Phospholipid Composition of the Plasma Membrane of the Green Alga, *Hydrodictyon africanum*

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A plasma-membrane fraction was isolated from the alga *Hydrodictyon africanum* by micro-dissection and its phospholipid components were analysed. Phosphatidylcholine was the major phospholipid of the preparation. Both phosphatidylserine and diphosphatidylglycerol were enriched in the fraction compared with the whole cell, but the relative amount of phosphatidylglycerol present was less than that in the whole cell. Phosphatidylinositol was absent from the plasma-membrane preparation.

The presence of cell walls makes breakage and subsequent subcellular fractionation of plant cells difficult. The plasma membrane breaks up during homogenization and cannot be easily distinguished from other broken membranes such as the tonoplast and smooth endoplasmic reticulum. Thus it has not been possible to obtain markers that would allow an unambiguous identification of the plasma membrane in fractionated preparations of plant cell membranes. The plasma membrane is important not only for the control of the internal environment of the cell, but also as the organelle across which the wall is deposited and at which some of the wall constituents are synthesized. We have tried a more direct approach to isolate it, and have used a giant algal cell, so that by micro-dissection a separation of the cell contents from the plasma membrane can be made without homogenization. By using a simple syringing technique (Northcote *et al.*, 1960) we have obtained a fraction from the coenocytic green alga *Hydrodictyon africanum*, consisting of the cell wall together with the adhering plasma membrane, which was isolated as a lamella rather than as broken vesiculated pieces. The morphology of this fraction was studied, and an analysis of its phospholipids after equilibration with [32 P]P_i was made, together with a total phospholipid analysis of the algal cells, and an analysis of the rate of incorporation of [32 P]P_i into the phospholipids of the cell membranes. Several analyses of the phospholipids of the green algae have been reported, especially of *Chlorella vulgaris* (Sastry & Kates, 1965) and *Euglena gracilis* (Calvayrac & Douce, 1970).

Materials and Methods

Biological material

Zygotes of the alga *Hydrodictyon africanum* (Pocock, 1960) obtained from the National Collec-

tion of Algae and Protozoa, Cambridge, U.K., were germinated in Bold's basal medium (autoclaved at 120°C for 20 min, and adjusted to pH 7.0) (Nichols & Bold, 1965), under natural lighting conditions in a window facing north. The young nets or coenobia (Pocock, 1960) were subcultured into fresh Bold's basal medium supplemented with 10% (v/v) soil extract (prepared by boiling loam with an equal volume of glass-distilled water, and filtering the resultant cold extract). Frequent subculturing was necessary to maintain maximum growth of nets. All cultures were sterile and free from other algal contaminants. The mature cells (coenocytes) grew to a size of 3–4 mm diameter, though cells with diameters of 1–2 mm were used as a routine.

Preparation of the wall-plasma-membrane unit

Mature cells (approx. diam. 2 mm) were syringed out by using approx. 3 ml of either glass-distilled water, a solution with a low salt concentration (0.4 M-sucrose in 2 mM-Hepes* buffer, pH 7.5), or a solution with a high salt concentration (0.4 M-sucrose in 25 mM-Tris/acetate buffer, pH 7.5). Syringing was followed by immediately fixing the cells, after various times of syringing, in 4% (w/v) glutaraldehyde, buffered with 0.1 M-sodium cacodylate, pH 7.2. After washing with cacodylate buffer, the cells were post-fixed with 1% (w/v) OsO₄ in 0.1 M-sodium cacodylate buffer, dehydrated in an ethanol series, infiltrated with several changes of propylene oxide and embedded in Araldite. Thin sections were stained with aq. 1% (w/v) KMnO₄ (Lawn, 1960), and viewed in an AEI EM 6B electron microscope.

Extraction of the lipid material

When the individual coenocytes had attained dimensions of 2 or 3 mm diameter, the nets were

* Abbreviation: Hepes, 2-(N-2-hydroxyethyl)piperazin-N'-yl)ethanesulphonic acid.

removed from the culture medium, washed in fresh medium and then glass-distilled water, and immediately extracted in chloroform/methanol/water (5:10:4, by vol.) (Bligh & Dyer, 1959), by homogenization in a glass homogenizer at 4°C. A further 5 vol. of chloroform and glass-distilled water were then added to the initial extraction medium, to obtain two clear phases, separated by cellular debris. The lower phase (chloroform) containing the lipid material, was removed and washed with three times its own volume of 0.7% (w/v) NaCl at least five times, to remove any non-lipid material. The chloroform phase was evaporated to dryness under N₂, and either analysed immediately or stored in chloroform at 0°C. All solvents were freshly distilled from glass apparatus.

