Induction of Cellular Efflux by a Galactosamine Polymer from *Neurospora crassa*

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A cationic polymer of D-galactosamine was isolated from culture filtrates of a colonial temperature-sensitive strain of *Neurospora crassa*. Adsorption of the polymer to the cell surface initiated immediate efflux of low molecular weight metabolites and subsequent loss of viability. The polymer appeared to bind to those sites on the cell surface that normally bind calcium ions. Chemical analysis of the polymer showed it to be partially *N*-acetylated. The polymer had an isoelectric point of 8.4. Thirty percent of the D-galactosamine residues contained free amino groups. A rapid assay that has potential application for monitoring the effect of a variety of other membrane-active factors on membrane permeability has been developed.

INTRODUCTION

Polycations have been shown to have deleterious effects on biological systems. Among the effects reported are: ultrastructural changes (Quinton & Philpott, 1973; Mayhew *et al.*, 1973), cell agglutination (Sirica & Woodman, 1971), and cytotoxicity and altered membrane permeability (Yphantis *et al.*, 1967; Jaspers *et al.*, 1975). Polycations are believed to exert their effects through ionic binding to negatively charged sites on the cell surface (Sela & Katchalski, 1959).

Galactosaminoglycan is a partially *N*-acetylated cationic polymer of D-galactosamine which can be isolated from cell walls and culture filtrates of several fungi, including *Neurospora crassa* (Distler & Roseman, 1960; Harold, 1962; Reissig & Glasgow, 1971). The biological role of the polymer isolated from *N. crassa* may involve the regulation of growth and hyphal morphology (Reissig & Glasgow, 1971; Springer & Srb, 1978). The polymer has been shown to adsorb to the cell surface of this fungus and to elicit alterations in both membrane permeability and viability (Glasgow & Reissig, 1974).

In the work described here we examined the effects on membrane permeability and viability of adsorption of galactosaminoglycan to *N. crassa* conidia. Exposure to galactosaminoglycan led to a decline in cell viability directly proportional to the duration of exposure to the polymer. Adsorption resulted in a rapid efflux of low molecular weight metabolites, which is believed to be responsible for the deleterious effect of the polymer on viability. The galactosaminoglycan-induced efflux phenomenon led to the development of a rapid assay which can potentially be used for the detection of a variety of membrane-active factors, even in crude preparations. We further report the chemical composition of the purified polymer.

METHODS

Strains and growth of cultures. The wild-type strain Tatum *a* (FGSC 621) and the colonial temperature-sensitive strain *cot-5* (FGSC 1362) were obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA 95521, USA. All strains were maintained on 1 × Vogel medium N supplemented with 2% (w/v) sucrose and solidified with 2% (w/v) agar as previously described (Vogel, 1964; DeBusk & DeBusk, 1980).

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Chemicals. The radiolabelled metabolites 3-*O*-methyl-D-[U-¹⁴C]glucose and D-[U-¹⁴C]glucosamine were obtained from New England Nuclear; L-[U-¹⁴C]phenylalanine, L-[U-¹⁴C]arginine and [U-¹⁴C]uridine were obtained from Schwarz/Mann (Spring Valley, NY, USA). Carrier amino acids were of the L-stereoisomer and were purchased from Calbiochem-Behring (San Diego, CA, USA). The following metabolites were obtained from Sigma: D-galactosamine.HCl, 3-methyl-2-benzothiazolinone hydrazone.HCl, trinitrobenzenesulphonic acid, chitosan (B-grade), poly-L-lysine, 3-*O*-methyl-D-glucose, uridine and cycloheximide. Lanthanum nitrate was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Isolation and purification of galactosaminoglycan. Erlenmeyer flasks (1 litre) containing 500 ml Vogel medium N (Vogel, 1964) supplemented with 2% sucrose were inoculated with conidia of the *cot-5* strain and incubated in the light on a rotary shaker (120 r.p.m.) at the permissive temperature (25 °C). Under these conditions, galactosaminoglycan production was maximal at 5–6 d. At this time, the media were separated from the cultures by filtration, chilled to 4 °C prior to the addition of 0.4 volumes of cold 95% (v/v) ethanol, and allowed to remain at 4 °C overnight. The resultant precipitate was collected by centrifugation (3000 g, 20 min, 4 °C). The precipitate was resuspended in 25 ml 10% (w/v) trichloroacetic acid, stirred overnight at 4 °C, and then centrifuged (10000 g, 1 h, 4 °C). The supernatant was dialysed against distilled water for 48 h at 4 °C with three changes of water. The non-dialysable material was lyophilized and stored at room temperature. This procedure yielded 60–80 mg pure galactosaminoglycan polymer per litre of culture filtrate.

