Interaction of Polygalactosamine with Conidia of *Neurospora crassa*

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Neurospora crassa produces a phase-specific cationic mucopolysaccharide composed primarily of galactosamine (galactosaminoglycan mucopolysaccharide; GAG-MP), which becomes part of the cell wall and is later secreted into the medium. Its appearance coincides with the onset of the restricted phase of growth, and causes efflux of small molecular weight metabolites when incubated with conidial cells. This activity may be a product of electrostatic interactions of the GAG-MP with the conidial plasma membrane. The activity is blocked by acetylation of the primary amines on the molecule, digestion by enzymes that hydrolyse carbohydrate linkages and the inclusion of NaCl (1 M) in the GAG-MP/conidia reaction mixture. The physiological activity of the GAG-MP mimics that of known depolarizing agents. A model is proposed in which GAG-MP depolarizes the plasma membrane at the onset of the restricted phase of growth, stimulating a cell surface enzyme to produce endogenous cyclic AMP which in turn switches on the enzymic and genetic machinery necessary for that phase of growth.

INTRODUCTION

The concept of genetic macroregulation of growth during the life cycle of *Neurospora crassa* has been proposed by Reissig & Glasgow (1971) and a mucopolysaccharide has been implicated in serving as a signal for restriction of growth. The mucopolysaccharide was identified as a partially acetylated cationic homopolymer of galactosamine (Reissig & Glasgow, 1971; Schmit *et al.*, 1975) termed galactosaminoglycan mucopolysaccharide (GAG-MP), and also as a component of the outer cell wall (Harold, 1962). It was produced and excreted into the culture medium during late-exponential phase (Reissig & Glasgow, 1971; Schmit *et al.*, 1975). Its presence coincided with the transition of the cellular metabolic emphasis from active growth to a resting or stationary phase. This transition may have represented the expression of genetic loci involved in stationary phase metabolism and the repression of genetic loci associated exclusively with active growth.

Reissig & Glasgow (1971) estimated the molecular weight of GAG-MP to be approximately 10^6 , and hence its interaction with the *N. crassa* cell would seem to be at the cell surface. Its ability to cause vacuolation of cellular cytoplasm presumably reflected the ability of GAG-MP to cause membrane permeability changes.

Changes in membrane permeability are frequently accompanied by a net depolarization of the plasma membrane, and Trevillyan & Pall (1979) have recently correlated depolarization of the plasma membrane in *N. crassa* with the rapid intracellular production of cyclic AMP. Cyclic AMP has been known to exert a pleiotropic effect at several metabolic levels and has been shown to regulate the synthesis of a number of enzymes subject to control by catabolite repression (Trevillyan & Pall, 1979). In addition, it plays a major role in the activation of enzymes, e.g. protein kinases, by phosphorylation activities. In yeast, adenylate cyclase, a membrane-bound enzyme which converts ATP to cyclic AMP, was found to be three to four times more active in cells entering the late-exponential or stationary phase than cells in the early-exponential phase (Scott & Tatum, 1970).

The data presented in this study suggest that GAG-MP interacts with cells at the level of the

plasma membrane. This interaction probably has a regulatory role, by altering the permeability properties of that membrane and, subsequently, may result in an elevation of intracellular cyclic AMP concentrations.

METHODS

Strains and growth conditions. *Cot*, a colonial, temperature-sensitive mutant of *Neurospora crassa* (FGSC 1362), was obtained from the Fungal Genetics Stock Center, Arcata, Calif. U.S.A. The mucopolysaccharide to be isolated was found in the culture medium during the restricted and stationary phase of growth of the organism (Mahadevan & Tatum, 1965). Conidia from 5–7 d old *cot* mutant cultures were inoculated by dusting conidia on to the surface of 500 ml sterile Vogel's minimal medium N plus 2% (w/v) sucrose (Vogel, 1964) in 1 litre foil-covered Erlenmeyer flasks. The flasks were shaken at 35 °C for 5 d and the mycelium-free medium was collected by filtration.

The wild-type strain (Tatum A) was from stocks maintained at Old Dominion University and was used in the preparation of the reagent cells employed in the efflux bioassay. Stocks of the *cot* mutant and the wild-type cultures were maintained on solid minimal medium N plus 2% sucrose.

