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MEMBRANE PHOSPHOLIPIDS OF NORMAL AND CROWN GALL CALLUS CULTURES

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Abstract—The relative proportions of different phospholipid species were determined in membrane-enriched fractions from normal and crown-gall callus cultures. In general the pattern of phospholipids was similar, but the tumour cultures contained relatively more phosphatidyl choline and less phosphatidic acid and phosphatidyl inositol than the corresponding normal cultures. These differences are probably not caused by phospholipase-D activity during homogenization.

Previous investigations have suggested the possibility that alterations in membrane properties are important in the transformation from normal to tumourous cells [1–3]; if this is the case, such alterations might be reflected in differences in the membrane constituents.

We have examined the distribution of phospholipids in membrane-enriched fractions of several crown-gall callus cultures together with corresponding cultures of normal origin. Normal and tumour cultures were isolated at the same time, in most cases from the same plant, and maintained under similar conditions. Data for sunflower seedling tissue and an auxin-habituated sunflower callus [4] are included for comparison.

Although the overall patterns of distribution of phospholipids in the normal and tumour membranes are similar, in each pair of isolates there was *ca* twice as much phosphatidic acid (PA) in the normal as in the tumour membranes (Table 1.) The normal membranes were also relatively richer in phosphatidyl inositol (PI) and with one exception poorer in phosphatidyl choline (PC) than the corresponding tumour membranes. It is not clear whether the higher content of PI in normal cultures is related to tumourigenesis or is due to the incorporation of inositol from the medium. The inclusion of inositol in normal cultures is necessary to maintain the

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Table 1. Relative proportions of phospholipids in membrane fractions derived from sunflower, carrot and tobacco callus and sunflower hypocotyl, expressed as precentage of the total in each sample

Species	Culture isolate		Phospholipid species PI	PS	PC	PG	PE	DPG	PA	F
Sunflower	Normal	NH1	11.6	3.3	40.1	2.6	33.1	0.9	5.4	3.0
	Tumour	TH1	8.5	2.2	49.2	2.3	32.3	1.1	2.5	1.9
	Normal	NH3	13.1	2.2	39.4	1.6	36.7	0.9	3.5	2.5
	Tumour	TH3	8.7	3.1	48.7	1.8	31.2	1.6	2.3	2.6
	Normal	NH6	9.7	3.2	39.9	3.6	32.6	0	6.9	3.8
	Tumour	TH6	8.1	2.6	50.0	1.5	32.7	1.2	2.8	1.1
Carrot	Normal	CA3N	8.2	4.9	37.5	2.6	27.9	1.2	16.5	1.2
	Tumour	CA3T	4.3	3.8	49.7	1.4	32.7	1.1	5.0	2.0
Tobacco	Normal	NR4N	9.7	2.1	50.3	3.6	28.5	1.1	3.4	1.2
	Tumour	NR2T	7.0	2.0	47.5	1.8	35.6	1.0	2.0	3.1
Sunflower	Habituated Callus	HaHl.1	10.0	2.8	44.3	2.8	30.6	1.2	5.2	2.6
Sunflower	Hypocotyl	—	7.1	3.5	48.8	1.7	23.9	0	11.1	3.7

PI = Phosphatidyl insotitol; PS = phosphatidyl serine; PC = phosphatidyl choline; PG = phosphatidyl glycerol; PE = phosphatidyl ethanolamine; DPG = diphosphatidyl glycerol; PA = phosphatidic acid; F = unknown.

Table 2. Specific activity of phospholipase-D in 5-week-old normal and crown-gall tumour cultures of sunflower, tobacco and carrot*

Species	Phospholipase-D activity (μg choline/mg protein/hr) Normal	Tumour
Sunflower	2.0[a]	17.4[g]
	4.4[a]	30.0[h]
	10.5[c]	11.6[d]
Tobacco	11.2[e]	22.4[f]
	11.2[e]	24.8[f]
Carrot	131.4[g]	258.0[h]

*Two separate determinations were made on sunflower NHI/THI isolates and tobacco NR4N and NR2T isolates. a = NHI, b = NTI, c = NH6, d = TH6, e = NR4N, f = NR2T, e = CA3N, f = CA3T.

growth-rate at a level comparable with that of the fast-growing tumour cultures.

The presence of PA in all the preparations examined might be interpreted as an artefact of homogenization and membrane preparation. Sastry and Kates [5] have shown that in *Phaseolus multiflorus* the PA component increased dramatically after homogenization as compared with the amount in intact leaves; this increase in PA was accompanied by an almost complete disappearance of PC and phosphatidyl ethanolanine (PE). The enzyme phospholipase-D (EC 3.1.44, phosphatidylcholine phosphatidohydrolase) which cleaves the terminal phosphate diester bond in glyceryl phosphates with the liberation of the corresponding alcohol moiety and the formation of phosphatidic acid, is widely distributed in higher plants [6–8]. Consequently, the observation that tumour tissues contain relatively less PA and more PC than the corresponding normal tissue is consistent with the explanation that the normal tissues contain a higher level of phospholipase-D which is active during the 20 min membrane preparation period, despite the use of conditions designed to minimize lipase activity.

To check this possibility, estimations of lipase activity were made on the normal and tumour lines of three culture isolates. Phospholipase-D activity was found in all of the callus tissues examined; the sp. act. varied considerably with the source, being highest in the carrot tissue (Table 2.). However, the enzyme activity was in all cases higher in the tumour extracts than in those of normal tissue. Furthermore, the increase in PA content in normal as compared with tumour tissues was insufficient to account for the decrease in PC (Table 1). It was also observed that substantial amounts of PA were present on the chromatograms during preliminary experiments in which callus cultures were directly extracted with lipid solvents without prior fractionation, indicating that PA was not a homogenization artefact.

