# STUDIES ON *LOLIUM MULTIFLORUM* ENDOSPERM IN TISSUE CULTURE

## I. NUTRITION

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*Abstract*

Stocks of *L. multiflorum* endosperm callus have been maintained in liquid suspension culture on a modified White's medium for 5 years. The mean doubling time under the conditions used is 3·2 days. Best growth is obtained on sucrose; fructose and glucose are good carbon sources, whereas growth is only moderate on an equimolar mixture of both. D-Galactose supports good growth after a long lag period but D-mannose, L-arabinose, D-xylose, and *myo*-inositol are not carbon sources. Cells grown on sucrose enter the stationary phase at a time coinciding with the disappearance of sucrose from the medium. The chemically defined Linsmaier–Skoog medium supports growth, but at a much lower rate than White's medium.

## I. INTRODUCTION

The usefulness and limitations of plant cells in tissue culture for biochemical and physiological studies have been discussed by Tulecke (1964) and by Puhan and Martin (1971). Our interest in the biochemistry of endosperm cell walls and in particular in the biosynthesis of a mixed-linked $\beta$-glucan found in some cereals (Clarke and Stone 1963) led us to consider the possibility of using cultured endosperm as a source of actively dividing cells. Endosperm of three gramineous species has been grown in tissue culture: viz. *Zea mays* L. (Straus and LaRue 1954), *Lolium perenne* L. (Norstog 1956), and *Lolium multiflorum* Lam. (Norstog *et al.* 1969). Macleod and McCorquodale (1958) had shown that seeds from *L. perenne* contain a water-soluble component with the characteristics of a $\beta$-glucan and for this reason the *Lolium* spp. were chosen for further investigation.

This paper describes aspects of the nutritional physiology of *L. multiflorum* in liquid culture and in Part II of this series (Mares and Stone 1973) the morphology and

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ultrastructure of the cultured endosperm is compared with that of endosperm from the developing seeds. The chemistry of *L. multiflorum* cell walls and aspects of the biosynthesis of the β-glucan components are reported elsewhere (Smith and Stone 1973*a*, 1973*b*).

## II. Materials and Methods

### (*a*) *Cell Cultures*

Cultures of callus derived from endosperm of *L. perenne* (Norstog 1956) and *L. multiflorum* (Norstog *et al.* 1969) were obtained from Dr. K. Norstog (Biology Department, North Illinois University, De Kalb, Illinois).

### (*b*) *Media*

The composition of the modified White's liquid medium used throughout this study (Table 1) was that recommended by Dr. K. Norstog (personal communication) except that the phosphate concentration has been increased. Glass-distilled water was used throughout. The pH of the solution containing vitamins, yeast extract, and sucrose was adjusted to 5·5 with a few drops of 1M HCl and filter-sterilized, whereas the solution of major and minor elements was autoclaved at 15 lb/in$^2$ for 30 min. Component solutions were then mixed aseptically.

The other liquid medium tested was the fully defined medium of Linsmaier and Skoog (1965) using Braun's supplement.

Table 1

Composition of the modified White's medium

| Component | Concn. (mM) | Component | Concn. (μM) |
|---|---|---|---|
| Major elements | | Minor elements | |
| $MgSO_4.7H_2O$ | 2·96 | $MnSO_4.4H_2O$ | 17·75 |
| $Na_2SO_4$ | 1·42 | $ZnSO_4.7H_2O$ | 1·74 |
| $Ca(NO_3)_2.4H_2O$ | 1·23 | $H_3BO_3$ | 8·08 |
| KCl | 0·94 | $CuSO_4.5H_2O$ | 0·1 |
| $NaH_2PO_4$ | 11·60* | $CoCl_2.6H_2O$ | 0·11 |
| $KNO_3$ | 0·79 | Ferric citrate | 40·7 |
| Other components | | Vitamins | |
| Indolylacetic acid | 5·7 μM | Nicotinic acid | 0·01 |
| Sucrose | 117 mM | Thiamine hydrochloride | 0·71 |
| Difco yeast extract | 0·5% | Calcium pantothenate | 0·53 |
| | | Pyridoxine | 1·21 |

* Concentration increased tenfold over White's medium as modified by Norstog.