Chemicals. L-[U-¹⁴C]Arginine, 300 mCi mmol⁻¹ (11.1 GBq mmol⁻¹) was purchased from Amersham and *N*-acetylglucosaminidase, β -glucuronidase (type H-1 from *Helix pomatia*), galactosamine. HCl, polylysine, gramicidin D, and valinomycin were purchased from Sigma. All chemicals used were of the highest purity available.

Mucopolysaccharide isolation and purification. Mucopolysaccharide was isolated from the medium of 5 d old liquid cultures of the *cot* mutant. The medium was filtered through Whatman no. 3 paper; subsequent purification of the mucopolysaccharide was performed essentially as described by Reissig & Glasgow (1971), with minor modifications. The purification procedure consisted of an initial precipitation of the mucopolysaccharide from the medium (cooled to 0 °C) with 2 vol. cold (–20 °C) 95% (v/v) ethanol. The precipitate was collected by centrifugation (13000 *g* for 40 min at 4 °C) and then dissolved, while cold, in 40 ml 1 M-NaCl for each 1 l of medium precipitated.

Deproteination was done by adding an equal volume of chloroform/isoamyl alcohol (24:1, v/v). This mixture was shaken vigorously for 15 min and centrifuged in a swinging-bucket clinical centrifuge (IEC) at 2000 to 3000 rev. min⁻¹ for 30 min, or until the aqueous phase was clear of chloroform and most of the denatured proteins. The aqueous phase was removed and the deproteination steps were repeated until protein was no longer visible at the interface. The aqueous phase was then precipitated with 2 vol. cold 95% ethanol, centrifuged and the precipitate dissolved in 1.0 M-NaCl (usually 50% to 75% of the original volume).

Contaminating RNA was digested by ribonuclease (50 μ g ml⁻¹) in 0.15 M-NaCl pH 5.0 for 30 min at 37 °C. The deproteination steps were then repeated to remove the RNAase and other remaining proteins. The aqueous phase was again precipitated with 2 vol. cold (–20 °C) 95% ethanol, centrifuged, and the precipitate redissolved in 9.0 ml dilute (0.01 M) NaCl. When solubilization was complete, 1.0 ml acetate/EDTA (3.0 M-sodium acetate in 0.001 M-EDTA, pH 7.0) and 1.3 vol. propan-2-ol were added while stirring rapidly. The supernatant fraction obtained was lyophilized and frozen until needed.

Determination of the chemical and physical properties of the mucopolysaccharide

Thin-layer chromatography. The mucopolysaccharide was hydrolysed in 6 M, constant-boiling HCl. (Pierce Chemical Co.) under N₂ at 100 °C for 18 h. The hydrolysis products were dried under a constant flow of dry N₂. To test for the identity of liberated amino sugars, cellulose TLC sheets (Eastman) were treated with 0.1 M BaCl₂, dried, and then spotted with the acid hydrolysis products. The sheets were developed in butanol-1-ol/pyridine/water (6:4:3, by vol.), dried and sprayed with ninhydrin (Yphantix *et al.*, 1967). Neutral sugars were assayed by spotting the acid hydrolysis products on to cellulose TLC sheets followed by development in butan-1-ol/acetic acid/water (12:3:5, by vol.). Silver reagent was used to detect the presence and location of reducing sugars (Wheat, 1966).

Colorimetric assay. The mucopolysaccharide was also analysed for the presence of galactosamine and glucosamine, by Wagner's colorimetric assay (Wagner, 1979).

Ion-exchange chromatography. The charge of the mucopolysaccharide molecule was determined by ion-exchange chromatography using Dowex-50W resin (Mesh size 20–50) in a column (0.5 × 10 cm) equilibrated with 0.4 M-HCl. The mucopolysaccharide sample (5 mg) was applied to the column and non-cationic molecules were eluted with 0.4 M-HCl. Cationic molecules were eluted from the resin by washing with 0.5 M-NH₄OH. Samples of each fraction were tested for the presence of mucopolysaccharide by Wagner's colorimetric assay (Wagner, 1979) and for activity in causing L-arginine efflux from reagent cells as described below.

Enzymic digestion of purified GAG-MP. The GAG-MP was digested individually by two enzymes as follows. (1) One unit (0.105 mg in 1 ml glass-distilled water) of *N*-acetylglucosaminidase was added to 1 ml GAG-MP solution (200 μ g ml⁻¹, pH 4.0). The solution was incubated for 1 h at 37 °C. (2) Two units (5 mg in 1 ml glass-distilled

water) of β -glucuronidase were added to 1 ml solution ($200 \mu\text{g ml}^{-1}$, pH 5.0). The solution was incubated for 1 h at 37°C . After the incubation period, 10% TCA (final concentration) was added to precipitate the protein (enzymes). GAG-MP was soluble in 10% TCA. The mixtures were then centrifuged to pellet the precipitated proteins and the supernatant fraction dialysed exhaustively against glass-distilled water, lyophilized, and resuspended in 1 ml water. Each sample of digested GAG-MP was then tested for efflux activity as described below.