Radiolabelled galactosaminoglycan was prepared as above from cultures to which 10 µCi of D-[U-¹⁴C]-glucosamine.HCl [7 mCi (259 MBq) mmol⁻¹] were added after 48 h incubation. The cultures were incubated for an additional 3–4 d.

Accumulation and efflux assays. The methods used for the conidial transport assays have been described by DeBusk & DeBusk (1980). Determination of radiolabelled substrate incorporated into macromolecules at any given incubation time is described by DeBusk *et al.* (1981). Assays were done at 25 °C for 3 h using the wild-type strain. In all cases the Vogel medium N present in the incubation medium was at a final concentration of 0.1 × rather than the standard 1 ×. The radiolabelled substrates L-[U-¹⁴C]phenylalanine, L-[U-¹⁴C]arginine, and 3-*O*-methyl-D-[U-¹⁴C]glucose were present in the incubation medium at a final concentration of 0.01 µCi (0.37 kBq) per 0.1 µmol per ml, and [U-¹⁴C]uridine was present at a final concentration of 0.005 µCi (0.185 kBq) per 0.1 µmol per ml. To study the effects of the polymers galactosaminoglycan, chitosan or poly-L-lysine on the accumulation or retention of radiolabelled phenylalanine, arginine, uridine or 3-*O*-methyl-D-glucose, the polymers were present at a final concentration of 20, 50, and 10 µg per ml of incubation medium, respectively, unless otherwise noted. When the effect on metabolite accumulation was monitored, the polymers were present in the incubation medium at the initiation of the transport assay. When the effect on metabolite retention was monitored, the polymers were added in concentrated form (200–300 µl) after accumulation had proceeded for 60 min in the presence of radiolabelled substrate. An equivalent volume of distilled water was added to the control flask. Where the effect of various salts was assayed, the salts were added in crystalline form before initiation of the transport assay or after 60 min of incubation of cells in the presence of radiolabelled substrate, as noted in the text. The final concentration of each salt is also noted at the appropriate point in the text.

Two modifications of the basic transport assay were used, (1) to assay biological activity of various fractions during the chemical characterization of the polymer, and (2) to develop the efflux assay designed to detect the effect of putative membrane-active factors on membrane permeability. To assay fractions for biological activity, a 2 ml assay was used. Conidia were suspended in 1 ml containing 0.2 mg (dry wt) conidia and 0.2 × Vogel medium N. The assay was initiated by adding the conidia to test tubes containing 0.5 ml of an L-[¹⁴C]phenylalanine solution [0.04 µCi (1.48 kBq) per 0.4 µmol per ml] and 0.5 ml of the test substance (in distilled water). The tubes were mixed briefly, incubated in a reciprocal-shake water bath for 1 h at 25 °C, and filtered onto nitrocellulose filters (Millipore, type AA, 0.8 µm pore size), washed, dried, and counted as usual (DeBusk & DeBusk, 1980).

For the efflux assay, conidia (5 mg dry wt) were developed to the post-conidial stage by incubation for 3 h at 25 °C in 50 ml containing 1% D-glucose and 1 × Vogel medium N. The post-conidial stage is an early developmental stage in which the activity of the amino acid transport systems is considerably amplified over that of the conidial stage so that an approximately tenfold increase in total amino acid accumulation is achieved (Tisdale & DeBusk, 1970). Radiolabelled L-arginine [0.01 µCi (0.37 kBq) per 0.1 µmol per ml final concentration] and cycloheximide (10 µg ml⁻¹ final concentration) were added after 3 h, and the cells were allowed to accumulate L-arginine for 60 min. The suspension was then stored at 4 °C for up to two weeks. The stored cells retained 98–99% of the accumulated arginine during the two week storage period. To test a substance for its effect on membrane permeability, 2 ml of the 0.1 mg ml⁻¹ radiolabelled conidial suspension were filtered onto nitrocellulose filters, washed twice with cold distilled water, and resuspended in test tubes containing 1 ml of 0.2 × Vogel medium N. When lanthanum nitrate was assayed, the medium N buffer was replaced with an equal volume of deionized water to avoid formation of lanthanum phosphate precipitates. Assays were initiated by the addition of 1 ml of test substance to the 1 ml cell/Vogel medium N suspension. The tubes were mixed, incubated for 60 min at 25 °C in a reciprocal-shake water bath, and then filtered, washed, dried and counted as usual.