### (*c*) *Carbon Sources*

The sucrose component of the modified White's medium was replaced by each of the following sugars: D-glucose, D-fructose, a mixture of D-glucose and D-fructose, D-mannose, D-galactose, *myo*-inositol, D-xylose, and L-arabinose and the growth of *Lolium* endosperm on each was measured relative to growth on a sucrose medium. The mixture of D-fructose and D-glucose contained 20 g of each sugar per litre of medium, whilst all other sugars were tested at a concentration of 40 g/l. Sugars were obtained from British Drug Houses, Poole, Dorset, U.K.

### (*d*) *Culture Conditions*

Liquid suspension cultures were maintained in 250-ml conical flasks containing 100 ml of medium. Flasks were inoculated with 15 ml of stationary phase culture and shaken on a horizontal rotary shaker (134 r.p.m.; 1·7 cm throw) in the dark at 25°C. Stocks were subcultured every 3–4 weeks, the inoculum being transferred by a spring-loaded syringe with a wide-bore needle to fresh media. This method of transfer enabled the delivery of a uniform and reproducible tissue sample.

### (*e*) *Measurements of Growth*

Growth was determined by packed cell volume (millilitres of cells/100 ml of culture) and dry weight (grams dry weight of cells/100 ml of culture). Duplicate samples (5 ml) were removed from each culture at various time intervals, the total volume of the sample was measured, and the cells were sedimented in graduated centrifuge tubes (1770 *g*, 10 min). The volume of cells was recorded, the cells washed once with distilled water, transferred to pre-weighed vials, and freeze-dried for dry weight determination. These parameters reflect increases in cell numbers, cell expansion, and accumulation or depletion of compounds in the cells (for example, starch).

Each growth curve is the mean of measurements made on four replicate cultures. A control growth curve, determined for cells grown on a modified White's medium which contained sucrose, served as a standard for growth comparisons in each experiment.

Growth on different media was measured for the duration of two successive periods of culture to assess the extent to which components in the parent medium were carried over in the initial inoculum.

### (*f*) *Disappearance of Sucrose from the Medium*

To measure the rate of sucrose disappearance from the medium three conical flasks containing 100 ml of medium (11·7 mM sucrose) were each inoculated with 15 ml of a stationary-phase culture and incubated as described. Packed cell volume and cell dry weight were measured throughout the period of culture. At intervals the extracellular medium was sampled and the polysaccharides present precipitated with 4 volumes of ethanol. The amount of non-precipitable carbohydrate present in the supernatant was measured as sucrose by the phenol–sulphuric acid method of Dubois *et al.* (1956) as modified by Immers (1964).

### (*g*) *Paper Chromatography*

Descending paper chromatography was performed on Whatman No. 3 chromatography paper which had been washed with water. Chromatograms were developed for 40 hr at room temperature in ethyl acetate–n-propanol–water, 4 : 1 : 2 by volume (upper phase). Reducing sugars were detected with alkaline $AgNO_3$ by the method of Trevelyan *et al.* (1950), as modified by Moscatelli (cited by Mayer and Larner 1959). Solutions were deionized with Bio-Rad AG 501-X8 ($H^+/HCO_3^-$ form) (Bio-Rad Laboratories, Richmond, California, U.S.A.) prior to chromatography.

## III. Results

### (*a*) *Liquid Suspension Culture*

White's medium as modified by Norstog (1956) and containing 1·16 mM $NaH_2PO_4$ was satisfactory for growing *L. multiflorum* and *L. perenne* endosperm on solid agar (0·9%, w/v) medium, but did not support growth in suspension culture. *L. multiflorum* endosperm grew rapidly in suspension culture if the phosphate concentration was increased tenfold to a level of 11·6 mM $NaH_2PO_4$ (Table 1). However, we were unable to grow *L. perenne* in suspension culture at this phosphate concentration or at 1·16 mM as used by Tulecke and Nickell (1960).

The ability of endosperm to grow in suspension culture when the phosphate concentration is increased may be related to the buffering capacity of the nutrient

medium. When the changes in pH of the culture medium were monitored throughout the growth cycle (Fig. 1), it was found that the pH fell from 5·3 to 4·9 by the end of the lag phase and then rapidly rose to 5·5 at the end of the exponential phase of growth.

