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THE LIPID COMPOSITION OF *ACER PSEUDOPLATANUS* CELLS GROWN IN CULTURE

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Key Word Index—*Acer pseudoplatanus*; Aceraceae; sycamore; cell cultures; lipid composition; fatty acids; phospholipid; triglyceride.

Abstract—Cells of *Acer pseudoplatanus* were grown in batch suspension culture for 22 days. The cultures were initiated at high cell density of 2×10^5 cells per ml of culture. Growth was characterised by a short lag phase, an exponential phase of rapid cell division and growth, and finally a stationary phase. Quantitative but not qualitative changes were observed in total lipid content, fatty acids and phospholipids at different stages of growth. Total lipids, phospholipids and fatty acids showed maximum concentrations in 12 day old cells. The major phospholipids isolated were phosphatidylcholine and phosphatidylethanolamine with minor amounts of phosphatidic acid and lysophosphatides. Other lipid components present were mono- and digalactosyl diglycerides, cerebrosides, sterol glucosides, free fatty acids and esterified sterol glucosides. The major constituent fatty acids were myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). During exponential cell growth the proportion of 16:0, 18:2 and 18:3 constituted nearly 90% of the total fatty acids. Triglycerides were the major repository of myristic acid (14:0) with substantial amounts of palmitic acid (16:0), whereas phospholipids contained 16:0, 18:2 and 18:3 in high amounts.

INTRODUCTION

Suspension cultures have been used increasingly in recent years to study a wide range of problems in plant biochemistry and physiology [1, 2]. Based on previous studies of carbohydrate metabolism with both batch and continuous cultures of sycamore cells [3, 4], the present studies were undertaken to investigate the interrelationship between carbohydrate metabolism and the donation of reduced pyridine nucleotides and carbon skeletons for lipid biosynthesis in plant cells undergoing rapid growth and division. This communication reports preliminary studies on the changes which occur in total lipids, fatty acids, phospholipids and the fatty acid composition of triglycerides and phospholipids during the growth of sycamore cells in batch suspension culture.

RESULTS AND DISCUSSION

The pattern of growth of sycamore cells in batch culture is shown in Fig. 1. As the cultures were initiated with a high cell density (200 000 cells/ml), the lag phase is of short duration (1–2 days) and the exponential to linear phase is well marked from day 4 to day 15 with the biomass (expressed as dry wt per ml culture) increasing threefold in 7 days. At least three cell generations passed during this period before the stationary phase was reached from day 15 onwards.

Total lipid content, total phospholipids and total fatty acids all showed 2–4 fold increases in concentration at day 12 when compared with their concentration at lag phase (Fig. 2). The rapid increase in the content of lipid-P and phospholipid fatty acids is attributed to active synthesis of acyl glycerol lipids during this period of active cell proliferation and growth. This view is supported by the observations that during this period the glycerophosphatide pool consisted of about 50–70% of the total lipids and that the free fatty acids were in low

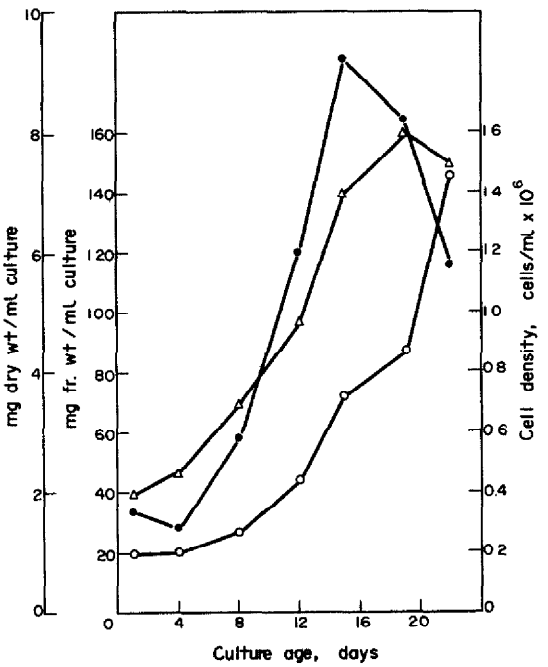

Fig. 1. Growth characteristics of sycamore cells maintained in batch suspension culture. The various parameters were measured as described in the text. Dry weight (●——●), fresh weight (○——○), cell density (△——△).