Gel permeation chromatography. The size of the mucopolysaccharide was determined on a Sephadex G-100 gel filtration column ($1.5 \times 60 \text{ cm}$), equilibrated with 0.01 M-Tris/HCl buffer (pH 8.0) and pumped with a Pharmacia P-3 pump to achieve a flow rate of 0.4 ml min^{-1} . The column was calibrated with Blue Dextran (mol. wt 2×10^6) to determine the void volume and phosphorylase (94000), bovine serum albumin (67000), RNAase (13700), tryptophan (204) and galactosamine.HCl (215) as molecular weight markers, the latter two indicating the inclusion volume of the column. A 5 mg sample of mucopolysaccharide in 1 ml buffer was applied to the column and followed by a continuous flow of buffer. Eluates from the column were monitored with an ISCO UV/visible monitor/recorder at 254 nm and 5 ml fractions were collected. The fractions were lyophilized, redissolved in 2 ml glass-distilled water and dialysed exhaustively against glass-distilled water in Spectrapore III dialysis tubing to rid the sample of small molecules (mol wt <3000). Samples of each fraction were tested for the presence of hexosamines (Wagner, 1979) and for activity in causing L-arginine efflux as described below.

Acetylation of the mucopolysaccharide. Acetic anhydride was used in the *N*-acetylation procedure described by Wheat (1966), where aqueous solubilized mucopolysaccharide ($200 \mu\text{g}$ per 4 ml H_2O) was treated with 0.4 ml saturated NaHCO_3 and 0.4 ml 2% acetic anhydride (in acetone) at 25°C for 2–5 min. After deionization by treatment with Dowex 50 H^+ form resin to remove sodium ions and non-acetylated GAG-MP, the mucopolysaccharide was precipitated with 3 vol. 95% ethanol, and stored at 4 – 10°C overnight. The precipitate was resuspended in glass-distilled water, dialysed exhaustively against glass-distilled water in Spectrapore III dialysis tubing and tested for L-arginine efflux activity as described below. The completeness of acetylation was measured by Wagner's assay (Wagner, 1979) or by testing for ninhydrin-positive material.

Physiological effect of GAG-MP on N. crassa

Preparation of reagent cells. Reagent cells were prepared as follows. Conidia from 7 d old cultures of wild-type *N. crassa* were transferred aseptically to sterile glass-distilled water (0°C), shaken vigorously and stored on ice for at least 1 h. During this time mycelial fragments floated to the top and conidia settled to the bottom of the tube. Mycelial fragments were aspirated from the top leaving a homogeneous conidial suspension. The dry weight of conidial cells per ml of suspension was determined by filtering a sample of cells on to a tared glass-fibre filter, drying, and reweighing. A volume of conidial cell suspension sufficient to give $0.2 \text{ mg dry wt cells ml}^{-1}$ (final concentration), was added to a sterile solution of 1% glucose and $1 \times$ Vogel's salts (Vogel, 1964) (pH 5.8) in glass-distilled water. The mixture was incubated for 3 h at 37°C with shaking. Cycloheximide (0.01 mg ml^{-1} final concentration) and L-[^{14}C]arginine ($0.01 \mu\text{Ci ml}^{-1}$ ($0.1 \mu\text{mol ml}^{-1}$), final concentration) were then added and this mixture was incubated for a further hour at 37°C with shaking. The reaction was terminated by transferring the reagent cells to an ice bath and storing at 0°C until used (G. DeBusk, personal communication).

Efflux bioassay. To determine the relative amounts of L-[^{14}C]arginine remaining in the reagent cells after treatment with various test solutions (e.g. the GAG-MP), 5 ml reagent cell suspension was first filtered on to a nitrocellulose filter ($2 \mu\text{m}$ pore size) by vacuum suction, and washed with glass-distilled water. The filter and cells were transferred to a flask containing 5 ml test solution and the cells were resuspended. Samples (1 ml) were removed at 2, 4, 8, and 16 min. Each sample was filtered through a nitrocellulose filter and washed with glass-distilled water, then placed in a scintillation vial and dried in an oven (60°C) overnight. Toluene-based scintillation fluid (Omnimix, ICN) was added to the vials, which were then capped, and counted in a Beckman LS-250 scintillation counter for a minimum of 10 min or 1000 counts. A diminished amount of radioactive L-[^{14}C]arginine retained by the cells during the incubation period indicated an efflux of L-[^{14}C]arginine from the cells.