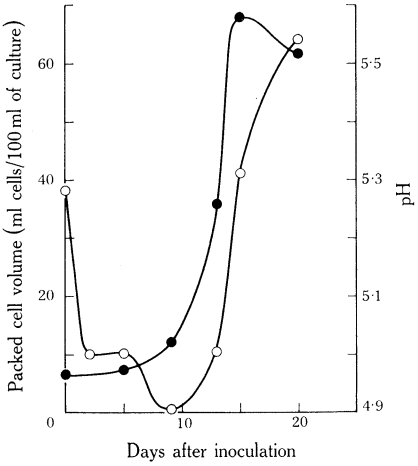



Fig. 1.—Changes in the pH (○) of the extracellular medium during growth of *L. multiflorum* endosperm. ● Packed cell volume.

In suspension culture, *L. multiflorum* endosperm cells tended to grow in aggregates which were difficult to disperse and count. Several attempts were made to disperse the aggregates to enable total and viable cell counts to be made. Incubation with protein and polysaccharide hydrolases and treatment by the chromium trioxide method of Henshaw *et al.* (1966) resulted either in complete disintegration of the cell walls or in no significant change in the state of aggregation.

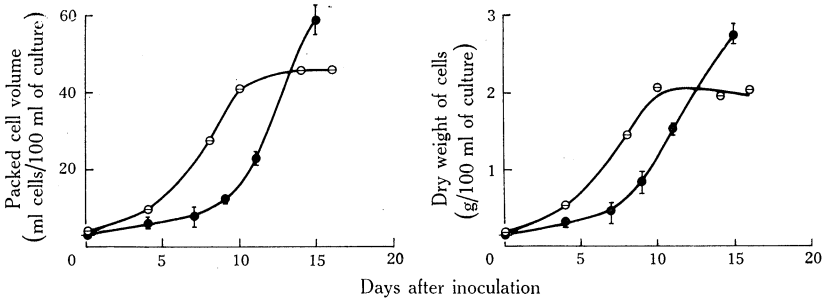



Fig. 2.—Growth response of *L. multiflorum* cells in a nutrient medium containing autoclaved sucrose (●) and in a nutrient medium containing filter-sterilized sucrose (⊖)

The ultrastructure of the endosperm cells in suspension culture has been examined by electron microscopy at different phases of growth and the results are reported in Part II (Mares and Stone 1973).

### (*b*) *Effect of Alteration of Sugar Carbon Source*

Growth responses were compared in media containing autoclaved sucrose or filter-sterilized sucrose (Fig. 2). The duration of the lag phase was considerably extended when autoclaved sucrose was used. Autoclaving results in some modification of the sugars in the medium and may cause a change in pH so that a longer conditioning period is required before the medium will support rapid cell growth. Consequently, in media used for subsequent growth determinations, sugars were filter-sterilized.

Growth responses obtained when sucrose was replaced by other sugars are shown in Figures 3(*a*)–3(*h*). *L. multiflorum* endosperm grew well in a modified White's medium containing D-glucose, D-fructose, or sucrose as the carbon source [Figs. 3(*a*) and 3(*b*)]. In the first subculture, there were no significant differences in the ability of the cells to grow in any one of these media. However, after the second transfer to fresh media maximum cell yields (packed cell volume and cell dry weight) for endosperm grown in glucose and in fructose media were noticeably lower than the maximum yield obtained from cells grown in a sucrose medium. These differences could not be attributed to variations in the preparation of media, since aliquots from the same batches of media were used for the two successive periods of cultivation. Surprisingly, when an equimolar mixture of glucose and fructose was tested [Fig. 3(*c*)], the yield of cells was lower than when either sugar was supplied alone.

The only other sugar found to support cell growth was D-galactose [Fig. 3(*d*)], although the onset of cell division was delayed when compared with the response for cells grown in a sucrose medium. For cells grown in D-galactose, the lag phase lasted for 12–14 days in both periods of culture, whereas the lag phase for sucrose-grown cells was 7 days after the initial transfer and 6 days after the second transfer of cells.