The procedure used to extract the lipids from the wall-plasma-membrane units was identical with that described for the whole cells. The separated wall-plasma-membrane units were carefully rinsed in glass-distilled water before extraction with chloroform/methanol/water (5:10:4, by vol.).

Separation of the phospholipids

The phospholipids were separated by two t.l.c. systems. One used a neutral solvent, chloroform/methanol/water (65:25:4, by vol.) in one dimension, and di-isobutyl ketone/acetic acid/water (8:5:1, by vol.) in the other (LePage, 1964). The second system used a basic solvent, chloroform/methanol/28% (v/v) NH₃ (13:7:1, by vol.), followed by chloroform/methanol/acetic acid/water (50:25:7:3, by vol.) (Bridges & Ricketts, 1966). The lipids were detected with I₂ vapour. Individual phospholipids were identified on the chromatograms by reinforcement of the lipids by using authentic standards (Lipid Products, Redhill, Surrey, U.K.). Specific stains were used to identify choline-containing lipids (Wagner *et al.*, 1961), amino-group-containing lipids (Marinetti, 1962), phosphatidylglycerol, phosphatidylinositol and glycolipids (periodate-Schiff reagent; Olley, 1968), phosphate-containing lipids (Dittmer & Lester, 1964) and glycolipids (the α -naphthol reagent; Siakotos & Rouser, 1965). Charring with 50% (v/v) H₂SO₄ was also used to detect any organic substances. The thin-layer plates consisted of a plastic-backed sheet of silica gel N (0.25 mm thick) (Machery-Nagel Polygram Sil G plates without gypsum used as a binder).

Phosphorus determinations

The thin-layer plates were washed consecutively in glass-distilled water, diethyl ether/methanol/chloroform (1:1:1, by vol.) and chloroform/methanol/water (2:2:1, by vol.) before use. After separation, the areas containing the phospholipids were cut out of the plate and the material was eluted

by vigorous shaking in chloroform/methanol/diethyl ether (1:1:1, by vol.).

Standard phosphate solutions (Na₂HPO₄·7H₂O) were treated with 18M-H₂SO₄/12M-HClO₄ solution (9:1, v/v) (0.2 ml) in a final volume of 3 ml. The dried samples were digested with the same H₂SO₄/HClO₄ mixture (0.3 ml) at 180°C for 1 h, and 2.8 ml of glass-distilled water was added. Standard solutions and samples were treated with 5% (w/v) ammonium molybdate solution (0.3 ml) and reducing agent [0.2 ml; 0.25 g of 4-amino-3-hydroxynaphthalene-1-sulphonic acid in 100 ml of 15% (w/v) NaHSO₃, to which was added 1 g of Na₂SO₃·7H₂O]. The solutions were incubated at 100°C for 7 min, and E₈₃₀ was measured on a Beckman double-beam spectrophotometer (Bartlett, 1959).

Incubations with ³²P

Approx. 20 mature nets (cell diam. 2 mm) were incubated in 100 ml of Bold's basal medium, pH 7.0, to which had been added 1 ml of [³²P]P_i (Na⁺ salt; sp. radioactivity 137 Ci/mg of P, 10 mCi/ml; The Radiochemical Centre, Amersham, Bucks., U.K.). The cells were grown in a growth chamber under constant light and temperature (2000 lx, 20°C). Each day the lipids from 20 cells were divided into two equal portions, and chromatographed in the two thin-layer systems. Radioautography was carried out by using short time-exposures (1–3 days) of the plates to Kodak Medical X-ray film BB54. The radioactive spots were scraped off the plate and counted for radioactivity on a Philips liquid-scintillation analyser (PW 4530), with a mixture of 50 mg of 1,4-bis-(5-phenyloxazol-2-yl) benzene plus 3.5 g of 2,5-diphenyloxazole in 1 litre of sulphur-free toluene as the scintillant. Efficiency of counting was greater than 98%.

