Biochem. J. (1975) **147**, 179–180
Printed in Great Britain

The Occurrence of a Phosphorylated Glycosphingolipid in *Aspergillus niger*

By PATRICK J. BRENNAN and JOHN ROE
Department of Biochemistry, University College, Dublin 4, Republic of Ireland

(*Received* 10 *December* 1974)

A novel type of water-soluble phosphorylated glycosphingolipid was isolated from *Aspergillus niger* by a simple procedure involving precipitation, DEAE-cellulose chromatography and preparative t.l.c. Besides ceramide and phosphorus it contains inositol, galactose, mannose and small amounts of glucosamine.

In a review of the lipids of fungi (Brennan *et al.*, 1974) we speculated on the origins of the high amounts of free ceramide and ceramide 1-phosphate isolated from some mould and yeast sources, from *Agaricus* and from representatives of the Phycomycetes and Fungi Imperfecti. It seemed possible that these were the products of the effects of alkali or autolysis on unrecognized complex glycophosphosphingolipids, since Steiner *et al.* (1969) have noted that in yeast a mannosylmonoinositolmonophosphorylceramide was a degradation product of mannosyldi-inositoldiphosphorylceramide, and it seemed that under certain conditions further degradation was feasible. On the basis of this reasoning we examined *Aspergillus niger* for the presence of complex glycosphingolipids and we report the isolation and some of the major structural features of a novel member of this lipid class.

Experimental

A. niger mycelium was supplied by Pfizer Chemical Corp., Ringaskiddy, County Cork, Ireland. The products obtained from this source appeared to be identical with those from *A. niger* A.T.C.C. 26522 grown under previously described conditions (Laine *et al.*, 1972).

Extraction of glycosphingolipid. Mycelium was initially refluxed with 50% (v/v) ethanol, a procedure which facilitated the subsequent extraction of the lipids with chloroform–methanol (2:1, v/v). Combined filtrates were concentrated and subjected to a Folch *et al.* (1957) wash. The upper aqueous phase was concentrated, and pyridine was added to give a final concentration of 30% (v/v). The solution was placed in an ice bath and 3 vol. of ice-cold ethanol was added (Carter *et al.*, 1958). The precipitate after flocculation was collected by centrifugation. This source contained about half of the recovered glycosphingolipids. More of the material was obtained by extracting the residual mycelium with 30% (v/v) pyridine (Steiner *et al.*, 1969). The pyridine extracts were concentrated, fresh pyridine was added to achieve dissolution and the ethanol precipitation process was applied. The combined precipitates were blown with N_2 to remove all traces of ethanol.

Purification of glycosphingolipid. The precipitate, suspended in chloroform–methanol–water, was applied to a column (4cm×35cm) of DEAE-cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.) in the acetate form (Brennan & Ballou, 1967). A linear gradient was applied composed of 500ml of chloroform–methanol–water (20:9:1, by vol.) in the mixing chamber and 500ml of 2M-ammonium acetate in chloroform–methanol–water (20:9:1, by vol.) in the reservoir chamber. This was followed by a 500ml step of 3M-ammonium acetate in chloroform–methanol–water (10:7:1, by vol.). Fractions (10ml) were collected and analysed for phosphorus and carbohydrate (Brennan & Ballou 1967). A careful emulsification step and dialysis (Brennan & Ballou, 1967) was used to remove ammonium acetate and to retain the water-soluble glycolipids. These were freeze-dried, dissolved in 30% (v/v) pyridine and applied to plates of silica gel H (0.75mm thick) for chromatography in chloroform–methanol–aq. 8% (v/v) NH_3 (9:7:2, by vol.). The plates were sprayed with water and the broad hydrophobic band was eluted from the gel with chloroform–methanol–water–pyridine (20:28:6:1, by vol.) (Steiner *et al.*, 1969). The eluate was dried, redissolved in 30% (v/v) pyridine and the glycolipids were recovered by precipitation with ethanol.