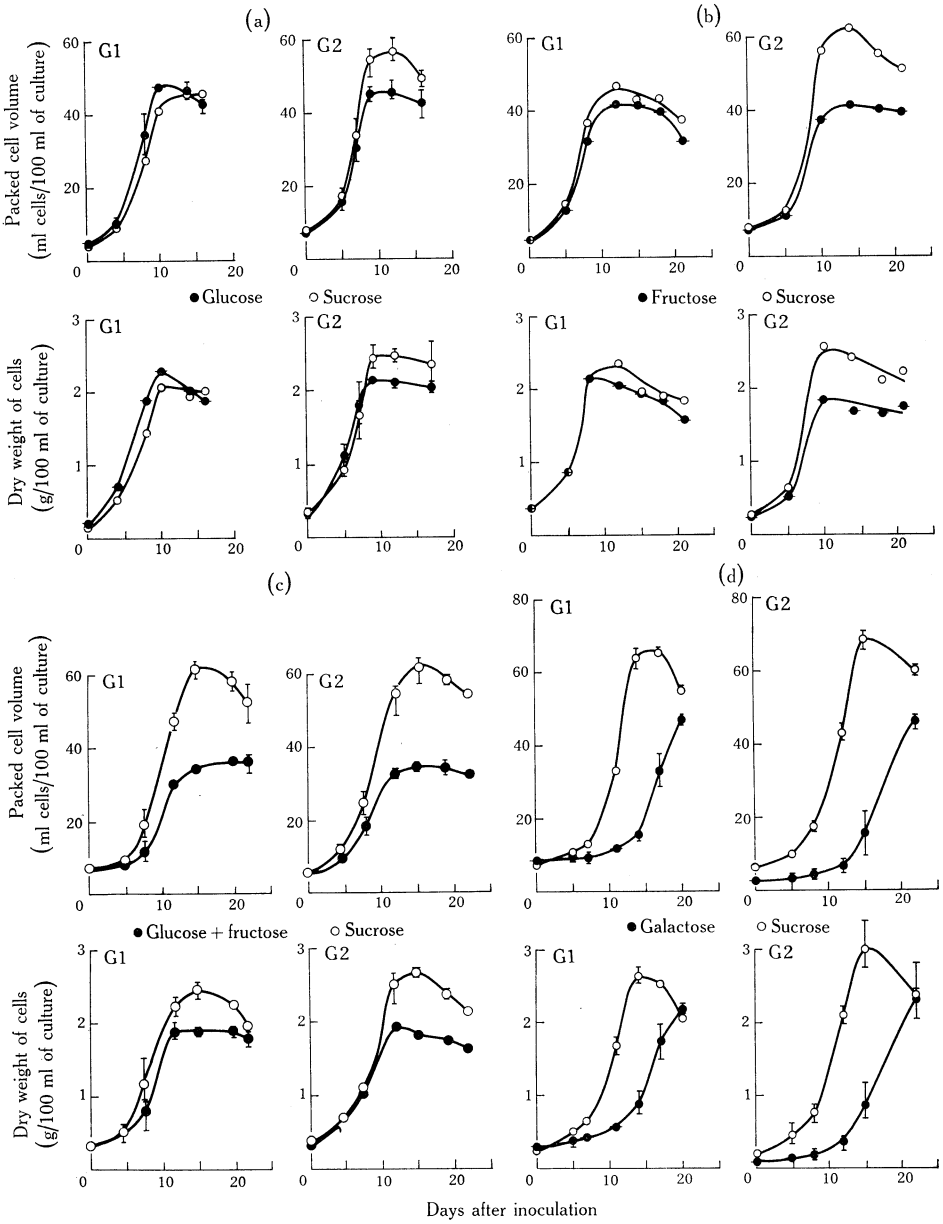
No significant growth could be measured when sucrose was replaced by D-mannose, *myo*-inositol, L-arabinose, or D-xylose [Figs. 3(*e*)–3(*h*), respectively].