Cytochrome oxidase assays

The mitochondrial marker enzyme, cytochrome oxidase, was assayed by oxidation of reduced cytochrome *c*. Various numbers of cells (15–120) of the alga were removed from the growth medium, laid on filter paper, and punctured with a pin. This emptied the vacuoles with very little disturbance of peripheral cytoplasm. The cells were suspended in 50 mM-potassium phosphate buffer (2.7 ml, pH 7.5), supplemented with 0.1 ml of 0.3% (w/v) digitonin, and sonicated for 30 s by using a Dawe Soniprobe (7532A) with a microtip attachment at 65 W output at 4°C. Cytochrome oxidase was assayed by measuring the oxidation of reduced cytochrome *c* at 550 nm (Hodges & Leonard, 1974). The oxidation was terminated by the addition of about 0.05 ml of 50 mM-NaCN. Wall-plasma-membrane units were assayed after sonicating them in the phosphate buffer/digitonin mixture.

Results

Preparation of the wall-plasma-membrane unit

The cytoplasm of the cells, coloured green by the chloroplasts, was swept out of the cells in the form of a sheet of one or two pieces by the syringing method. The appearance of the cells during syringing (Plates 1*a* and 1*b*) showed that a shear plane developed between the cytoplasm and the plasma membrane owing to the adhesion of a continuous chloroplast chain (Plate 1*b*). The high-salts syringing medium broke the chloroplast outer membranes; glass-distilled water caused the chloroplasts to swell and burst very rapidly (Plate 1*a*). The best conditions were those with the low-salts medium (Plates 1*b*, 1*c* and 1*d*), which gave good preservation of the chloroplasts and other membranous organelles, although there was a partial loss of the plasma membrane from certain areas of the wall-plasma-membrane unit. This loss occurred with all three conditions used for syringing.

Phospholipid separation and measurement

The amount of phospholipids from each net was variable (about 1–2 mg of phospholipid/g fresh wt.), since small differences in cell size changed the volume of the vacuole, a major contribution to the fresh weight of the cell. Separation of the polar lipids of the alga in the LePage (1964) t.l.c. system is shown in Fig. 1 and the composition of the phospholipids of the cell is shown in Table 1. The results shown represent the mean values obtained for four separate determinations. The chromatographic properties of certain phospholipids differed markedly from that expected (Fig. 1 and Plate 2), especially phosphatidylserine and cardiolipin. The original method discussed by Skipski & Barclay (1969) used silica gel G with

Fig. 1. Diagram to show the separation of polar lipids on t.l.c.

T.l.c. was performed on plastic-backed sheets of silica gel N without gypsum binder: solvent 1, chloroform/methanol/water (65:25:4, by vol.); solvent 2, di-isobutyl ketone/acetic acid/water (8:5:1, by vol.). The cross-hatched areas indicate the polar lipids of extracts of whole cells that were present in such amounts that they were readily detected by I_2 vapour. The other areas indicate the polar lipids present in whole cell and plasma-membrane extracts that were only detectable by autoradiography of [^{32}P]phospholipids (cf. Plate 2). 0, Origin; 1, lysophosphatidylcholine; 4, phosphatidylinositol; 5, phosphatidylcholine; 7, phosphatidylglycerol; 9, diglycosyl diglyceride; 10, phosphatidylserine; 11, phosphatidylethanolamine; 15a, monoglycosyl diglyceride; 15b, diphosphatidylglycerol; 16–18, pigments. The other spots 2, 3, 6, 8, 12–14 and 19 were unidentified.

Table 1. Major phospholipids in the whole cells and in the wall-plasma-membrane unit of *Hydrodictyon africanum*

The analyses were made by determination of the amount of P in the separated phospholipids either by spectrophotometric or radioactive measurements. The results are mean values $\pm$ S.E.M. for four independent determinations.