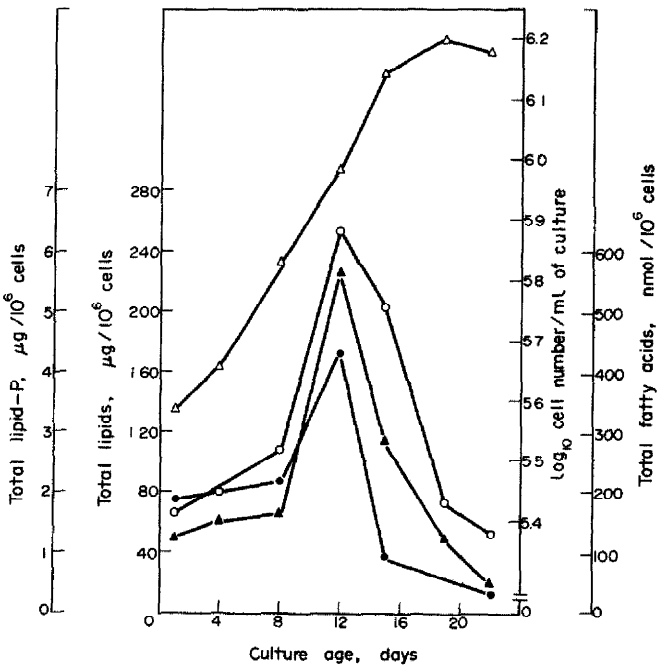

Fig. 2. Changes in total lipid content, phospholipids and fatty acids during the growth of sycamore cells in batch suspension culture. The standard deviation on each interval mean is less than 5%. Total lipid-P (●——●), total lipids (○——○), cell number (△——△), total fatty acids (▲——▲).