### (*c*) *Comparison of Modified White's Medium and the Fully Defined Medium of Linsmaier and Skoog*

The endosperm cells did not grow well in the medium of Linsmaier and Skoog (Fig. 4). The rate of growth during the exponential phase was less than during the corresponding period for cells grown in the modified White's medium and growth was even further diminished in the Linsmaier and Skoog medium after the second transfer of cells. The cells in the Linsmaier and Skoog medium were very white in appearance, in contrast to the buff colour of cells grown in the modified White's medium which contained yeast extract.

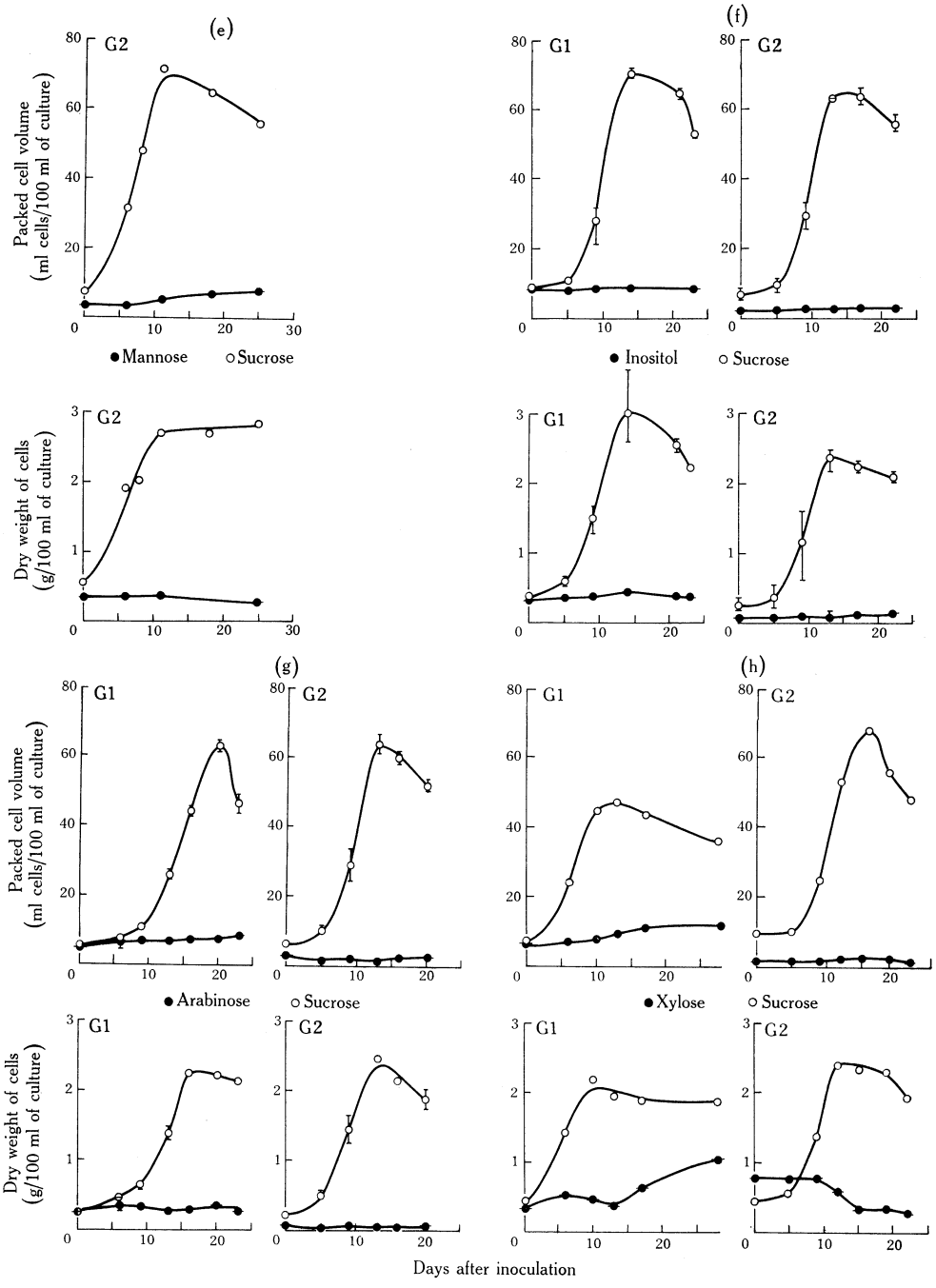
### (*d*) *Disappearance of Sucrose from the Medium*

Only after the onset of the exponential phase of growth was there any marked reduction in the sucrose concentration of the medium (Fig. 5). The cessation of growth corresponded to the virtual disappearance of carbohydrate (measured as sucrose) from




Figs. 3(*a*)–3(*d*).