RESULTS

Isolation of purified GAG-MP

It has been shown that the maximal amount of biologically active material was produced by the *cot* mutant by the sixth day of growth in liquid shake cultures (J. Jensen, personal communication). The isolation procedure was designed to separate proteins and nucleic acids from carbohydrates that were secreted into the growth medium (Marmur, 1963) and therefore allowed for maximal purification of the GAG-MP (Table 1). The repeated deproteination treatment with chloroform/isoamyl alcohol both before and after the RNAase digestion allowed for the maximal removal of proteins. Ribonuclease and propan-2-ol treatments removed the remaining RNA and DNA, respectively, leaving medial polysaccharides in the remaining solvent. In the

Table 1. *Chemical analysis of purified GAG-MP from the culture medium of the cot mutant of Neurospora crassa*

See Methods for details of assay.

Compound	Amount detected (%)
Galactosamine	81.0
Unidentified material	15.6
Amino acids/proteins	3.4
Glucosamine	0.0
Neutral sugar	0.0

final supernatant, following lyophilization, the yield of purified GAG-MP averaged 5 mg of a dry powdery material per litre of medium.

Chemical and physical characterization of purified GAG-MP

Hexosamine determination. Using the galactosamine-glucosamine differential assay of Wagner (1979) and galactosamine.HCl as a standard, it was found that purified GAG-MP was composed of approximately 81% (w/w) galactosamine. This figure may be low due to the potential for the secondary and tertiary structure of the molecule to hinder reaction of all primary amine sites necessary for complete colorimetric detection.

Acid hydrolysis and TLC analysis of products. The TLC analyses of the acid hydrolysis products (see Methods) were sensitive to greater than 5 ng amino sugars and 10 ng neutral sugars. Neutral sugars and glucosamine could not be detected in the acid hydrolysis products of the purified GAG-MP, whereas galactosamine could be detected along with some other ninhydrin-positive material.

Efforts to quantify the galactosamine concentration in the acid hydrolysate yielded a value 45% lower than that present in the unhydrolysed sample of GAG-MP. Acid hydrolysis of a known concentration of monomeric galactosamine confirmed extensive degradation of galactosamine by this procedure, hence a correction factor had to be applied to the calculation of galactosamine concentration in the acid-hydrolysed GAG-MP. Glucosamine was similarly degraded by acid hydrolysis and thus any small amount present in GAG-MP might not be expected to be revealed on the thin-layer chromatograms.

Ion-exchange chromatography. If, as suggested (Reissig & Glasgow, 1971), the purified GAG-MP is a polymer of amino sugars, it should behave as a polycationic molecule. Therefore, a sample of purified GAG-MP was dissolved in glass-distilled water, applied to a cation-exchange resin (Dowex-50W) column and eluted with an acidic solution. The resin was then stripped by the addition of a basic solution. The initial eluate from the ion exchange was totally devoid of both hexosamines and efflux activity, whereas the NH_4OH eluate contained both hexosamines and efflux activity. This observation would be expected if purified GAG-MP were a polymeric hexosamine.

Enzymic digestion of purified GAG-MP. Although the active component isolated by the procedure described has already been shown to be a partially acetylated cationic homopolymer of galactosamine (Reissig & Glasgow, 1971), we decided to confirm the identity of the membrane-active component in our preparation. Enzymes that hydrolyse carbohydrate linkages, *N*-acetylglucosaminidase, and β -glucuronidase, completely destroyed the biological activity of the purified GAG-MP (data not shown). These findings suggest that the efflux activity of the GAG-MP is due primarily to the carbohydrate components of the molecule.