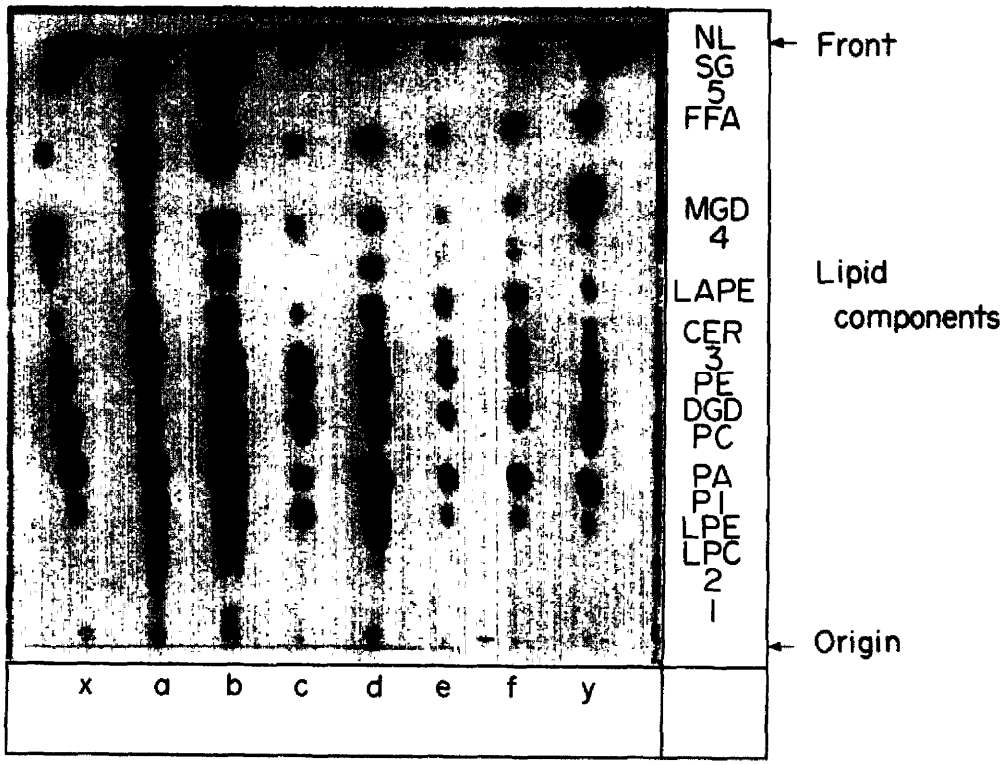

Fig. 3. Thin-layer chromatography of total lipid extracts (100–200 μg) from *Acer pseudoplatanum* cells grown in batch suspension culture. Samples were chromatographed from cells that were 1 (f), 4 (e), 8 (d), 12 (c), 15 (b) and 22 (a) days old. Constituents were identified by co-chromatography with authentic standards and comparison with extracts from leaves of *Acer pseudoplatanus* (x and y); LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine, PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; DGD, digalactosyldiglyceride, PE, phosphatidylethanolamine; LAPE, N-acyl lysophosphatidylethanolamine; MGD, monogalactosyldiglyceride; FFA, free fatty acids; SG, sterol glucoside; NL, neutral lipids; 1, 2, 3, 4 and 5 unidentified.

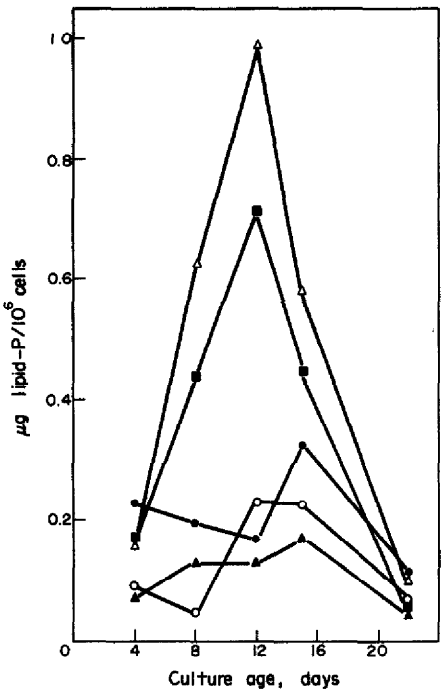

Fig. 4. Changes in the phospholipid composition of sycamore cells grown in batch suspension culture. Values are means of four analyses (2 separate experiments). Standard deviation of each interval mean is less than 10%. Phosphatidylcholine (△——△), phosphatidylethanolamine (■——■), phosphatidic acid (●——●), lysophosphatidyl ethanolamine (○——○), *N*-acyl lysophosphatidylethanolamine (▲——▲).

concentration (Figs. 2 and 3). Additional support comes from the work of Simola & Sopanen [5] who showed that in sycamore cells grown in batch culture, the activity of α-glycerophosphatase is inversely related to the changes in phospholipids and fatty acids observed in our experiments.

With the approach of stationary phase (by day 15) the total lipid content dropped by 25%, the phospholipids by 75% and the fatty acid content by 50%. The rapidly changing metabolic status of the culture was demonstrated further as the cells progressed into stationary phase when the total lipid content dropped by a further 65% and nearly approached the concentration observed in lag phase cells. Similar decreases were observed in the levels of phospholipids and fatty acids. The final concentrations here were even lower than those observed in the lag phase cells. These latter observations suggest that the cell population contained, at this stage, cells in different phases of growth.

Qualitative analysis of the total lipid extracts from 1, 4, 8, 12, 15 and 22 day cultures showed a consistent pattern of lipid components (Fig. 3). Major lipid components identified were LPE, PA, PC, DGD, PE, cerebroside, sterol glycoside, MGD, free fatty acids and triglycerides. Thus only five phospholipids appeared consistently throughout the growth period and the lipid-P of each showed that PC and PE were the major components to show a significant increase from day 4 to day 12 cultures (Fig. 4), which is in agreement with earlier observations on total phospholipids. With the onset of stationary phase, the concentrations of both decreased to levels observed in cells at lag phase. It is noteworthy that concentrations of PA actually show a decrease during this exponential phase, but then peak later around day 15 cultures and then decrease as stationary phase is approached. The presence of two lysophosphatides (LPE and LAPE) is of interest as these components tended to show an increase in level only after about 12 days in culture. Of the non-phospholipid components, the glycolipids, MGD and DGD and sterols appeared in substantial amounts (10–15% of total lipids) towards the late exponential phase and stationary phase. Although no attempt was made to quantitate them, it is speculated that the presence of the glycolipids is due to plastid formation through the effect of light on the growing cells.