Viability assays. Wild-type conidial suspensions (0.1 mg dry wt ml⁻¹) were prepared as for the accumulation and efflux assays. Cells were incubated at 25 °C with gentle shaking for up to 4 min in 0.1 × Vogel medium N

containing 20 µg galactosaminoglycan per ml of incubation medium. After exposure to galactosaminoglycan, conidia were aseptically filtered onto nitrocellulose filters, washed with 10 ml sterile distilled water, serially diluted in 0.1 × Vogel medium N, and plated onto colony-producing medium containing 1 × Vogel medium N, 1.5% (w/v) sorbose, 0.15% sucrose and 2% agar. Colonies were scored after 4 d at 25 °C. Where the ability of the representative salt KCl to prevent loss of viability following galactosaminoglycan treatment was examined, conidia were treated as above and at the end of each treatment period an equal volume of 2.4 M-KCl was added to the incubation medium containing galactosaminoglycan to give a final concentration of 1.2 M-KCl. After 15 min, conidia were filtered, washed, diluted and plated as above.

Galactosaminoglycan binding assays. Conidia (0.1 mg dry wt ml⁻¹, final concentration) were incubated for up to 1.5 h in 0.1 × Vogel medium N containing [¹⁴C]galactosaminoglycan at a concentration of 5 µg per ml of incubation medium (320 c.p.m. per 5 µg). The preparation of radiolabelled galactosaminoglycan is described above, under 'Isolation and purification of galactosaminoglycan'. The assays were done at 25 °C in a reciprocal-shake water bath. At intervals, 5 ml samples were withdrawn, filtered, and washed as for accumulation and efflux assays. Radioactivity was detected by liquid scintillation spectroscopy. Non-specific binding of galactosaminoglycan to the nitrocellulose filters was determined using an incubation medium prepared as above but lacking conidia. The non-specific binding was subtracted from the experimental determinations. When the effect of lanthanum nitrate on galactosaminoglycan binding was examined, the 0.1 × Vogel medium N was replaced with deionized water.

Characterization of galactosaminoglycan. The isoelectric point of purified [¹⁴C]galactosaminoglycan was determined by isoelectric focusing for 48 h at 15 °C (300 V, 6 mA) on an LKB 810 electrofocusing column according to the method of Vesterburg (1972). The fractions, collected at the rate of 1 ml min⁻¹, were assayed for both pH and radioactivity.

For amino sugar analysis, non-radiolabelled galactosaminoglycan was hydrolysed in 4 M-HCl at 100 °C in sealed tubes purged with nitrogen. Hydrolysis was for 16 h, which resulted in the maximum release of amino sugar. The acid hydrolysate was applied directly to the resin of an amino acid analyser (Beckman model 120B) and eluted with borate buffer (pH 7.6) as described by Weber & Winzler (1969). Hexosamine analysis was done on lyophilized acid hydrolysates according to the total hexosamine procedure of Ludowieg & Benmaman (1967). The quantity of *N*-unsubstituted D-galactosamine was determined by nitrous acid deaminative degradation which was combusted at pH 4 (Tsuji *et al.*, 1969; Dische & Borenfreund, 1950; Lindahl *et al.*, 1973).

For amino acid analysis, non-radiolabelled galactosaminoglycan was hydrolysed for 22 h in 6 M-HCl at 100 °C in evacuated tubes purged with nitrogen, lyophilized, and fractionated on an amino acid analyser (Beckman model 120B) using an AA 15 resin and the buffer system described by Spackman (1964). Ascending paper chromatography was done on Whatman no. 1 filter paper with 1-propanol/ethyl acetate/water (7:1:2, by vol.) as the solvent.

Neutral sugars were estimated by the phenol/sulphuric acid method (Dubois *et al.*, 1956), individual hexoses by gas-liquid chromatography of their alditol acetates (Lehnhardt & Winzler, 1968), uronic acids by a carbazole procedure (Davidson, 1966), and free amino groups by the trinitrobenzenesulphonic acid procedure (Habeeb, 1966); total phosphate was analysed by the ashing procedure outlined by Ames (1966).