Molecular sieving studies with GAG-MP. Separation of GAG-MP by Sephadex G-100 column chromatography showed a heterogeneous size distribution of polygalactosamine polymers (Fig. 1). Analysis of samples of each fraction revealed only two sizes of polygalactosamine polymers capable of causing efflux of L-arginine from reagent cells (Fig. 1). The two molecular weights of

Fig. 1. Analysis of size distribution of polygalactosamine of purified GAG-MP from the culture medium of the *cot* mutant of *Neurospora crassa* on a Sephadex G-100 column, and efflux activity after 16 min incubation versus size. ○, Concentration of GAG-MP; ●, percentage efflux activity. The samples in each fraction were first dialysed against glass-distilled water before being tested for efflux activity. Blue Dextran (mol. wt 2×10^6) was used to measure the V_0 (void volume). Tryptophan (mol. wt 215), indicated as A, and galactosamine.HCl (mol. wt 204), indicated as B, were used to measure the V_e (inclusion volume).

active polymers were approximately 85000 and 4800, as determined by comparative column runs with known molecular weight markers.

Sephadex column chromatography yielded molecular weight estimates that may be considered only as rough values for the fractions analysed. Tryptophan (mol. wt 204) and galactosamine.HCl (215) have similar molecular weights, yet galactosamine.HCl eluted five fractions later than tryptophan (Fig. 1). It is suggested therefore that the polygalactosamine polymers might be retained on the column longer than indicated by their molecular weights (i.e. the estimates of molecular weight for the polygalactosamines may be low). It should be noted that GAG-MP appeared to have a deleterious effect on Sephadex and repeated use of the same column in separation of GAG-MP resulted in loss of separation capacity. The interaction(s) of GAG-MP with Sephadex were not determined, but caution in re-use of such column materials is suggested.

Despite such problems, there are clearly two size ranges of polygalactosamine (GAG-MP) that effect the membrane permeability of *N. crassa* conidia. The lower and intermediate molecular weight material, containing higher total concentrations of galactosamine, appeared to have only minimal effect on membrane permeability of the reagent cells (Fig. 1).

Physiological activity of GAG-MP on reagent cells

Effects of increasing concentrations of GAG-MP on efflux activity. The reagent cells used in the efflux bioassay were tested for their stability and were found to be stable for up to 14 d when maintained at 0°C. In addition, the radiolabelled arginine preloaded in the reagent cells represented more than 95% of the total extractable radiolabel.

The effects of increased concentrations of GAG-MP on the reagent cells were tested by monitoring the amount of L-[^{14}C]arginine left in the cells after 2, 4, 8 and 16 min of incubation (Fig. 2). Incubation of reagent cells with increasing concentrations of GAG-MP, from $1 \mu\text{g ml}^{-1}$ to $20 \mu\text{g ml}^{-1}$, caused an increasingly rapid loss of radiolabel from the cells. The efflux of radiolabel appeared to consist of two steps, a rapid initial loss of radiolabel, followed by a second slower loss of radiolabel. However, plotting the data on semilog paper revealed that the rate approaches an exponential curve, suggesting that the reaction is of the first order.

Plotting the initial rate of efflux against the concentration of GAG-MP reveals that the rate of efflux apparently becomes independent of GAG-MP concentration at concentrations greater

Fig. 2

Fig. 2. Effects of increasing concentrations of GAG-MP on efflux of L-[^{14}C]arginine from reagent cells of *Neurospora crassa*. GAG-MP concentrations ($\mu\text{g ml}^{-1}$) were: 0 (H_2O control; $\circ$), 1 ($\square$), 2 ($\triangle$), 5 ($\blacktriangle$), 10 ($\blacksquare$), 20 ($\bullet$).

Fig. 3

Fig. 3. Initial rate of L-[^{14}C]arginine efflux from reagent cells of *Neurospora crassa* with increasing concentrations of GAG-MP. The rate represents the slope of the line between the data points at time 0 and 2 min in Fig. 2.

than $20 \mu\text{g ml}^{-1}$ (Fig. 3). A double reciprocal plot of Fig. 3 gave two values: (1) a maximum attainable rate of efflux at infinitely high concentrations of purified GAG-MP [$670 \text{ ng L-}^{14}\text{C}\text{]arginine (mg conidial cells)}^{-1} \text{ min}^{-1}$] (this value was based on the calculation of the amount of L-arginine accumulated and did not take into account the dilution of the specific activity of radiolabel by endogenously synthesized L-arginine); and (2) a concentration of GAG-MP that provided for an efflux rate one-half of the maximum rate attainable ($2.86 \mu\text{g ml}^{-1}$). Based on the number of cells in the assay ($3\text{--}4 \times 10^6$ cells) and the estimated molecular weights of the purified, biologically active GAG-MP polymers (4800 and 85000), an estimate of the number of molecules of GAG-MP required to achieve an efflux rate one-half the maximum attainable rate per germinated conidium can be generated. To achieve one-half the maximum attainable efflux rate would require 1.0×10^8 molecules of 4800 molecular weight per conidial cell, or 5.7×10^6 molecules of 85000 molecular weight per conidial cell. Further interpretation of these values is not possible, since it is unknown whether both sizes are interacting with the cells by equivalent mechanisms, or if both sizes are binding to one or more cell types, i.e. germinated microconidia or macroconidia. It is also unknown whether every molecule in solution binds to the cells or whether some equilibrium is attained between bound and free GAG-MP.