Fig. 3.—Comparison of growth curves obtained for cells grown on sucrose and cells grown on the following media: (*a*) D-glucose; (*b*) D-fructose; (*c*) a mixture of D-glucose and D-fructose; (*d*) D-galactose; (*e*) D-mannose; (*f*) *myo*-inositol; (*g*) L-arabinose; (*h*) D-xylose. G1, first generation; G2, second generation. The *myo*-inositol medium was prepared by autoclaving a separate solution of *myo*-inositol (40 g) in 300 ml of water and aseptically mixing this with the mineral salt and vitamin components.


(e)
(f)
(g)
(h)
G1
G2
Packed cell volume (ml cells/100 ml of culture)
Dry weight of cells (g/100 ml of culture)
● Mannose ○ Sucrose
● Inositol ○ Sucrose
● Arabinose ○ Sucrose
● Xylose ○ Sucrose
Days after inoculation

Figs. 3(*e*)–3(*h*).

the culture medium; the concentration of non-precipitable carbohydrate was reduced during the period of exponential growth from 103 mM to 2 mM, after which little change was observed.

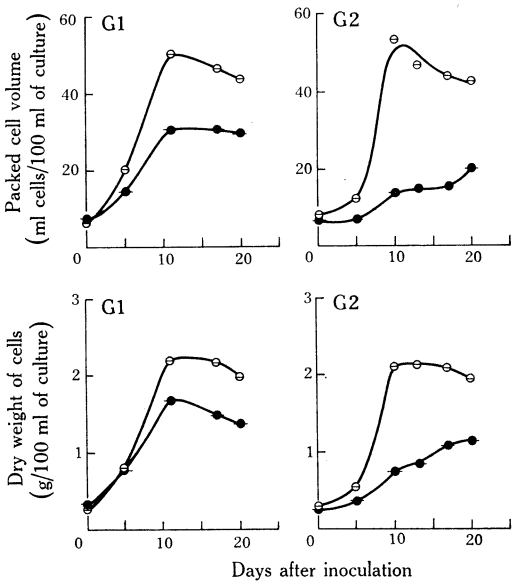



Fig. 4.—Comparison of growth curves obtained for cells grown on the fully defined medium of Linsmaier and Skoog 1965 (●) and on the modified White's medium (⊖). G1, first generation; G2, second generation.

When the sugar composition of the medium was examined by paper chromatography 8 days after inoculation, the major component was sucrose, but smaller

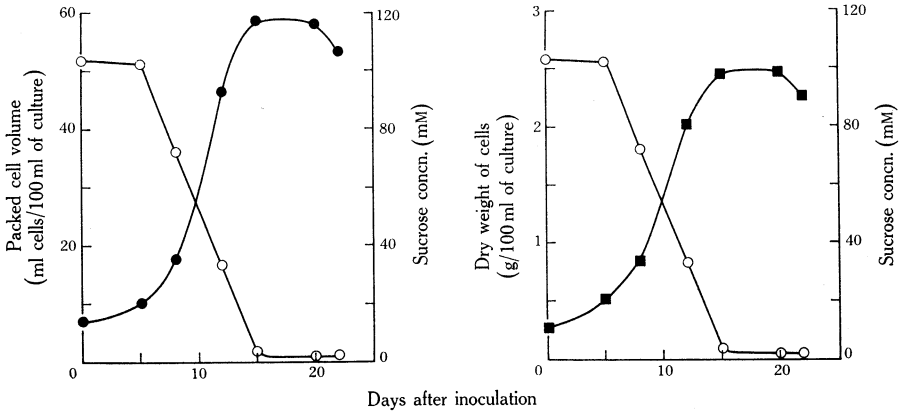



Fig. 5.—Disappearance of sucrose from the culture medium. ● Packed cell volume. ○ Sucrose concentration. ■ Dry weight of cells.

amounts of glucose, fructose, and traces of two slower-moving oligosaccharides were also present.