RESULTS

Effects of galactosaminoglycan on membrane permeability

The effect of galactosaminoglycan on membrane permeability was monitored by incubating wild-type *N. crassa* conidia with radiolabelled metabolites in the presence and absence of galactosaminoglycan. Several low molecular weight metabolites were tested: the neutral amino acid L-phenylalanine, the basic amino acid L-arginine, the sugar 3-*O*-methyl-D-glucose, and the pyrimidine uridine. The effect of galactosaminoglycan on the accumulation and retention of phenylalanine and arginine is characteristic of the effects seen with each of the metabolites tested (Fig. 1). No accumulation of either phenylalanine or arginine occurred in the presence of galactosaminoglycan. Furthermore, when conidia were allowed to transport amino acid before the addition of galactosaminoglycan to the incubation medium, efflux of accumulated amino acid occurred upon addition of the polymer. Efflux was rapid, with maximum loss occurring within 4 min following the addition of galactosaminoglycan (data not shown). Efflux was minimal at polymer concentrations below 2 µg per ml of incubation medium (Fig. 2).

A proportion of the accumulated amino acid was not subject to efflux and was retained by conidia (Fig. 1). The retained amino acids were present in macromolecular form, suggesting that amino acid which has been incorporated into protein is not subject to efflux upon treatment with galactosaminoglycan. When incorporation into protein was prevented by inclusion of the

Fig. 1

Fig. 2

Fig. 1. Effect of galactosaminoglycan on the accumulation and retention of L-phenylalanine and L-arginine by wild-type *N. crassa*. Conidia ($0.1 \text{ mg dry wt ml}^{-1}$) were incubated at 25°C in $0.1 \times$ Vogel medium N containing radiolabelled L-phenylalanine (a) or L-arginine (b) ($0.01 \text{ } \mu\text{Ci per } 0.1 \text{ } \mu\text{mol per ml}$) in the absence of galactosaminoglycan (●), in the presence of galactosaminoglycan ($20 \text{ } \mu\text{g ml}^{-1}$) added at the time the assay was initiated (■), or in the presence of galactosaminoglycan added 60 min after assay initiation (○). Samples (0.5 mg dry wt) were removed at 20 min intervals, washed with three volumes of ice-water, dried, and counted in a gas-flow proportional counter.

Fig. 2. Effect of galactosaminoglycan concentration on the ability of wild-type *N. crassa* to retain L-phenylalanine. Conidia were incubated and assayed as described for Fig. 1. Various concentrations of galactosaminoglycan were added 60 min after assay initiation: no addition (●), $0.5 \text{ } \mu\text{g}$ galactosaminoglycan per ml of incubation medium (○), $1 \text{ } \mu\text{g ml}^{-1}$ (▲), $2 \text{ } \mu\text{g ml}^{-1}$ (△), and 3, 4, 5, 10, 15 or $20 \text{ } \mu\text{g ml}^{-1}$ (■).

protein synthesis inhibitor cycloheximide in the incubation medium, complete efflux of accumulated phenylalanine or arginine was observed (data not shown).

Effect of cations on galactosaminoglycan activity

The simultaneous presence of one of several salts in the incubation medium eliminated both the ability of galactosaminoglycan to prevent the accumulation of radiolabelled low molecular weight metabolites and the ability to cause efflux of previously accumulated metabolites (Fig. 3). The simultaneous presence of 1.2 M-KCl , a representative of the various salts tested, allowed accumulation and prevented efflux of each of the metabolites previously described, albeit at reduced rates. The ability of KCl to prevent galactosaminoglycan-induced efflux was directly related to the duration of exposure of conidia to the polymer prior to the addition of the salt (Fig. 4). The concentration of KCl used in these experiments was that which allowed maximal accumulation of the test metabolite during a standard 3 h assay in the presence of $20 \text{ } \mu\text{g}$ galactosaminoglycan per ml of incubation medium. Maximum accumulation ranged from 90 to 98% of the level attained in the presence of the salt alone.