	Whole cell		Radioactivity in wall- plasma-mem- brane unit (% of total ^{32}P)
	Chemical composi- tion (% of total P)	Radio- activity (% of total ^{32}P)	
Phosphatidylinositol	4 $\pm$ 1	8 $\pm$ 1	Trace
Phosphatidylcholine	40 $\pm$ 4	35 $\pm$ 2	46 $\pm$ 2
Phosphatidylglycerol	25 $\pm$ 2	24 $\pm$ 2	6 $\pm$ 0
Phosphatidylserine	—	Trace	7 $\pm$ 0
Phosphatidylethanol- amine	15 $\pm$ 2	18 $\pm$ 2	9 $\pm$ 1
Diphosphatidylglycerol	4 $\pm$ 1	3 $\pm$ 1	11 $\pm$ 1

gypsum as a binder. With this phosphatidylserine was retarded. However, when there was no gypsum present, the phosphatidylserine moved much more rapidly. This also happened to a lesser extent with cardiolipin, when faster migration rates than expected were found in systems with no gypsum present. Little difference in the chromatography of the major phospholipids phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol and phosphatidylethanolamine was observed. The anomalous behaviour of phosphatidylserine in this system proved of considerable importance, since phosphatidylserine, although a minor component of the whole tissue, was a major component of the wall-plasma-membrane unit.

Labelling with ^{32}P

The time-course of the incorporation of P_i into the individual phospholipid pools was investigated in four separate experiments (Fig. 2). On subculturing

Fig. 2. Time-course of the incorporation of $[^{32}\text{P}]\text{P}_i$ into the phospholipids of *Hydrodictyon africanum*

The solid lines represent the observed incorporation of the ^{32}P into the individual phospholipids extracted from ten coenocytes of a mature coenobium, and separated by t.l.c. ●, Phosphatidylcholine; ▲, phosphatidylglycerol; ■, phosphatidylethanolamine; ○, phosphatidylinositol. The broken lines represent a theoretical rate of incorporation of radioactivity into the phospholipid to give a final composition of the phospholipid of 40% phosphatidylcholine, 25% phosphatidylethanolamine, 15% phosphatidylglycerol and 5% phosphatidylinositol. This was the composition of the major phospholipids of the coenocytes found by chemical analysis of four separate experiments.

the cells into fresh medium containing ^{32}P , a peak of incorporation into phosphatidylglycerol was found. After about 4 days, the rate of entry of ^{32}P into phosphatidylglycerol diminished, and the relative amounts of the phospholipids measured by the radioactive ^{32}P were equivalent to those obtained by the non-radioactive analyses. This pattern of incorporation is similar to that reported for *Chlorella vulgaris* (Sastry & Kates, 1965).

According to the concept of membrane flow (Northcote, 1971), there is a sequence of membrane formation, from the endoplasmic reticulum to the Golgi membranes and the plasma membrane. Thus a steady state of incorporation of radioactivity into the plasma membrane was ensured by continuing the incubations several days after the attainment of steady-state incorporation found for the total phospholipid of the whole cell. A period of 20 days' incubation was used, since the overall steady state was attained after about 8–13 days.

Hydrodictyon africanum contained substantial amounts of phosphatidylcholine (35–40% of the phospholipid P; spot 5, Fig. 1), as well as phosphatidylglycerol (25%; spot 7, Fig. 1) and phosphatidylethanolamine (15–18%; spot 11, Fig. 1), with smaller amounts of phosphatidylinositol (4–8%; spot 4, Fig. 1) and diphosphatidylglycerol (3%; spot 15, Fig. 1). Only traces (less than 1%) of phos-

phatidylserine were found (Tables 1 and 2). Phosphatidylserine is only a minor component of most plant cells (Galliard, 1973). Phosphatidylglycerol is found in chloroplasts in higher plants (Calvayrac & Douce, 1970), although it may be distributed among other organelles (Mazliak & Abdelkader, 1970). The initial incorporation of radioactivity into phosphatidylglycerol may indicate that the ^{32}P label entered the chloroplast lipids, but some other membrane such as the endoplasmic reticulum could act as the primary acceptor of ^{32}P for phospholipid assembly.

Cytochrome oxidase assays

Cytochrome oxidase activities obtained from whole cells are shown in Fig. 3. There was no detectable cytochrome oxidase activity in wall-plasma-membrane units prepared from 50, 100 or 200 similarly sized cells.