Effect of salts on the biological activity of GAG-MP. Reissig (1974) and J. Jensen & G. DeBusk (personal communication) have provided substantial evidence that interaction of GAG-MP with conidia occurs via electrostatic binding of GAG-MP molecules to anionic components at the cell surface. Indeed, the action of purified GAG-MP can be blocked with increasing concentrations of NaCl (Fig. 4). For example, the addition of NaCl (1 M) to a suspension of reagent cells completely blocked the activity of GAG-MP at a concentration ($20 \mu\text{g ml}^{-1}$) sufficient to cause maximum efflux of L-arginine from these cells; however, the sensitivity of the GAG-MP/conidia reaction to increasing concentrations of NaCl was biphasic. In a concurrent study, J. Jensen & G. DeBusk (personal communication) observed a similar situation and proposed that there may be high- and low-affinity receptors for GAG-MP on the conidial surface. They proposed that the low-affinity receptors may release bound GAG-MP readily in the presence of low concentrations of salt, whereas the high-affinity receptors may require a greater concentration to release the bound GAG-MP. In light of our finding that two sizes of active GAG-MP polymers may exist, an alternative model is plausible. Both 4800 dalton and 85000 dalton polymers could bind to cells by ionic interactions, and the binding constants may

Fig. 4. Percentage efflux of radiolabelled L-arginine from reagent cells of *Neurospora crassa* after a 16 min incubation period with GAG-MP ($10 \mu\text{g ml}^{-1}$) and increasing concentrations of NaCl. Reagent cells resuspended in glass-distilled water only were used as a control to assess radioactivity remaining in cells where no efflux (0% efflux) occurred.

Table 2. *Effect of various compounds on the efflux of L-[^{14}C]arginine from reagent cells of *Neurospora crassa**

The percentage efflux of radiolabelled arginine from reagent cells was calculated from the amount of radioactivity remaining in the cells after 16 min incubation at 37°C . Glass-distilled water was used as a control, i.e. no efflux.

Test solution	Concentration	Percentage of L-arginine effluxed
Water	—	0.0
GAG-MP	$20 \mu\text{g ml}^{-1}$	95.0
Acetylated GAG-MP	$100 \mu\text{g ml}^{-1}$	0.0
GAG-MP + 2.5 M-sucrose	$20 \mu\text{g ml}^{-1}$	95.0
GAG-MP + 1 M-NaCl	$20 \mu\text{g ml}^{-1}$	0.0
Polylysine	$50 \mu\text{g ml}^{-1}$	95.6
Polylysine	$20 \mu\text{g ml}^{-1}$	91.5
Polylysine	$10 \mu\text{g ml}^{-1}$	64.4
Polylysine + 0.1 M-NaCl	$20 \mu\text{g ml}^{-1}$	37.6
Polylysine + 1 M-NaCl	$20 \mu\text{g ml}^{-1}$	11.1
Acetylated polylysine	$100 \mu\text{g ml}^{-1}$	0.0
Sucrose	0.05–2.5 M	0.0
NaCl	0.05–2.5 M	0.0
Gramicidin D	1 mM	90.1
Valinomycin	1 mM	94.6
2,4-Dinitrophenol	1 mM	92.7

show differential sensitivities to NaCl concentrations as dictated by their size and number of ionic determinants, i.e. primary amines.