During all culture periods small amounts of 10:0, 12:0, 16:1 and 16:2 fatty acids were detected. They accounted however for less than 1% of the total fatty acid complement. The medium chain saturated fatty acid, myristic acid (14:0) and long chain saturated acid, stearic (18:0) also comprised only a small proportion of the total fatty acids determined during growth (Table 1). In contrast palmitic acid (16:0) constituted 20–25% of the total fatty acids up to 15 days of culture and then increased to about 35% in stationary phase cells (day 22).

The most striking changes were noted in the unsaturated acids, linoleic (18:2) and linolenic (18:3) acids. During the period of active cell proliferation and growth, 18:2 and 18:3 constituted 40–50% and 20–25% respectively of the total fatty acids (Table 1). Thereafter 18:2 showed a rapid decrease till the end of stationary phase, whereas 18:3 continued to increase till day 19 and then decreased to a level similar to that observed in lag phase cells.

Calculated on the basis of nanomoles of fatty acid per cell, significant amounts of 14:0, 16:0, 18:2 and 18:3 were synthesized in cells of day 1 cultures (Table 1) but

Table 1. Contents of total fatty acids in cells of *Acer pseudoplatanus* grown in batch suspension culture

Growth Period (Day)	14:0 (myristic) nmol $\times 10^{-6}$ cells	mol %	16:0 (palmitic) nmol $\times 10^{-6}$ cells	mol %	18:0 (stearic) nmol $\times 10^{-6}$ cells	mol %	18:1 (oleic) nmol $\times 10^{-6}$ cells	mol %	18:2 (linoleic) nmol $\times 10^{-6}$ cells	mol %	18:3 (linolenic) nmol $\times 10^{-6}$ cells	mol %
1	8.5	6.8	31.4	25.0	5.2	4.1	6.0	4.8	50.3	40.0	21.6	17.2
4	6.9	4.6	36.0	23.9	2.8	1.8	7.7	5.1	71.0	47.2	26.2	17.4
8	2.1	1.3	37.8	23.2	3.8	2.3	6.4	3.9	78.1	48.0	34.5	21.2
12	10.8	1.9	132.2	23.2	5.8	1.0	14.9	2.6	277.9	48.7	128.6	22.6
15	4.7	1.6	63.4	22.3	2.1	0.7	5.2	1.8	147.3	51.9	61.2	21.6
19	3.1	2.5	33.0	26.6	0.7	0.6	0.9	0.7	54.5	43.9	31.8	25.6
22	4.0	8.9	15.7	34.5	0.6	1.3	0.9	2.0	16.7	36.7	7.3	15.9

Trace amounts (<0.5%) of 10:0, 12:0, 16:1 & 16:2 were detected in all samples. Fatty acids were analysed as methyl esters by GLC on a column of 10% diethylene glycol succinate polyester on supelcoport at 195°. Values are means of four analyses (2 separate experiments) and the standard deviations were less than 10%.

Table 2. Fatty acid composition of triacylglycerols and phospholipids of *Acer pseudoplatanus* cells grown in batch suspension culture

Fatty acid	Day 1		Day 4		Day 8		Day 12		Day 22	
	Triacylglycerol	Phospholipid	Triacylglycerol	Phospholipid	Triacylglycerol	Phospholipid	Triacylglycerol	Phospholipid	Triacylglycerol	Phospholipid
	Mole%									
14:0	85.3	0.6	5.3	6.7	26.7	12.8	67.7	3.9	89.8	0 6
16:0	6.9	24.2	36.8	24 9	23.7	22 3	8 6	21.0	5 1	37 9
18:0	2.3	1.4	4.3	3.9	3.8	1.2	tr*	0.9	1.1	tr
18:1	2.3	4.8	10.1	4.7	7.6	2 9	tr	2.3	2.3	1.3
18:2	3.1	48.3	33.8	44.2	28.9	43.1	15.8	48 6	1.1	39.4
18:3	tr	20.8	9.7	15.7	9.3	17.7	7.9	23.3	tr	15.9
20:1	tr	tr	tr	tr	tr	tr	tr	tr	tr	2.4
Fractional mole %	13.0	60.0	4.2	59.4	4.9	63.1	1 9	50.8	4.0	34.7

Lipid components were separated by TLC as described in text. Values are means of two separate analyses.
* tr, trace amounts detected (<0.5%).

the most notable increases in amounts were seen in 16:0, 18:2 and 18:3, with palmitic acid increasing by 4-fold at day 12 and linoleic acid (18:2) and linolenic acid (18:3) both increasing by nearly 6-fold in amounts during this same period of active cell division and growth. As this phase of active growth decelerates, the amounts of all these fatty acids drop by at least half and as the cells reach stationary phase, the final concentrations are 2–3 fold lower than in cells at lag phase.