Labelling of the wall-plasma-membrane unit with ^{32}P

$[^{32}\text{P}]\text{Phospholipids}$ extracted from the wall-plasma-membrane unit were separated by t.l.c. (Plate 2). The radioactivity of each spot is shown in

Table 2. Analysis of all the ^{32}P -containing material from the wall-plasma-membrane unit and the whole cells

The values are means $\pm$ S.E.M. of four independent determinations. For further details see the text and Fig. 1.

No. of area on t.l.c. plate (Fig. 1)	Radioactivity (%)		Identity
	Wall-plasma-membrane unit	Whole cells	
0	3 $\pm$ 0	Trace	Origin
1	Trace	2	Lysophosphatidylcholine
2	Trace	Trace	Unknown
3			
4	Trace	8 $\pm$ 1	Phosphatidylinositol
5	46 $\pm$ 2	35 $\pm$ 2	Phosphatidylcholine
6	Trace	—	Unknown
7	6 $\pm$ 0	24 $\pm$ 2	Phosphatidylglycerol
8	Trace	—	Unknown
9	—	—	Diglycosyl diglyceride
10	7 $\pm$ 0	Trace	Phosphatidylserine
11	9 $\pm$ 1	18 $\pm$ 2	Phosphatidylethanolamine
12	—	—	Unknown
13	Trace	1	Unknown
14	Trace	2	Unknown
15a	—	—	Monoglycosyl diglyceride
15b	11 $\pm$ 1	3 $\pm$ 1	Diphosphatidylglycerol
16	2 $\pm$ 1	Trace	Pigments and material running at the solvent front
17	2 $\pm$ 1	Trace	
18	2 $\pm$ 1	Trace	
19	3 $\pm$ 0	2	Unknown
20	Trace	—	Neutral lipid

EXPLANATION OF PLATE I

Electron microscopic examination of the wall-plasma-membrane unit at various stages of its preparation

The scale lines on the photographs represent 1 μ m. (a) Cell fixed in glutaraldehyde at an intermediate stage of the removal of the contents. The cytoplasm was removed by syringing with glass-distilled water. The cleavage plane between the cytoplasm and the plasma membrane can be clearly seen. The chloroplasts at this stage, when water was used, were swollen and they eventually broke up. Magnification $\times 12\,666$. (b) Cell fixed in glutaraldehyde at an intermediate stage of the removal of its contents by syringing with a low-salt solution (0.4M-sucrose/2mM-Hepes, pH 7.5). A chloroplast chain, without any swelling, was washed away from the plasma membrane. Magnification $\times 6666$. (c) Cell fixed after the contents had been washed away from the plasma membrane. The plasma membrane remained adhering to the inner surface of the wall, apparently anchored at some points. Magnification $\times 7666$. (d) Wall-plasma-membrane unit prepared in the same way as that described for Plate 1(c). The bilamellar nature of the plasma membrane can be clearly seen. Magnification $\times 57\,333$.

EXPLANATION OF PLATE 2

Radioautogram of a t.l.c. separation of [^{32}P]phospholipids of the wall-plasma-membrane unit

Experimental details are given in the caption to Fig. 1.

Fig. 3. Cytochrome oxidase activity of whole cells

The cells (0.1–0.2 mm diam.) were broken and the activity was measured by the oxidation of reduced cytochrome *c*. The results are means of two separate experiments.

Tables 1 and 2. The results shown represent the mean values obtained for two separate experiments. The absence of occluded ³²P radioactivity from the diglycosyl diglyceride component of the non-radioactive carrier lipid (Plate 2) demonstrated that the extracts had been washed free of [³²P]P_i, and that the radioactive spots represented covalently linked ³²P and not some occlusion artifact.