Analytical methods. The glycolipid was hydrolysed in 1ml of 2M-HCl at 100°C for 3h or in 6M-HCl at 110°C for 6h. The hydrolysate was extracted with 3×0.5ml of chloroform–methanol (2:1, v/v). The chloroform phase was subjected to t.l.c. in chloroform–methanol–aq. 4% (v/v) NH_3 (40:10:1, by vol.), and the plate was sprayed with a solution of 0.25% (w/v) ninhydrin in acetone to identify long-chain bases. The aqueous phase from the hydrolysate was repeatedly dried over N_2 to remove HCl

and chromatographed on Whatman no. 1 filter paper in butan-1-ol–pyridine–water (6:4:3, by vol.) or in pyridine – ethyl acetate – acetic acid – water (36:36:7:21, by vol.). Sugars and polyols in this fraction were located with an $AgNO_3$–NaOH dip reagent (Brennan & Ballou, 1967). The glycolipid was also methanolysed in Teflon-lined screw-capped tubes with 1M-HCl in methanol at 75°C for 16h. The methylated fatty acids in the hexane extract of the methanolysate were subjected to g.l.c. on a column of 3% OV-1 operating isothermally at 240°C. The methanol fraction from the methanolysate was neutralized by the addition of Ag_2CO_3 and the trimethylsilyl derivatives were prepared (Vance & Sweeley, 1967). The products were chromatographed on a column of 3% OV-1 operating at 240°C for the detection of long-chain bases and at 160°C for the detection of sugars.

Results

From 2kg wet weight of mycelium, 12g of the combined precipitate was obtained and this contained 20mg of phosphorus. DEAE-cellulose chromatography of this material showed a single phosphorus- and carbohydrate-containing peak which began to emerge from the column when the concentration of ammonium acetate had reached 2M. About 70mg of the glycolipid was obtained by preparative t.l.c. The glycolipid was readily soluble in 30% pyridine, less so in water and dilute alkali and it was insoluble in the usual chloroform–methanol mixtures. T.l.c. in chloroform–methanol–aq. 8% (v/v) NH_3 (9:7:2, by vol.) showed a double band with the following reagents: a water spray, I_2 vapour, the Molybdenum Blue reagent for phosphorus (Dittmer & Lester, 1964), the orcinol reagent for carbohydrate (Christie, 1973) and the periodate–Schiff reagent for carbohydrate (Shaw, 1968) (Plate 1*a*). The glycolipid was hydrolysed in aq. 2M-HCl and the lipid fraction from the hydrolysate was subjected to t.l.c. Exposure of the plate to ninhydrin showed the definite presence of long-chain bases, and g.l.c. of this fraction after trimethylsilylation showed three major individual components. G.l.c. of the methylated fatty acids from the glycolipid showed one major peak with a retention time of 27min compared with a retention time of 5min for α-hydroxymethylstearate. The retention time for this component was increased to 33min after treatment with the trimethylsilylating reagent. These results suggest that the fatty acids of the glycolipid consist of mostly one hydroxylated long-chain fatty acid. Moreover, t.l.c. of the methylated fatty acid showed an R_F of 0.2, compared with an R_F of 0.6 for non-hydroxylated fatty acids, a feature typical of hydroxylated fatty acids (Brennan *et al.*, 1970).