The fatty acid composition of triglyceride and phospholipid fractions constituted nearly 50–70% of the total fatty acids during the phase of active cell division. The phospholipid fraction alone constituted 50–60% of the total fatty acids present in cells at lag phase through into exponential phase and then dropped by nearly half as the cells approached stationary phase. The triglycerides contained 13% of the total fatty acids in lag phase cells and showed a continuous decrease right through phases of active cell division and growth. But as the stationary phase was reached, the proportion of fatty acids in triglyceride tended to rise. Low amounts of 10:0, 12:0, 16:1 and 16:2 were always detected in the fractions.

The mole ratio of fatty acids in triglyceride to phospholipids was also the highest at lag phase (0.22) and showed a continuous decrease right through to the phase of cell proliferation, thus in day 12 cultures the ratio dropped to 0.04. As the cells were transformed into a stationary phase condition, the ratio again increased nearly three fold to 0.12.

The triglyceride fraction showed the most interesting changes in fatty acid composition. At lag phase myristic acid (14:0) and palmitic acid (16:0) accounted for nearly 92% of the total (Table 2), whereas the unsaturated fatty acids (18:2 and 18:3) constituted a very low proportion. It is interesting to note that as the cells enter exponential phase, there is a drastic drop in proportion of 14:0 in the triacylglycerol fraction and a concomitant increase in 16:0, 18:1, 18:2 and 18:3. But as the cells advance well into the phase of active growth, the proportion of myristic acid again tended to rise by a factor of 5 to 12 and at the same time 16:0, 18:1 and 18:2 dropped in proportion nearly 2–5 fold with 18:3 unaffected. As the cells enter stationary phase, the proportion of 14:0 almost equals that at lag phase, and low proportions of 16:0, 18:1, 18:2 and 18:3 are observed.

In contrast the proportion of myristic acid in phospholipids at lag phase is very low and there is little evidence of significant change in this as the cells divide and grow. However the proportions of palmitic acid is higher and accounts for about 20–23% starting from lag phase till the end of active cell division. But at the end of stationary phase its proportion had increased to about 38%. Of the other long chain fatty acids in phospholipids, stearic acid hardly showed any significant changes right through the exponential phase and in fact was reduced to very low proportions in day 12 and day 22 cultures. The proportion of long chain monoene, oleic acid (18:1), in phospholipids was low and did not show much variation during the entire growth period. The amount of linoleic (18:2) and linolenic (18:3) acids in phospholipids at lag phase was about 70% and was in direct contrast to their low levels in triacylglycerol during the same culture period. Although their combined amounts dropped by 10% in the day 4 cultures, these cells, in a high state of division and growth (day 8 to day 12), showed that their membrane phospholipids were highly unsaturated (60–70%).

The data are in general agreement with other published work on the lipids of cultured plant cells, bearing in mind species specific differences [6–8]. The patterns of growth and metabolism observed in suspension cultures are by the nature of the system transient, a point which must be considered in the interpretation of data [2]. Bearing this in mind the exponential growth phase nonetheless afforded us cell cultures characterized by rapidly dividing and growing cells with good synchrony as reported previously [9]. This phase was marked by net synthesis of lipids, although the total lipid content did not exceed 4% of the dry matter of the cells. Phospholipids and fatty acids reached a maximum in day 12 cultures. The data are indicative of increased membrane synthesis and function during this phase of rapid cell division and growth.