Several agents were tested for their ability to prevent efflux of radiolabelled amino acid. In each case a range of concentrations ($0\text{--}3.0 \text{ M}$) was tested. When the salts KCl, LiCl, MgCl_2 ,

Fig. 3

Fig. 4

Fig. 3. Ability of KCl to interfere with the effects of galactosaminoglycan on amino acid accumulation and retention by wild-type *N. crassa*. Conidia were incubated and assayed as described for Fig. 1. (a) Effect of KCl on the ability of galactosaminoglycan-treated conidia to accumulate L-phenylalanine. The following additions were made to the incubation medium before the initiation of the transport assay: none (control) (■), 1.2 M-KCl (●), 20 µg galactosaminoglycan ml⁻¹ + 1.2 M-KCl (□), or 20 µg galactosaminoglycan ml⁻¹ (○). (b) Effect of KCl on the ability of galactosaminoglycan-treated conidia to retain L-arginine. The following additions were made 60 min after the transport assay was initiated: none (control) (■), 1.2 M-KCl (●), 20 µg galactosaminoglycan ml⁻¹ + 1.2 M-KCl (□), or 20 µg galactosaminoglycan ml⁻¹ (○).

Fig. 4. Ability of KCl to prevent efflux of accumulated L-phenylalanine following exposure of wild-type conidia to galactosaminoglycan. Conidia were incubated and assayed as described for Fig. 1. Galactosaminoglycan (20 µg ml⁻¹) was added 60 min after the transport assay was initiated. KCl (1.2 M) was added simultaneously with galactosaminoglycan (□), or 0.5 (▲), 1 (△), 2 (▼), or 3 (●) min after the addition of galactosaminoglycan. ■, Control (no additions); ○, no KCl added.

MgSO₄ or NaCl were added to a conidial suspension that had been exposed to galactosaminoglycan for 30 s, further efflux was halted and accumulation resumed (data not shown). The maximal effective concentration was 1.0–1.2 M for all salts except NaCl, which was maximally effective at 1.4 M. A combination of half the maximal concentration of KCl plus half the maximal concentration of NaCl prevented efflux as effectively as did the maximal concentration of either salt. The importance of the ionic nature of the agent was demonstrated by the failure of the non-ionic osmoticum sorbitol to prevent efflux, even at a concentration of 3.5 M. Furthermore, 0.4 M-K₂SO₄ was as effective as 1.0 M-KCl or 1.0 M-MgSO₄, suggesting that the cationic, rather than the anionic, moiety of the salt molecule was the more important in preventing galactosaminoglycan-induced cellular efflux.

Effects of other polycations on membrane permeability

The polysaccharide chitosan and the amino acid polymer poly-L-lysine (mol. wt 20000) were tested for their ability to prevent the accumulation of radiolabelled amino acids by *N. crassa*

conidia. Both polymers gave results similar to those in Fig. 1. Additionally, efflux of accumulated arginine was tested by using poly-L-lysine with results similar to those obtained with galactosaminoglycan (Fig. 3*b*). The simultaneous presence of 1.0 M-KCl in the incubation medium prevented each of the membrane permeability effects observed with these polycations.

Adsorption of galactosaminoglycan to the conidial surface

The ability of galactosaminoglycan to bind to the surface of *N. crassa* conidia was tested by incubating conidia (0.1 mg dry wt ml⁻¹) with radiolabelled galactosaminoglycan and measuring the label associated with the conidia as a function of duration of exposure to the polymer. Samples were removed at frequent time intervals. Maximal binding was observed by 0.5 min, and no additional binding was observed after 1.5 h incubation. A conidial density of 0.1 mg (dry wt) bound 0.8 µg galactosaminoglycan. Increasing the concentration of radiolabelled galactosaminoglycan fourfold did not increase the amount of polymer bound within a 5 min period. The simultaneous addition of 1 M-KCl and radiolabelled galactosaminoglycan reduced binding of the polymer to less than 10% of that seen with conidia incubated with galactosaminoglycan alone. The addition of 1 M-KCl to conidia that had been allowed to bind the polymer for 30 s resulted in the rapid release of the polymer from the conidial surface. KCl was as effective in releasing galactosaminoglycan that had bound to the conidial surface for 3 min as it was in releasing polymer that had bound for only 0.5 min.

The binding specificity of galactosaminoglycan was investigated using lanthanum, a calcium antagonist (Takata *et al.*, 1966; Weiss, 1974). The simultaneous presence of 5×10^{-4} M-lanthanum nitrate eliminated the effects of galactosaminoglycan on amino acid accumulation and efflux without itself affecting membrane permeability. A concentration of 2×10^{-3} M-lanthanum nitrate completely prevented the binding of galactosaminoglycan to the conidial surface.