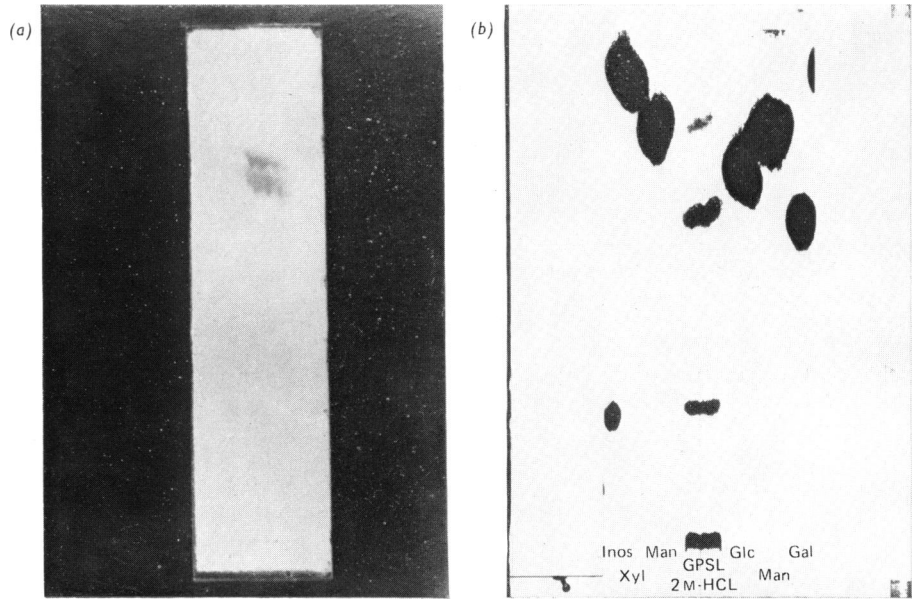
Hydrolysis of the glycolipid with 2M-HCl and paper chromatography of the water-soluble products showed the presence of inositol, galactose, small amounts of mannose and unhydrolysed material (Plate 1*b*). There appeared to be approximately three times more galactose present than mannose. Hydrolysis of the glycolipid with 6M-HCl showed additional small quantities of glucosamine.

These observations prove the existence in *A. niger* mycelium of a water-soluble glycophosphosphingolipid with a distinctive sugar composition. This compound and the less glycosylated lipid-soluble variety described by Lester and colleagues (Steiner *et al.*, 1969; Smith & Lester, 1974) form a distinct group of mycoglycosphingolipids, which are apparently structurally related to the phytoglycosphingolipids of higher plants (Carter *et al.*, 1965).

We are grateful to the National Science Council for their financial support, to the Pfizer Chemical Corporation for the supply of mycelium and to Miss Patricia Carty for skilled technical assistance.

References

Brennan, P. J. & Ballou, C. E. (1967) *J. Biol. Chem.* **242**, 3046–3056
Brennan, P. J., Lehane, D. P. & Thomas, D. W. (1970) *Eur. J. Biochem.* **13**, 117–123
Brennan, P. J., Griffin, P. F. S., Losel, D. M. & Tyrell, D. (1974) *Progr. Chem. Fats Other Lipids* **14**, part 2, 51–89
Carter, H. E., Celmer, W. D., Galanos, D. S., Gigg, R. H., Lands, W. E. M., Law, J. H., Mueller, K. L., Nakawayama, T., Tomizawa, H. H. & Weber, E. (1958) *J. Amer. Oil Chem. Soc.* **35**, 335–343
Carter, H. E., Johnson, P. & Weber, E. J. (1965) *Annu. Rev. Biochem.* **34**, 109–142
Christie, W. E. (1973) *Lipid Analysis*, pp. 197–201, Pergamon Press, Oxford
Dittmer, J. C. & Lester, R. L. (1964) *J. Lipid Res.* **5**, 126–127
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
Laine, R. A., Griffin, P. F. S., Sweeley, C. C. & Brennan, P. J. (1972) *Biochemistry* **11**, 2267–2270
Shaw, N. (1968) *Biochim. Biophys. Acta* **164**, 435–436
Smith, S. W. & Lester, R. L. (1974) *J. Biol. Chem.* **249**, 3395–3405
Steiner, S., Smith, S., Waechter, C. J. & Lester, R. L. (1969) *Proc. Nat. Acad. Sci. U.S.* **64**, 1042–1048
Vance, D. E. & Sweeley, C. C. (1967) *J. Lipid Res.* **8**, 621–630



EXPLANATION OF PLATE 1

T.l.c. of the glycosphingolipid from A. niger in chloroform–methanol–aq. 8% (*v*/*v*) NH_3 (9:7:2, *by vol.*) (*a*) *and paper chromatography in butan-1-ol–pyridine–water* (6:4:3, *by vol.*) *of the products from* 2 M-*HCl hydrolysis of the glycosphingolipid* (*b*)

The t.l.c. plate was stained with the periodate–Schiff reagent. The chromatograms were stained with the $AgNO_3$–NaOH reagent. Gal, galactose; Glc, glucose; Man, mannose; Inos, inositol; Xyl, xylose; GPSL, glycophosphosphingolipid from *A. niger*.