A SUCCINYLATED MANNAN IN THE MEMBRANE SYSTEM OF MICROCOCCUS LYSODEIKTICUS

Peter Owen and Milton R. J. Salton
Department of Microbiology, New York University School of Medicine, New York, NY 10016

Received February 17,1975

SUMMARY: A mannan has been isolated from membranes of Micrococcus lysodeikticus (NCTC 2665) grown in the presence of [1,4 ^{14}C] succinic acid. Hydrolysis of this polysaccharide with 0.1 N NaOH yielded a compound soluble in both diethyl ether and distilled water. This component was identified as [^{14}C] succinic acid by paper chromatography of both the free acid and of its corresponding hydroxamic acid derivative. Semiquantitative analysis indicated an ester-linked succinic acid content for mannan of approximately 2.5%.

INTRODUCTION: Micrococcus lysodeikticus, unlike most gram-positive bacteria, does not possess a membrane teichoic acid except when grown under conditions of limiting Mg^{2+} (1). This organism does, however, synthesize a mannose polymer (2, 3). Recent work in this laboratory has shown that this polysaccharide represents over 90% of the total membrane-bound carbohydrate and that it is localized largely in the mesosome of this organism (4). Characterization of mannan purified from isolated mesosomal vesicles (5) and from whole cells of M. lysodeikticus (6, 7) has shown that it possesses acidic properties which are lost upon mild base hydrolysis. A full characterization of the ionic properties of this molecule would thus be important since the possibility exists that an acidic mannan may represent a teichoic acid analogue for M. lysodeikticus. In the present study we report the presence of ester-linked succinate residues in this polymer.

MATERIALS AND METHODS: Cells of Micrococcus lysodeikticus (NCTC 2665) were grown on peptone-water-yeast extract medium for 24 h as previously described (8). Culture medium (750 ml) was supplemented with

100 μCi [1,4 ^{14}C] succinic acid (20.4 mCi/mmole, Amersham/Searle). Total membrane fractions (i. e. plasma plus mesosomal membranes) were prepared from washed cells by lysozyme digestion and osmotic lysis, and were subsequently washed six times in 50 mM Tris-HCl buffer, pH 7.4, at 4°C (9). The method for preparation of isolated mesosomal membranes followed that described by Owen and Freer (10).

Details of the purification of membrane-bound mannan by essentially non-degradative procedures will be given in detail elsewhere (5). In brief, mannan was solubilized from washed membranes by ionic-heat shock treatment (4), concentrated with an Amicon ultrafiltration unit and then precipitated with 75% ethanol. Mannan isolated from mesosomal vesicles by this method has been shown to contain 94% mannose, 1% lipid and no detectable phosphorus (4, 5). [^{14}C] -labeled mannan prepared from total membranes was further purified by incubation at 23°C for 24 h with a twenty-fold weight excess of concanavalin A in 18 mM phosphate buffer, pH 7.2, containing 1.0 M NaCl. The resultant precipitate was washed twice with phosphate buffer containing 1.0 M NaCl, once in phosphate buffer alone and then lyophilized.

Both labeled and unlabeled mannan preparations at a concentration of 1 mg/ml were hydrolyzed with 0.1 N NaOH for 1 h at 100°C under a nitrogen atmosphere. Hydrolysates were then made 0.5 N with respect to HCl and extracted four times with six-fold volumes of diethyl ether (11). Ether was subsequently evaporated and the residue dissolved in distilled water. Hydroxamic acid derivatives of the ether soluble extract of base-hydrolyzed mannan and of succinic acid were prepared according to the method described by Keller and Ballou (12). Free acids were subjected to ascending chromatography on prewashed (13) Whatman SG 81 silica gel impregnated paper using solvent I - butanol/formic acid/water, 4/1/5 by volume and solvent II - ethanol/ammonia/water, 16/1/3 by volume; ascending chromatography of corresponding hydroxamate derivatives was performed on Whatman No. 1 chromatography paper with solvent III - 2-propanol/ammonia, 2/1 by volume and solvent IV - water-saturated isobutyric acid. Acids were detected with either bromocresol purple (14), bromophenol blue (15) or with methanolic aniline-xylose (16) and hydroxamate derivatives with 1% ethanolic ferric chloride (17). Carbohydrates were localized on chromatograms with aniline hydrogen phthalate (18) and determined quantitatively in solution by the anthrone method of Morris (19) using mannose as standard. Radioactive components were localized by cutting chromatograms into 1/8th inch strips, immersing each in Aquasol scintillation fluid (New England Nuclear) and making radioactive measurements over 4-min intervals in a Nuclear Chicago Mark I scintillation counter.