The loss of efflux activity of a $20 \mu\text{g ml}^{-1}$ concentration of GAG-MP in the presence of increasing salt concentrations was not the result of an osmotic stabilizing effect of the salt preventing cell lysis in the presence of GAG-MP. Concentrations of sucrose up to 2.5 M were tested with GAG-MP at a concentration of $20 \mu\text{g ml}^{-1}$. The result was a 95% efflux of intracellular L-[^{14}C]arginine (Table 2), the same as in the absence of sucrose. These results reaffirmed the conclusion that interaction of GAG-MP with the conidial surface is electrostatic in nature and can be blocked by a sufficiently high ionic strength (Table 2). As controls, sucrose (0.05–2.5 M) and NaCl (0.05–2.5 M) were used by themselves to test for their ability to cause efflux of radiolabel from reagent cells, and neither compound resulted in loss of radiolabelled L-arginine (Table 2). Thus, the efflux caused by GAG-MP was not due to an osmotic imbalance in the reagent cells.

Efflux activity of other compounds. Polylysine (mol. wt 15 000–30 000), a cationic homopolymer of lysine, may be considered a molecular analogue of GAG-MP. Both are large, positively

charged molecules capable of electrostatic interaction. At very low concentrations (20–50 $\mu\text{g ml}^{-1}$) polylysine exerted the same biological activity as GAG-MP on reagent cells, causing efflux of over 90% of intracellular L-[^{14}C]arginine within 16 min. High concentrations of NaCl (1 M) also blocked this effect when incubated with the conidial cells prior to the introduction of polylysine (Table 2). Acetylation of polylysine and GAG-MP by the procedure of Wheat (1966) prevented both molecules from causing efflux of intracellular L-[^{14}C]arginine (Table 2).

The mechanism by which the electrostatic interaction of GAG-MP with the membrane allows for the efflux of low molecular weight metabolites might very well be due to a net depolarization of the membrane resulting in the apparent permeability changes. To test this hypothesis, gramicidin D and valinomycin (ionophores) and 2,4-dinitrophenol (a proton conductor), all known for their depolarizing effect on *N. crassa* plasma membranes (Slayman, 1965; Trevillyan & Pall, 1979), were tested, each at 1 mM, for their effects on L-arginine efflux from conidial cells. In all three cases, over 90% of the intracellular L-[^{14}C]arginine was effluxed from the cells within 15 min of incubation (Table 2). Although these results did not conclusively show that GAG-MP was a depolarizing agent, it did indicate that, under the conditions of the efflux bioassay, the physiological activity of the GAG-MP mimicked that of known depolarizing agents.

DISCUSSION

The biochemical and physiological characteristics of the mucopolysaccharide found in the outer cell wall fraction of *N. crassa* (Harold, 1962; Mahadevan & Tatem, 1965; Reissig & Glasgow, 1971) during late-exponential phase, and later excreted into the media of growing cultures, have been of extreme interest in uncovering the biological role of this molecule. It was found that the galactosamine polymers isolated from the two sources (cell wall and medium) were structurally equivalent (J. Jensen & G. DeBusk, personal communication). They are highly positively charged molecules with a heterogeneous molecular size.

Reissig & Glasgow (1971) recovered 50% of the dry weight of their purified, acid-hydrolysed mucopolysaccharide as galactosamine. They stated that this shortage may be due to destruction and incomplete hydrolysis of the macromolecule. In their chemical determination of purified medial GAG-MP, J. Jensen & G. DeBusk (personal communication) detected 97.4% galactosamine, 0.71% amino acids, 0.068% neutral sugars, and 0.075% unknown ninhydrin-positive material. In this study, mucopolysaccharide (GAG-MP) was found to be primarily composed of galactosamine (81%) with some associated protein(s). The variations in chemical composition of the biologically active GAG-MP in these three studies may be due to differing isolation procedures or differing methods of chemical analysis.

The present study has shown that biological activity was dependent on the concentration and molecular size of the GAG-MP. GAG-MP molecules of two distinct sizes were identified that were capable, in small concentrations, of causing efflux of intracellular L-arginine. Molecules of intermediate and lower molecular weights showed no efflux activity, even in higher molar concentrations.

As yet, the mechanism by which GAG-MP interacts at the cell surface, or why particular molecular sizes cause permeability changes, is uncertain. In their work on determining pore size in the *Neurospora* cell wall, Trevithick *et al.* (1966) suggested that a molecule as large as mol. wt 4750 was capable of penetrating the cell wall through pores. Hence, the smaller GAG-MP molecule (mol. wt 4800) may be small enough to penetrate the cell wall and yet still be large enough to cause membrane leakiness. The larger GAG-MP molecule (mol. wt 85000) may be able to 'blanket' a portion of the cell surface, affecting numerous sites where the plasma membrane comes in contact with the external environment, and affect enough of the membrane to cause a permeability change. The lower and intermediate molecular weight molecules, incapable of causing efflux, may penetrate or blanket the cell wall, respectively, but may not be able to affect a large enough area of the membrane to cause the depolarization necessary for membrane leakiness.