Effect of galactosaminoglycan on viability

When wild-type conidia were treated with galactosaminoglycan before plating onto colony-producing medium, viability was inversely related to the duration of exposure to the polymer (Fig. 5). When galactosaminoglycan-treated conidia were subsequently treated with KCl, the ability of the salt to prevent loss of conidial viability was also inversely related to the duration of exposure to the polymer (Fig. 5).

Development of a rapid assay for the detection of membrane-active agents

The positively charged amino acid L-arginine is not readily lost from *N. crassa* conidia following accumulation. The results presented here with galactosaminoglycan, however, showed that arginine could be rapidly lost from cells treated with an agent that alters membrane permeability. These data suggested a ready assay for the detection of biological compounds that alter membrane permeability. The ability of a test substance or crude extracts to alter permeability was assayed by incubating the substance or extract with conidia that had been preloaded with radiolabelled arginine. After a 60 min incubation, the conidia were filtered, washed, and assayed for radiolabel. A typical set of assay results is shown in Table 1. The assay was very effective in monitoring the presence of galactosaminoglycan in various fractions during purification of the polymer and also served as a preliminary assay for the identification of active fractions of dialysed culture media from human cystic fibrosis fibroblasts (unpublished results). The efflux effects observed with both galactosaminoglycan and cystic fibrosis medium were reversed by the addition of KCl in a manner similar to the results shown in Fig. 3.

Chemical characterization of galactosaminoglycan

The ethanol/trichloroacetic acid treatment of culture filtrates from the colonial temperature-sensitive strain *cot-5* grown in the presence of D-[¹⁴C]glucosamine yielded radiolabelled galactosaminoglycan polymer for chemical analysis. Purity of the polymer was determined by isoelectric focusing. A single peak, containing both radiolabel and biological activity, focused at a pI of 8.4. The purified polymer was composed of 97.2% D-galactosamine (as anhydrous D-

Fig. 5

Fig. 6

Fig. 5. Ability of KCl to prevent loss of viability of conidia treated with galactosaminoglycan. Conidia were incubated as described for Fig. 1. Galactosaminoglycan ($20 \mu\text{g ml}^{-1}$) was added for 0, 0.5, 1, 2, 3 or 4 min. The conidia were then filtered, washed three times with sterile ice-water, plated, and scored for viability after 4 d at 25°C (●). Where the effect of KCl was monitored, 1.2 M-KCl was added after each galactosaminoglycan treatment (○); the conidia were incubated an additional 15 min and then filtered, washed, plated, and scored for viability. Viability is expressed as a percentage of the total colonies recovered when cells were not exposed to galactosaminoglycan.

Fig. 6. Gel permeation chromatography of deaminative cleavage products of galactosaminoglycan. [^{14}C]Galactosaminoglycan (1 mg ; 64000 c.p.m.) was dissolved in $1 \text{ ml } 0.28 \text{ M-acetic acid}$ containing 2.9 M-NaNO_2 followed by the addition of $0.01 \text{ ml } 6 \text{ M-acetic acid}$. After 20 min incubation at 20°C , a molar excess of ammonium sulphamate was added to terminate the reaction. The entire sample was applied to a Bio Gel P-4 column ($1.6 \times 85 \text{ cm}$). The column was eluted with water, 2.7 ml fractions were collected, and 1 ml samples were counted for radioactivity. The trisaccharide maltotriose was used as a standard and eluted at the position of the arrow. The void and total volumes of the column were 54 and 165 ml, respectively.

Table 1. *Effect of galactosaminoglycan on the ability of wild-type conidia to retain L-arginine*

Conidia (5 mg dry wt) were incubated for 3 h at 25°C at a final concentration of $0.1 \text{ mg dry wt ml}^{-1}$ in Vogel medium N containing 1% D-glucose. Radiolabelled L-arginine ($0.01 \mu\text{Ci per } 0.1 \mu\text{mol per ml}$) and cycloheximide ($10 \mu\text{g ml}^{-1}$) were added, and the conidia were allowed to accumulate arginine for 60 min. Samples (2 ml) were filtered onto nitrocellulose, washed, and resuspended in $0.1 \times$ Vogel medium N containing no addition, galactosaminoglycan ($20 \mu\text{g ml}^{-1}$), 1 M-KCl , or galactosaminoglycan plus KCl. After 60 min incubation at 25°C in a reciprocal-shake water bath, the conidia were filtered, washed, dried, and counted using a gas-flow proportional counter.