RESULTS AND DISCUSSION: Approximately 12% of the radioactivity incorporated into cells of M. lysodeikticus grown in the presence of [1,4 ^{14}C] succinic acid was recovered in the total membrane fraction. In turn, about 14% of the membrane-bound radioactivity could be accounted for as mannan which was released from the membrane by ionic-heat shock and

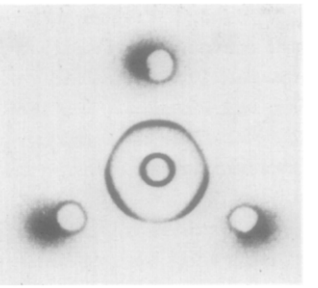
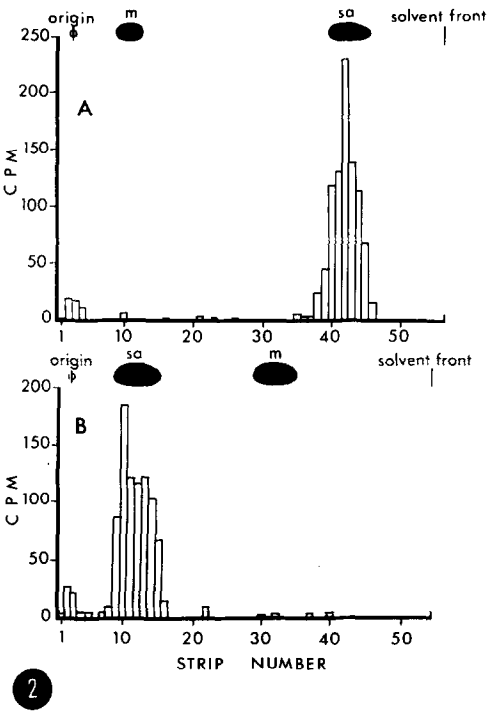


Figure 1. Autoradiogram of the gel diffusion pattern obtained following diffusion of [^{14}C] - labeled mannan (2 mg/ml, outer wells) isolated from cells labeled with [1,4 ^{14}C]-succinic acid against concanavalin A (10 mg/ml, delineated center well).

purified by ethanol precipitation. Ethanol precipitated, [^{14}C] -labeled mannan was tested against the lectin concanavalin A in agar gel and the autoradiogram is shown in Fig. 1. One major precipitin band was obtained and it showed a line of identity with purified unlabeled mannan isolated from the mesosome fraction. Subsequent purification of [^{14}C] -labeled mannan by coprecipitation with concanavalin A yielded a fraction (180,000 cpm) accounting for 64% and 95% of the radioactivity present in the ionic-heat shock fraction and in the ethanol precipitate respectively.

Evidence obtained from polyacrylamide gel electrophoresis (6), from DEAE cellulose chromatography (7) and from electrophoresis in agar (5) has indicated that mannan isolated from whole cells (6, 7) and from isolated mesosomal vesicles of this organism (5) is an acidic polymer whose ionic characteristics are lost following mild alkaline hydrolysis. This property has been attributed to the presence of charged fatty acids covalently attached to the polysaccharide molecule (7). In the present study we observed that mild base hydrolysis followed by acidification and exhaustive ether extraction yielded a product soluble in both ether and distilled water which accounted for approximately 7% of the total radioactivity observed for intact mannan. Chromatography of this material (Fig. 2) revealed the presence of a major radioactive peak accounting for over 90% of the observed radioactivity. This compound gave a positive reaction with detection



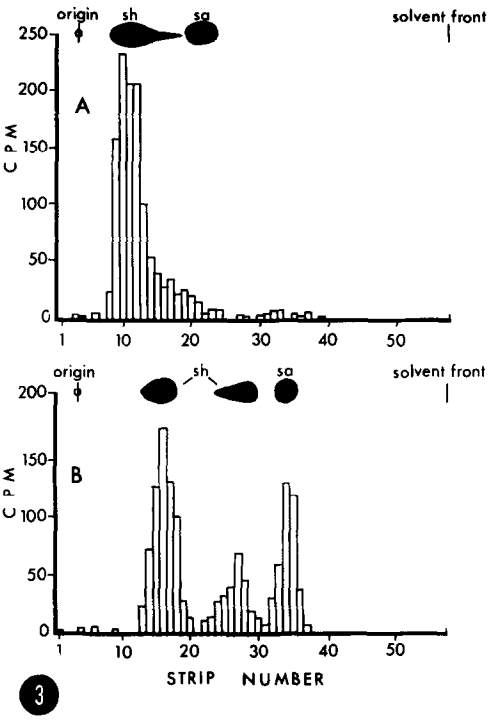


Figure 2. Paper chromatograms of an ether extract of base-hydrolyzed [^{14}C] -mannan isolated from cells labeled with [1,4 ^{14}C] -succinic acid. The ether extract was dissolved in distilled water and subjected to chromatography in solvent I (A) and in solvent II (B). The chromatographic behavior of authentic succinic acid (sa) and of mannose (m) are shown for comparison.

Figure 3. Paper chromatograms of the hydroxamate derivatives of an ether extract of base-hydrolyzed [^{14}C] -labeled mannan. Derivatives were subjected to chromatography in solvent III (A) and in solvent IV (B). The chromatographic behavior of authentic succinohydroxamate (sh) and of free succinic acid (sa) are shown for comparison.