Earlier work with plant cell cultures [3, 10, 11] has shown that during the early period of growth there is relatively a greater increase in capacity of the pentose phosphate pathway than in the Embden–Meyerhof Parnas pathway. It was postulated [3] that in the early stages of the growth of cells in culture, the tricarboxylic acid cycle is geared to the production of substrates for biosynthesis, and the pentose phosphate pathway acts in concert with this system by providing the NADPH necessary for this biosynthesis. The major synthesis of lipid seen in the early stages of culture growth in the present work may therefore be linked to the changing pattern of carbohydrate metabolism through the supply

of carbon precursors and reduced pyridine nucleotide. Further studies on these interrelationships are now in progress.

Another aspect of the carbon flow from precursors into stable products of lipid biosynthesis which is of particular interest may be seen in the present data in relation to the rapidly changing mole fatty acid ratios between triacylglycerides and phospholipids. It is suggested that a very active pool of triacylglycerides is present (rich in 14:0) and that their fatty acids are broken down very rapidly by some mechanism and then quickly resynthesized directly into phospholipids. The possibility of short and medium chain fatty acids being used for direct chain elongation and desaturation has been suggested by Stumpf and coworkers [12]. Also chain elongation and desaturation of fatty acid may occur while attached to phospholipids as shown by several workers [13]. The presence of high amounts of phosphatidylcholine and phosphatidylethanolamine in actively dividing cells in day 12 cultures is in agreement with the above suggestions. Other possibilities of carbon flow through β-oxidation to C_2 units followed by resynthesis and desaturation could be considered but must remain speculative until further work with labelled intermediates.

EXPERIMENTAL

Batch cultures. The *Acer pseudoplatanus* cell cultures were subcultured every 22 days and grown as described elsewhere [4]. The stock cultures were maintained under fluorescent light at 25° ± 2° in 250 ml or 500 ml conical flasks in an orbital incubator shaker operating at about 120 rpm. The culture medium was that of Stuart & Street [14] with 20 g/l of sucrose as the carbon source. New stocks were initiated by inoculating old stock into new medium to give an initial cell density of about 200 × 10^3 cells/ml culture medium.

Measurement of growth parameters. Fr. wt, dry wt and cell density were determined as described by Henshaw *et al.* [15].

Extraction and analysis of lipids. Sufficient cells to give 2–3 g fr. weight were harvested over a single layer of miracloth in a Hartley funnel supported in a Buchner flask. Excess culture medium was removed by suction. Cells were washed twice in 2 vol of ice cold distilled H_2O, and immediately homogenized in 3 ml boiling 2-PrOH; the homogenate was centrifuged at 1500 *g* for 5 min and the supernatant was decanted. The residual pellet was reextracted twice with 3 ml of the same solvent, followed by extractions with 3 ml, 1 ml and 1 ml of $CHCl_3$–MeOH–H_2O (1:2:0.8) [16]. The combined extracts were taken to dryness under red pres and the residue then suspended in 3.8 ml $CHCl_3$–MeOH–H_2O (1:2:0.8). The mixture was diluted with 1.0 ml each of $CHCl_3$ and H_2O, mixed, and the biphasic system separated by centrifugation. The $CHCl_3$ phase was removed and the upper phase re-extracted twice with 1 ml $CHCl_3$; the combined $CHCl_3$ extracts were diluted with an equal volume of C_6H_6 and brought to dryness under a stream of N_2. Tert-butyl hydroxytoluene (100 μg. Sigma Chemical Co.) was added to the lipid residue which was then dissolved in a known volume of $CHCl_3$ and stored at −20° until ready for analysis. TLC was on Si gel H (0.5 mm) developed unidirectionally, first in $CHCl_3$–MeOH–conc NH_3–H_2O–water (80:10:3.5:2), then in $CHCl_3$–MeOH–HOAc–H_2O (80:10:1.5:0.7) [17, 18]. The chromatograms were viewed under UV after spraying with Rhodamine 6G in Me_2CO. The lipid spots were identified by comparing their R_f values with those of authentic standards and also with lipids isolated from sycamore leaves on Si gel column chromatography. Separate chromatograms were sprayed with one of the following: 0.3% ninhydrin in H_2O saturated BuOH for amino groups; Dittmer & Lester reagent for detection of phosphorus [19, 20]; α-naphthol reagent followed by conc. H_2SO_4 and heating at 100° for glycolipids, sterols and steryl esters; and finally charring at 200° to visualize all lipid components [21]. For quantitation of lipid classes, triglycerides and phospholipids, the total lipids were fractionated by TLC on Si gel H using the double solvent system as described elsewhere [18]. Bands corresponding to total phospholipids and triglycerides were scraped from the chromatograms and the lipids transesterified in 5% HCl in MeOH, together with a known amount of methyl heptadecanoate as internal standard. The fatty acid methyl esters of each fraction and the total lipids were analysed by GLC. Dual 0.64 cm × 2.28 m stainless steel columns were packed with 10% DEGS-PS on 80/100, A/W Supelcoport. The column was operated at 195° with a N_2 carrier gas pressure of 2.0 kg/cm^2. Peaks were identified by comparison of their R_t's with those of authentic standards. The percentage composition by weight of each fatty acid component was determined by the retention time-peak height method of Carrol [22]. Lipid-P was determined on total lipids and fractionated phospholipids by the modified microprocedure of Bartlett [23]. All experiments were repeated at two different times on separate cultures and at least two analyses were performed on all extracts. All procedures were, as far as possible, carried out in an atmosphere of N_2. All lipid data are expressed on a per cell basis to obtain a meaningful concept of the changing metabolic status of the cells in culture.