Addition	L-Arginine retention (c.p.m.)
None	6030
Galactosaminoglycan	200
1 M-KCl	6190
Galactosaminoglycan + 1 M-KCl	5980

galactosamine. HCl), 0.7% amino acid, and less than 0.1% neutral sugar. No D-glucosamine, uronic acid or phosphate was detected. The D-galactosamine residues were 70% N-substituted, presumably by N-acetylation. After nitrous acid degradation, which cleaved the polymer at D-galactosamine residues bearing free amino groups, the major deamination product detected was of trisaccharide size (Fig. 6). These results suggest that N-unsubstituted D-galactosamine residues are regularly distributed throughout the polymer and that the polymer is probably comprised of small repeating units. When chromatographed on carboxymethylcellulose

equilibrated with 0.1 M-Tris/acetate buffer pH 7.3 and developed with a linear NaCl gradient (0–2 M), the polymer eluted as a single peak at an ionic strength of 0.82 M.

Gel filtration and polyacrylamide gel electrophoresis of the undegraded polysaccharide suggested the molecular weight was polydisperse within a range of 70 000–120 000 (data not shown).

DISCUSSION

Galactosaminoglycan, a polymer of D-galactosamine, was isolated from culture filtrates of a colonial temperature-sensitive strain of *N. crassa*, *cot-5*, grown at the permissive temperature, which allows normal hyphal morphology. Galactosaminoglycan has been isolated by Reissig and coworkers (Reissig & Glasgow, 1971; Glasgow & Reissig, 1974) from another colonial temperature-sensitive strain of *N. crassa*, *cot-1*, grown at the non-permissive temperature, which promotes hyphal branching, leading to colonial morphology. The single major difference between the two polymers appears to be the extent to which the D-galactosamine residues are *N*-substituted, presumably by acetylation. Galactosaminoglycan purified from *cot-5* cultures grown at the permissive temperature contained 70% of the residues *N*-substituted whereas only 25% of the D-galactosamine residues were *N*-substituted in the polymer produced by *cot-1* cultures grown at the non-permissive temperature. The difference in *N*-substitution may reflect simply a strain difference or the fact that the differing growth conditions lead to differing *N*-acetylation levels of the polymer which in turn lead to alterations in hyphal morphology. Springer & Srb (1978) have correlated such alterations with the *N*-acetyl content of galactosaminoglycan.

Despite the difference in the extent of *N*-substitution, the two polymers are very similar in their effects on membrane permeability and viability. In the present study, treatment of *N. crassa* conidia with galactosaminoglycan led to loss of viability and to an increase in membrane permeability as measured by the rapid efflux of low molecular weight metabolites. The loss of intracellular small molecules, and presumably ions as well, is believed to underlie the decline in viability following galactosaminoglycan treatment. Adsorption to the conidial surface was essential to the polymer's ability to affect membrane permeability and viability. Binding sites for galactosaminoglycan on the cell surface were rapidly saturated in the presence of lethal concentrations of galactosaminoglycan. The addition of any one of several ionic agents interfered with adsorption and concomitantly interfered with the deleterious effects of the polymer on both permeability and viability. Treatment of cells with the calcium antagonist lanthanum also prevented galactosaminoglycan from binding and from exerting its effects on membrane permeability; lanthanum achieved this at a much lower concentration than the other salts, suggesting that the binding sites for galactosaminoglycan on the cell surface are those normally occupied by calcium.

It has been proposed that the bulk of the ionic interaction between galactosaminoglycan and the cell surface is non-specific and that lethality is initiated by the adsorption of galactosaminoglycan to specific lethal receptors (Glasgow & Reissig, 1974). In the present study, galactosaminoglycan had little effect on conidial viability or membrane permeability at subthreshold concentrations. These results suggest that a specific quantity of the polymer must bind to the cell surface in order to exert its effects. In addition, both poly-L-lysine and chitosan were observed to have conidial membrane permeability effects which mimicked those of galactosaminoglycan. The results indicate that the inability of conidial membranes to retain small molecules may be a general response to the presence of polycations in media of low ionic strength. Yeast cells have also been shown to lose low molecular weight cytoplasmic constituents in the presence of polycations (Yphantis *et al.*, 1967; Jaspers *et al.*, 1975). The present results favour a mechanism of action based upon the cumulative binding of polycations to the cell surface.

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