The observations above support the view that the differences in phospholipid distribution in membranes of normal and crown-gall tumour tissue cultures are real and not due to lipase activity. However, it is evident that the role, mode of action, and even the nature of phospholipase-D are at present quite uncertain [8]; until these uncertainties are resolved, some consideration must always be given to the possibility of phospholipase when interpreting data on the distribution of phospholipids.

EXPERIMENTAL

Normal and crown-gall cultures of sunflower (*Helianthus tuberosus* L. cv Russian Giant) were initiated and maintained as previously described [4]. Similar methods were used to obtain culture isolates from *Nicotiana rustica* L. (stem) and *Daucus carota* L. cv Chantenay (root). Lipid extracts were prepared from 5–6-week-old cultures.

Membrane preparation. Callus tissue (*ca* 100 g fr. wt) was homogenzied in a Polytron blender for 10 sec in a medium containing 0.35 M sucrose, 0.05 M Tris, 1 mM Na_2EDTA pH 7.4. These conditions minimize the activity of phospholipase-D [9]. The homogenate was filtered through muslin and centrifuged at 22500 *g* for 10 min. The pellet, which on electron microscope examination was found to contain plastids, intact mitochondria and debris, was discarded.

The membrane-enriched fraction was obtained from the supernatant either by direct centrifugation at 36500 *g* for 15 min or by centrifugation at 2000 *g* for 5 min following the addition of cold TCA to a final conc of 5%. Preliminary expts showed that TCA precipitation increased the yield without altering the phospholipid distribution; this method was used for all subsequent determinations of the phospholipid distribution. The pellet obtained by 36500 *g* centrifugation consisted largely of convoluted and often concentrically arranged membrane vesicles; no intact mitochondria or plastids were observed. All procedures were carried out at 0–4°; the total time between homogenization and TCA addition was 20–22 min.

Extraction and separation of phospholipids. Lipids were extracted from the membrane pellet with successive 100 ml portions of *iso* PrOH, $CHCl_3$–*iso*PrOH (2:1) and $CHCl_3$ MeOH (2:1) by stirring at room temp. for 30 min each time. The use of *iso* PrOH minimizes lipase activity [10, 11]. The combined extracts were dried *in vacuo* at 40° and the residue taken up in $CHCl_3$. After removal of non-lipid material by partitioning against 1% NaCl soln and subsequent drying, lipid extracts were examined by Si gel TLC [12]. Chromatograms were developed in the first dimension with $CHCl_3$–MeOH–NH_4OH (65:30:4) and in the second dimension with $CHCl_3$ MeOH HOAc–H_2O (85:15:10:3) [13]. Phospholipids were identified by co-chromatography with authentic compounds, spraying with ninhydrin and comparison with published R_f values. The individual lipid spots were eluted from the plates [12] after localization with I_2 vapour, and P contents were determined [14] in triplicate for each phospholipid.

Phospholipase-D activity. A modification of the method of ref. [15] was used. 5–6-week-old tissue (10 g) was homogenized in the French press with two vols of H_2O at 0°. Triplicate 0.6 ml aliquots of the homogenate were immediately transferred to stoppered vials containing 2 mg lecithin (Sigma, L-α-lecithin, type III-E, from egg yolk), 1.75 ml of 0.2 M acetate buffer pH 5.6 and 0.15 ml of M $CaCl_2$. At zero time 0.5 ml of Et_2O was added and the stoppered vials incubated at 25° with vigorous shaking. The reaction was stopped after 0 and 15 min by the addition of 0.5 ml N HCl and the unreacted lecithin and phosphatidic acid removed by repeated partitioning against Et_2O. After evapn of the aq. phase to dryness *in vacuo*, choline was extracted with 2 ml of 0.1 M Pi buffer pH 7 and determined after filtration [16], using the zero time sample as a reagent blank.

The protein content of the callus homogenate was estimated after TCA precipitation [17].

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REFERENCES

1. Braun, A. C. and Wood, H. N. (1962) *Proc. Natl. Acad. Sci. U.S.A.* **48**, 1776.
2. Wood, H. N. and Braun, A. C. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 1532.
3. Stonier, T. and Yang, H. (1971) *Physiol. Plant.* **25**, 474.
4. Butcher, D. N., Sogeke, A. K. and Tommerup, I. C. (1975) *Protoplasma* **86**, 295.
5. Sastry, P. S. and Kates, M. (1964) *Biochemistry* **3**, 1280.
6. Kates, M. (1970) *Adv. Lipid Res.* **8**, 197.
7. Kleinig, H. and Kopp, C. (1978) *Planta* **139**, 61.
8. Roughan, P. G. and Slack, C. R. (1976) *Biochim. Biophys. Acta* **431**, 86.
9. Galliard, T., (1974) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. XXXI, p. 520. Academic Press, New York.
10. Nichols, B. W. (1963) *Biochim. Biophys. Acta* **70**, 417.
11. Toyosawa, I. and Nishimoto, U. (1967) *Agric. Biol. Chem.* **31**, 275.
12. Skipsky, P. V., Peterson, R. F. and Barclay, M. (1964) *Biochem. J.* **90**, 374.
13. Renkonen, O. and Varo, P. (1967) in *Lipid Chromatographic Analysis* (Marinetti, ed.) Vol. I, p. 41 Dekker, New York.
14. Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466.
15. Kates, M. and Sastry, P. S. (1969) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. XIV, p. 197. Academic Press, New York.
16. Appleton, D. A., La Du, B. N., Levy, B. B., Steel, J. M. and Brodie, B. B. (1953) *J. Biol. Chem.* **205**, 803.
17. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randell, R. J. (1951) *J. Biol. Chem.* **193**, 265.