Abbreviations. PS, phosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; LAPE, *N*-acyl lysophosphatidylethanolamine; MGD, monogalactosyldiglyceride; DGD, digalactosyldiglyceride; FFA, free fatty acid; TG, triacylglycerol; CER, cerebroside; SG, sterol glucoside; ESG, esterified sterol glucoside; 10:0, Capric acid, 12:0, lauric acid; 14:0 myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

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REFERENCES

1. Street, H. E. (1973) in *Biosynthesis and its Control in Plants* (Milburn, B. V., ed.) pp. 93–125. Academic Press, London.
2. King, P. J. and Street, H. E. (1973) *Plant Tissue & Cell Culture*, (H. E. Street, ed), p. 269. Blackwell Scientific Publ.
3. Fowler, M. W. (1971) *J. Exp. Bot.* **22**, 715.
4. Fowler, M. W. and Clifton, A. (1974) *European J. Biochem.* **45**, 445.
5. Simola, L. K. and Sopanen, T. (1970) *Physiol. Plantarum* **23**, 1212.
6. Tattrie, N. H. and Veliky, I. A. (1973) *Can. J. Botany* **51**, 513.
7. Song, M. and Tattrie, N. (1973) *Can. J. Botany* **51**, 1893.
8. Staba, E. J., Shin, B. S. and Mangold, H. K. (1971) *Chem. Phys. Lipids* **6**, 291.
9. King, P. J., Cox, B. J., Fowler, M. W. & Street, H. E. (1974) *Planta (Berl.)* **117**, 109.
10. Wilson, S. B. (1971) *J. Exp. Botany* **22**, 725.
11. Thorpe, T. A. and Laishley, E. J. (1973) *J. Exp. Botany* **24**, 1082.
12. Jacobson, B. S., Kannangara, C. G., Stumpf, P. K. (1973) *Biochem. Biophys. Res. Commun.* **52**, 1190.
13. Pugh, E. L. and Kates, M. (1973) *Biochim. Biophys. Acta* **315**, 305.
14. Stuart, R. and Street, H. E. (1969) *J. Exp. Botany* **20**, 556.
15. Henshaw, G. G., Jha, K. K., Mehta, A. R., Shakeshaft, D. J. and Street, H. E. (1966) *J. Exp. Botany* **17**, 362.
16. Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911.
17. De Silva, K. N. L. S., Weinberger, W., De la Rothe, I. and Kates, M. (1975) *Can. J. Botany* **53** (In press).
18. De Silva, K. N. L. S. (1974) Ph.D. Thesis, Unversity of Ottawa, Ottawa, Canada.

19. Dittmer, J. C. and Lester, R. L. (1964) *J. Lipid. Res.* **5**, 126.
20. Vaskovsky, V. E. and Kostestsky, E. Y. (1963) *J. Lipid. Res.* **9**, 396.
21. Kates, M. (1972) *Techniques of Lipidology*, pp. 436–441. North Holland, Amsterdam.
22. Carrol, K. K. (1961) *Nature (London)* **191**, 377.
23. Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466.