## IV. Discussion

In liquid culture, *L. multiflorum* endosperm cells grow rapidly with a mean doubling time of 3·2 days (calculated from the collected data for growth on modified White's medium with sucrose as carbon source). The endosperm tissue grows in large cellular aggregates of variable size which are difficult to disperse into single cells for counting. As shown in Part II (Mares and Stone 1973) individual cells store both starch and protein bodies and in many morphological features resemble native endosperm cells.

The continuation of growth of *Lolium* endosperm in culture is clearly limited by the availability of carbohydrate as a source of organic carbon for energy and biosynthesis, as has also been observed for tobacco (Upper *et al.* 1970) and sycamore (Simpkins and Street 1970) suspension cultures.

The best growth was obtained on sucrose. D-Fructose and D-glucose are good substrates, but growth was only moderate on an equimolar mixture of both D-glucose and D-fructose. D-Galactose supported good growth after a long lag period, but D-mannose, L-arabinose, D-xylose, and *myo*-inositol were not substrates. Straus and LaRue (1954) showed that for maize endosperm grown on solid agar media, sucrose supported best growth; fructose or glucose could replace sucrose, but lactose, galactose, arabinose, mannose, and xylose did not support growth. As might be expected from the ubiquitous presence of sucrose in plants, a number of plant tissues in culture exhibit a similar preference for sucrose, but vary with respect to their ability to use other sugars and disaccharides for growth (Carew and Staba 1965; Nickell and Maretzki 1970; Simpkins *et al.* 1970; Puhan and Martin 1971).

The observation that equimolar mixtures of glucose and fructose are inferior as carbon sources to sucrose, or to glucose and fructose alone, is similar to that made by Simpkins and Street (1970) for sycamore suspension cultures. These workers found that 2% sucrose and 2% fructose support better growth than 2% glucose, or a mixture containing 1% glucose and 1% fructose. This effect was more marked when the level of kinetin was increased from 0·25 mg/l to 2·5 mg/l. In all experiments the sugars had disappeared from the medium by 21 days. It was therefore suggested that the differences in growth of sycamore cells on the various sugars and mixtures of sugars could not be attributed to differences in rate of uptake but rather to some rate-limiting steps in the subsequent metabolism of the sugars. However, in other cell types differences in transport mechanisms may also be involved. Thus in sugar-cane parenchyma cells separate mechanisms are involved in the absorption of glucose and fructose on one hand (Gayler and Glasziou 1972; Maretzki and Thom 1972) and for sucrose on the other.

It was found that galactose supports good growth of endosperm cells, but only after a prolonged lag period. This lag period was reduced somewhat but not eliminated after the second transfer to a galactose medium.

The fully defined medium of Linsmaier and Skoog (1965) supported growth, although the rate of growth during the exponential phase was less than for cells growing in White's medium. The yield of cells and the rate of growth on Linsmaier and Skoog's medium was further diminished following a second transfer to this medium, suggesting that cultures were becoming deficient in essential growth factors provided by the yeast extract or vitamin components of the White's medium (see

Table 2). However, comparison of the composition of the two media indicates that altered concentrations of certain nutrient salts or the higher osmolarity of the medium could also have contributed to the poorer growth.

TABLE 2

SUMMARIZED COMPARISON OF THE COMPOSITIONS OF THE MODIFIED WHITE'S AND THE LINSMAIER AND SKOOG MEDIA

| Factor | Linsmaier and Skoog medium | Modified White's medium |
|---|---|---|
| 1. Sucrose concentration | 3% w/v | 4% w/v |
| 2. Supplements | Cytidine 3′-monophosphate<br>Guanosine 3′-monophosphate<br>Asparagine<br>Glutamine<br>Kinetin | Yeast extract<br>Pyridoxine<br>Nicotinic acid<br>Calcium pantothenate |
| 3. Inorganic salt concentrations | See Linsmaier and Skoog (1965) | See Table 1 |
| 4. Osmolarity | 0·200 osmolar | 0·051 osmolar |

## V. ACKNOWLEDGMENTS

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