose should be included to identify non-specific uptake. A number of key experiments are evident from the last paragraphs of 11.4. To these might be added certain ^{14}C-pulse/chase experiments; studies of efflux from preloaded (^{14}C) material; and fractionation of protoplasts to localize the activity of enzymes concerned with glucose, fructose and sucrose activity. It has been suggested, however, that some key enzymes are only weakly bound to the tonoplast. There is, therefore, merit in incubating isolated vacuoles in relevant substrates (e.g. sucrose phosphate and F6P using both ^{14}C and ^{32}P labels) to determine vectorial release of products rather than relying upon results from homogenates.

14.7 Conclusion

A modified hypothesis for accumulation of sugars in the grape pericarp is as follows:

> The pathway of carbohydrate movement during the accumulating stage of ripening in the grape berry is in the first place apoplasmic, by which accumulation in the tissue precedes concentrative uptake in the cell. Sucrose is unloaded from the phloem into the intercellular space where transfer through the apoplasm by diffusion, and inversion, occurs. Glucose and fructose traverse the plasmalemma. Then, at the tonoplast, sucrose phosphate is synthesized vectorially from activated glucose and F6P (derived from fructose and/or glucose) by metabolism unaffected by pool equilibria (14.3). The sucrose phosphate is dephosphorylated, the sucrose inverted, and glucose and fructose accumulate inside the vacuole.

As a development from Fig. 7.1b, the components of uptake identified with the tonoplast are depicted schematically in Fig. 14.1.

KEY :

HK : hexokinase

PGI : phosphoglucose isomerase

PGM : phosphogluco-mutase

PP : UDPG pyrophosphorylase

SPS : Sucrose phosphate synthase

——— : ENZYMES (PGI, SPS) ASSOCIATED WITH TONOPLAST

– – – – : ENZYMES POSSIBLY INTEGRAL TO THE COMPLEX

•••••► : Movement of metabolites

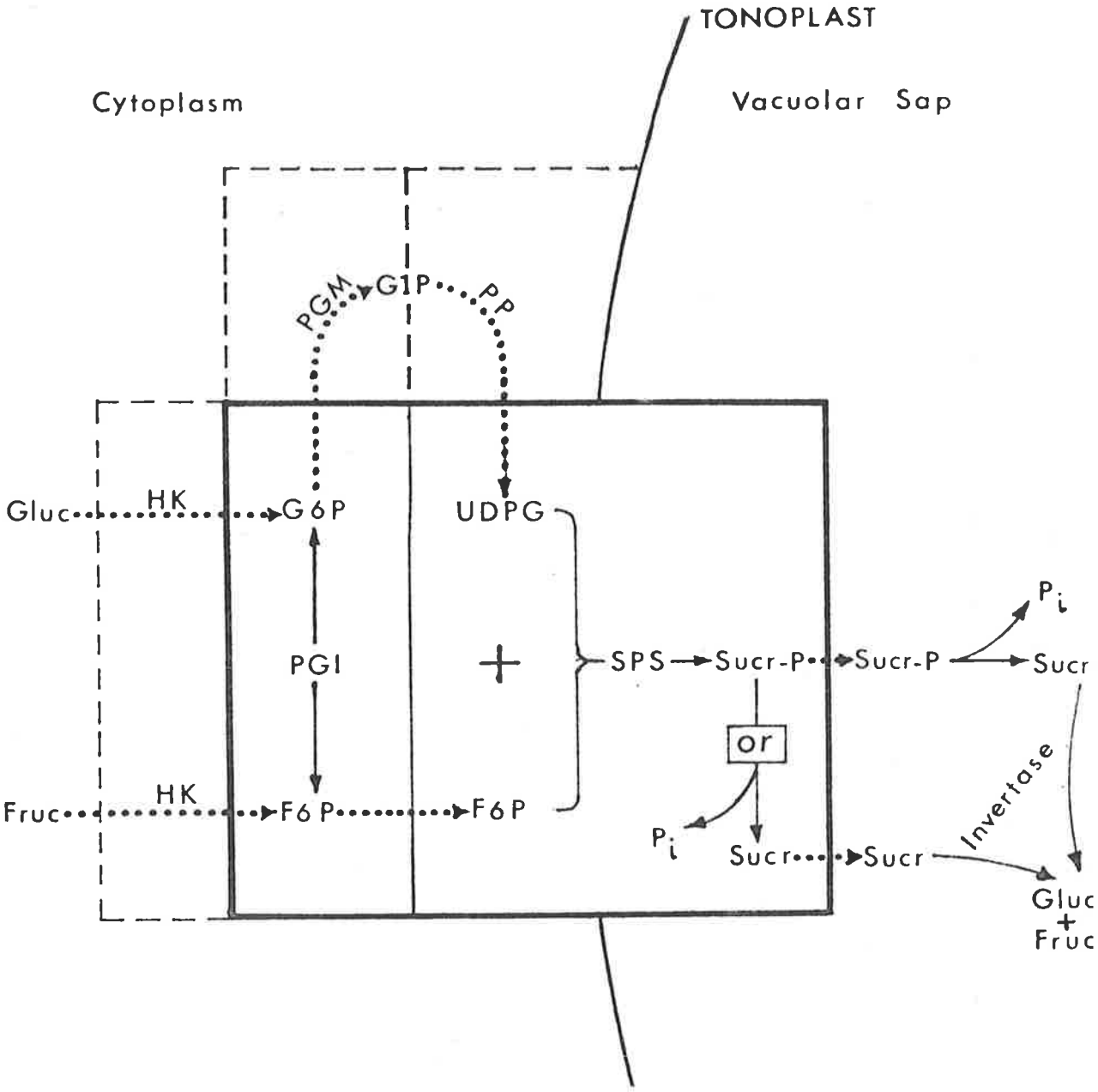


Fig. 14.1 Elements of a group translocator proposed to be at the tonoplast, being a multienzyme complex of sucrose phosphate synthase and phosphoglucose isomerase. The other enzymes, HK, PGM and PP may also be integral components.

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