---

reagents for aliphatic acids (14-16) and cochromatographed with authentic succinic acid. The remaining radioactivity could be accounted for by material remaining at the origin and may represent a slight carry over of residual mannan. No labeled free mannose was detected.

Confirmation of the presence of succinyl residues was obtained by chromatography of the corresponding hydroxamic acid derivatives. In solvent III (Fig. 3A) the major radioactive peak corresponded to an area of the

chromatogram staining purple with ferric chloride (17) and showed similar chromatographic properties to authentic succinohydroxamate. The slight tailing of the peak could in part be accounted for by the presence of unreacted succinic acid. Following chromatography in solvent IV (Fig. 3B) three distinct radioactive peaks were observed. Two of these corresponded to regions of the chromatogram staining purple with ferric chloride; the third showed similar chromatographic properties to free succinic acid. The observation that the hydroxamate derivative of authentic succinic acid also gave two similar components staining positively with ferric chloride following chromatography in this solvent confirms the earlier observations of Fink and Fink (20) and suggests the occurrence of acid hydrolysis in this solvent system.

Succinyl residues, detected as their hydroxamate derivatives, were also found in unlabeled mesosomal mannan. Visual comparison of the color reactions produced between ferric chloride and known quantities of authentic succinohydroxamate following chromatography in solvent III allowed a semi-quantitative estimate of the succinic acid content of this polymer. A value of approximately 2.5% was obtained. Mannan obtained from mesosomal vesicles is known to contain 94% mannose and 1% associated lipid (4, 5). Allowing for a small degree of protein contamination, succinyl residues would bring the recovery of identifiable components to about 100%.

The occurrence of succinyl residues in bacterial polysaccharides is comparatively rare and its detection in the membrane mannan of M. lysodeikticus accounts for the acidic properties of this polymer. The possession of this polysaccharide in this organism is of interest as it appears to be devoid of the other common type of acidic polymer (teichoic acid) found in walls and membranes of gram-positive bacteria. The only other instances

in which succinyl residues have been reported are in the teichoic acid of Actinomyces violaceus (21), the lipopolysaccharide of Mycobacterium phlei (12) and more recently in a glycerophosphate-oligosaccharide isolated from Escherichia coli (22).

ACKNOWLEDGEMENTS: This research was supported by a grant (BMS75-03934) from the National Science Foundation. P. O. was also in receipt of a NATO Research Fellowship (B/RF/3636).

REFERENCES

1. Ellwood, D. C., and Tempest, D. W. (1972) Adv. Microbiol. Physiol. 7, 83-117.
2. Scher, M., and Lennarz, W. J. (1969) J. Biol. Chem. 244, 2777-2789.
3. Lahav, M., Chiu, T. H., and Lennarz, W. J. (1969) J. Biol. Chem. 244, 5890-5898.
4. Owen, P., and Salton, M. R. J. (1974) 74th Ann. Meeting of the American Society for Microbiology, P80.
5. Owen, P., and Salton, M. R. J. (1975) Manuscript in preparation.
6. Schmit, A. S., Pless, D. D., and Lennarz, W. J. (1974) Ann. N. Y. Acad. Sci. 235, 91-104.
7. Powell, D. A., Duckworth, M., and Baddiley, J. (1974) FEBS Lett. 41, 259-263.
8. Owen, P., and Freer, J. H. (1970) Biochem. J. 120, 237-243.
9. Salton, M. R. J. (1967) Trans. N. Y. Acad. Sci. Ser. II 29, 764-781.
10. Owen, P., and Freer, J. H. (1974) Biochem. J. 129, 907-917.
11. Beck, W. S. (1962) Methods in Enzymology, 5, 581-587, Academic Press, New York.
12. Keller, J. M., and Ballou, C. E. (1968) J. Biol. Chem. 243, 2905-2910.
13. Isherwood, F. A., and Hanes, C. S. (1953) Biochem. J. 55, 824-830.
14. Reid, R. L., and Lederer, M. (1951) Biochem. J. 50, 60-67.
15. Kennedy, E. P., and Barker, H. A. (1951) Anal. Chem. 23, 1033-1034.
16. Saarnio, J., Niskasaari, E., and Gustafsson, C. (1952) Suomen Kemistilehti 25B, 25.
17. Sherma, J., and Zweig, G. (1971) Paper Chromatography and Electrophoresis, 11, 172-199, Academic Press, New York.
18. Cummins, C. S., and Harris, H. (1956) J. Gen. Microbiol. 14, 583-600.
19. Morris, D. L. (1948) Science, 107, 254-256.
20. Fink, K., and Fink, R. M. (1949) Proc. Soc. Exptl. Biol. Med. 70, 654-656.
21. Naumova, I. B., Belozerskii, A., and Shafikova, F. A. (1962) Dokl. Akad. Nauk. SSSR 143, 730-733.
22. Van Golde, L. M. G., Schulman, H., and Kennedy, E. P. (1973) Proc. Natl. Acad. Sci. USA 70, 1368-1372.