Discussion

It has been possible by breakage of plant cells to isolate the majority of organelles in a relatively undamaged form (Price, 1974). However, the conditions during the cell disruption are too drastic to preserve the integrity of the external plasma membrane, which vesiculates. Most studies of the plasma membrane of plant cells have been attempts to separate these fragments by density-gradient centrifugation, often by using markers such as enzymic activities or chemical labelling techniques as aids in identification (Keenan *et al.*, 1973; Wallach & Lin, 1973; Demandre, 1975). Even if identification was unequivocally possible, the vesicles have lost most of the native extended sheet-like structure. Use of protoplasts (Cocking, 1974), which are osmotically fragile, overcomes some of the problems of cellular disruption, but it is difficult to obtain uniform preparations of viable protoplasts free from lysed cells, wall debris and adsorbed degradative enzymes. In addition, the enzymes that digest away

the wall will also remove covalently bound carbohydrate present at the outer surface of the membrane, and this may cause modification to biological function. Density-gradient-centrifugation methods may also co-purify tonoplast membrane fragments with the plasma-membrane vesicles.

During cell disruption by homogenization, the walls of cells may acquire membranes that adhere to them. The origin of these membranes is in doubt, and it seems probable that non-specific disruption of any organized tissue will result in the deposition of membrane fragments from the organelles on to the cell walls. However, the phospholipid composition of these wall preparations has been attributed to the presence of the plasma membrane (Bartholomew & Mace, 1972). In the present work it has proved possible to disrupt the cells by a controlled micro-manipulative syringing process and to characterize the resulting cell-wall-plasma-membrane unit in the electron microscope. By using different conditions it was seen that a shear plane developed between the cytoplasm, which was washed away as a sheet, and the plasma membrane, which was left adhering to the wall. The wall stabilized the membrane, and large areas of membrane lying along the inner surface of the wall, apparently anchored at discrete points, were obtained. Recovery of the plasma membrane was not quantitative, since certain areas seemed to vesiculate. Thus it was necessary to compromise between the maximum removal of the cytoplasm from the cells and vesiculation which the syringing process might have induced. This was monitored at every stage of the preparation.

It was necessary to prevent the disruption of the internal organelles of the cell so that the possibility of membrane fragments being adsorbed on to the wall preparation was prevented. Chloroplasts are abundant in *Hydrodictyon* and they are labile organelles, but with appropriate conditions their integrity was maintained. An advantage of the syringing method of cellular disruption was that the removal of the internal components of the cell was very rapid. These components often include potent degradative enzymes. An especial problem with plant tissues is the presence of phenolic compounds in the vacuole. In normal procedures for cellular disruption these remain in close proximity to the membranes during the relatively slow homogenization and centrifugation steps. Syringing rapidly diluted and removed them. Careful examination of the plasma membrane left on the wall showed that it had a clear bimolecular-leaflet-type structure that resembled the plasma membrane of intact cells.

The present work has characterized the phospholipid content of the whole cells and compared this with that of the wall-plasma-membrane unit. One major disadvantage of the micro-manipulative method for the preparation of the wall-plasma-

membrane unit was the low yield, since only one cell could be treated at a time. The sensitivity of the method was therefore increased by using ^{32}P , which enabled the phospholipid composition of the plasma membrane to be measured. The low amounts of phosphatidylglycerol found in the unit compared with that found in the whole cells showed that very little contamination of the wall-plasma-membrane preparation with chloroplast fragments had occurred. However, the large amounts of diphosphatidylglycerol present suggested mitochondrial contamination. This was surprising, since the presence of mitochondria was not apparent when the unit was examined with the electron microscope; there was also no detectable cytochrome oxidase activity in the preparation. Most of the work which indicates that diphosphatidylglycerol is an exclusively mitochondrial lipid has used animal tissues (Colbeau *et al.*, 1971; Getz *et al.*, 1968), in which cellular contents of the precursor phosphatidylglycerol are low. In contrast, plant tissues have very high contents of cellular phosphatidylglycerol. This is very similar to the situation in bacteria, where diphosphatidylglycerol is a major constituent of the membrane and occurs with phosphatidylglycerol (Shaw, 1974). The considerable enrichment of phosphatidylserine in the wall-plasma-membrane unit is, however, similar to that which occurs in animal cells, where phosphatidylserine is predominantly associated with the plasma membrane, and to a lesser degree the membranes of the Golgi apparatus (Pfleger *et al.*, 1968; Ray *et al.*, 1969; Keenan & Morré, 1970).

Perhaps more than other cell organelles, the physiological functioning of the plasma membrane in plants may be severely curtailed when its complex organization is destroyed by the standard methods of plant-cell fractionation. The preparation described here avoids this problem.

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