Biological activity of the GAG-MP, manifested in its ability to cause efflux of radiolabelled

metabolites from conidial cells, may be blocked in three ways: by acetylation, by enzymic digestion, and by addition of NaCl to the efflux assay. Inactivation of GAG-MP by acetylation indicated that the primary amine portion of the molecule was actively involved in biological activity, and supports the hypothesis that GAG-MP interacts with the conidial surface by an electrostatic attraction similar to that of polylysine. Digestion by enzymes that hydrolyse carbohydrate linkages caused complete loss of activity. These results suggested a major involvement of the carbohydrate portion of the molecule (a galactosamine polymer) in biological activity. The biphasic appearance of the effects of increasing concentrations of NaCl on blocking the ability of GAG-MP to cause efflux of L-arginine, and the detection of two distinct sizes of active GAG-MP molecules, has led to the suggestion that there may be common binding sites on the cell surface. These sites may differentially bind the two sizes of active GAG-MP, each binding being differentially sensitive to NaCl. Considering the differing number of primary amines on each molecular species it might not seem unreasonable to propose that lower concentrations of salts might displace the smaller (mol. wt 4800) molecule, while much higher NaCl concentrations would be required to release the bound (mol. wt 85000) GAG-MP molecule. This study shows that GAG-MP reaches a saturation in binding at higher concentrations, and the drastic effects of high concentrations of the GAG-MP may be a result of cumulative binding. The large cationic polymers of GAG-MP may be binding to the anionic phospholipid portion of the membrane, distorting the membrane organization and allowing for leakage of small metabolites.

In previous studies, polycations were shown to be effective in causing ultrastructural changes (Quinton & Philpott, 1973) and membrane leakiness (Yphantis *et al.*, 1967). This study has shown that high concentrations of the cationic polymer of galactosamine (GAG-MP) caused permeability changes in the *Neurospora* cell surface and R. DeBusk (personal communication) concluded that this efflux was specific for molecules of small molecular weight.

Large transport fluxes of metabolizable compounds occur across the *N. crassa* membrane as a result of depolarization of the membrane (Pall, 1977). The actions of valinomycin and gramicidin D, known ionophores, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, have long been recognized as depolarizing agents (Trevillyan & Pall, 1979) and the data presented here indicated that the physiological activity of these compounds mimics the action of GAG-MP in causing membrane leakiness.

These concentrations of GAG-MP used in this study represented an approximate 1000-fold increase in concentration from normal physiological concentrations of GAG-MP in *N. crassa* cultures. Only 5 mg of purified polysaccharide was obtained from 1 l of *cot* media, and *cot* was an overproducer of the substance. However, by using these very high concentrations of GAG-MP, it has been possible to implicate an electrostatic interaction of GAG-MP at the cell surface. Under normal growth conditions, the mucopolysaccharide would not be present in sufficient quantities to be cytotoxic to the cell.

Pall (1977) has shown that, in *N. crassa*, membrane depolarization produces an increase in cyclic AMP levels by stimulating the membrane-bound adenylate cyclase. It is suggested that additional cyclic AMP may be produced in the cell in response to the GAG-MP/plasma membrane interaction. The elevated levels of cyclic AMP may activate genetic and enzymic machinery now necessary for this phase of development in the fungus. Marked and transient increases in cyclic AMP concentrations are found in wild-type *N. crassa* cells in restricted phase metabolism (Terenzi *et al.*, 1976), and Trevillyan & Pall (1979) found that increases in cyclic AMP decrease cell membrane permeability, increase cell wall thickness and effect other cell surface changes. Such changes may perhaps be necessary to maintain cell surface integrity now threatened by the presence of GAG-MP. In the work of Terenzi *et al.*, (1976), it was found that cyclic AMP stimulated the transition from vegetative hyphae to conidiating aerial hyphae, a phenomenon associated with the transition from the restricted to the stationary phase of growth (Schmit *et al.*, 1975).

Although the biological role of the GAG-MP is as yet not clearly understood, a model may be postulated whereby GAG-MP may act to depolarize the plasma membrane, which in turn stimulates a cell surface enzyme to produce endogenous cyclic AMP in the restricted phase of

growth. The cyclic AMP in turn switches on the enzymic and genetic machinery necessary for that developmental phase of metabolism.

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