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A Potential Macroregulatory Mechanism for Growth Regulation in *Neurospora crassa*

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A POTENTIAL MACROREGULATORY MECHANISM FOR GROWTH REGULATION IN *Neurospora crassa*

by

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ABSTRACT

A POTENTIAL MACROREGULATORY MECHANISM FOR GROWTH REGULATION IN Neurospora crassa

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Neurospora produces a phase specific, cationic mucopolysaccharide composed primarily of galactosamine (GAG-MP) which becomes part of the cell wall and is later secreted into the media. 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 hydrolyze carbohydrate linkages and the inclusion of NaCl (1M) in the GAG-MP/conidia reaction mixture. The GAG-MP's physiological activity 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 cAMP which in turn switches on the enzymatic and genetic machinery necessary for that phase of growth.

DEDICATION

I dedicate this work to Mary Jane and Joseph Patrick Mooney whose moral, spiritual and financial support have made this work possible.

ACKNOWLEDGEMENTS

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I would like to thank Dr. Lloyd Wolfinbarger for his continual guidance, intellectual stimulation and professional appraisal of this study and manuscript. I thank Dr. Johnson, the Biological Sciences Support Facility, and Dr. Sitz for use of their equipment and/or materials. And I would like to express my great appreciation to Nadean Salalila for her speedy and accurate preparation of this manuscript.

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INTRODUCTION

The concept of genetic macroregulation of growth during the life cycle of Neurospora crassa has been proposed by Reisig and Glasgow (28) 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 (15, 28), termed galactosaminoglycan mucopolysaccharide (GAG-MP), and as a component of the outer cell wall (11). It was produced and excreted into the culture media during late log phase (28, 31). 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.

Reisig and Glasgow (28) estimated the molecular weight of GAG-MP to be approximately 10^6 daltons 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 (28).

Changes in membrane permeability are frequently accompanied by a net depolarization of the plasma membrane and Trevillyan and Pall (39) have recently correlated depolarization of the plasma membrane in N. crassa with the rapid intracellular production of cyclic adenosine monophosphate (cAMP). Cyclic AMP has been known to exert a pleiotrophic

effect at several metabolic levels and has been shown to regulate the synthesis of a number of enzymes subject to control by catabolic repression (39). In addition, it played a major role in the activation of enzymes, i.e., protein kinases, by phosphorylation activities. In yeast, adenylate cyclase, a membrane bound enzyme which converts ATP to cAMP, was found to be 3 to 4 times more active in cells entering the late log or stationary phase than cells in the early log phase (34).

The data presented in this study suggests that GAG-MP interacts with cells at the level of the plasma membrane. This interaction probably mediates a regulatory role by altering the permeability properties of that membrane and, subsequently, may result in an elevation of intracellular cAMP concentrations. The interaction of GAG-MP with the membrane was ionic in character and exhibited saturation kinetics for biological activity. In addition, the alteration in membrane permeability appeared to be specific in permitting the loss of a variety of small molecular weight metabolites.

MATERIALS AND 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, California. The mucopolysaccharide to be isolated was found in the cot culture media during the restricted and stationary phase of growth of the organism (15). Conidia from 5 to 7 day old cot mutant cultures were dust inoculated into 500 ml of sterile Vogels minimal media N plus 2% (w/v) sucrose in 1 liter foil-covered, Erlenmeyer flasks. The flasks were shaken at 35 C for 5 days and the mycelium-free media 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 of this study. Stocks of the cot mutant and the wild type cultures were maintained on solid minimal media N plus 2% (w/v) sucrose.

Chemicals

L-(U-^{14}C) Arginine, 300 mCi/mmole, was purchased from Amersham (Arlington, Ill.) and N-acetylglucosaminidase, Ribonuclease A (Type 1A, from Bovine Pancreas), β-glucuronidase (Type H-1 from Helix pomatia), Protease (Type VIII from B. subtilis), galactosamine·HCl, poly-lysine, gramacidin D, and valinomycin were purchased from Sigma Chemical Co. (St. Louis, Mo.). All chemicals used in this study were of the highest

purity available.

Mucopolysaccharide Isolation and Purification

Mucopolysaccharide used in this study was isolated from the media of 5 day old liquid cultures of the *cot* mutant. The media was filtered through a Whatman #3 paper and subsequent purification of the mucopolysaccharide was performed essentially as described by Reisig and Glasgow (28) with minor modifications. The purification procedure consisted of an initial precipitation of the mucopolysaccharide from the media (cooled to 0 C) with 2 volumes of cold (-20 C) 95% ethanol. The precipitate was collected by centrifugation (10,000 rpm for 40 min at 4 C) and then dissolved, while cold, in 40 ml of 1M NaCl for each 1 liter of media precipitated.

Deproteination was performed with the addition of 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 at 2,000 to 3,000 rpm 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 volumes of cold 95% ethanol, centrifuged and the precipitate dissolved in 1.0M NaCl (usually 50% to 75% of original volume).

The RNase digestion of contaminating RNA was achieved by the addition of ribonuclease (5X, crystalized, Sigma Chem. Co.) to a 50 µg/ml final concentration in dissolved 0.15M NaCl (pH 5) and incubation of the solution at 37 C for 30 min. The deproteination steps were then repeated to remove the RNase and other remaining proteins.

The aqueous phase was again precipitated with 2 volumes of cold (-20 C) 95% ethanol, centrifuged and the precipitate redissolved in 9.0 mls of dilute (0.01M) NaCl. When solubilization was complete, 1.0ml of acetate-EDTA (3.0 M sodium acetate in 0.001M EDTA, pH 7.0) and 1.3 volumes of isopropanol were added while stirring rapidly. The supernatant obtain was lyophilized and frozen until needed.

Determination of the Chemical and Physical Properties of the Mucopolysaccharide

A. Thin layer chromatography

Determination of the chemical composition of the mucopolysaccharide was achieved by acid hydrolysis and thin layer chromatography. The mucopolysaccharide was acid hydrolyzed in 6N, constant-boiling HCl (Pierce Chem. Co.) under N_2 at 100 C for 18 h. The hydrolysis products were then dried under a constant flow of dry N_2. To test for the identity of liberated amino sugars, cellulose thin layer chromatography sheets (Eastman) were treated with 0.1M $BaCl_2$, dried and then spotted with the acid hydrolysis products. The sheets were developed in an n-butanol/pyridine/water (6/4/3;v/v/v) solvent, dried and sprayed with ninhydrin (42). Neutral sugars were assayed by spotting the acid hydrolysis products onto cellulose thin layer chromatography sheets (Eastman) followed by development in an n-butanol/acetic acid/water (12/3/5;v/v/v) solvent. Silver reagent was used to detect the presence and location of reducing sugars (42).

B. Colorimetric assay

The mucopolysaccharide was also analyzed for the presence of galactosamine and glucosamine using Wagner's colorimetric assay (42). The "total hexosamine assay" was used to determine total hexosamine

present in the mucopolysaccharide using galactosamine·HCl and glucosamine as standards with absorbance measured at 535 nm. The "galactosamine-glucosamine differential assay" permitted determination of galactosamine as some percentage of total hexosamines with a linearly increasing color formation correlating to increasing galactosamine concentration (from 2 μg/ml to 40 μg/ml). Galactosamine·HCl was used as a standard with absorbance measured at 530 nm.

C. 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 x 10 cm) equilibrated with 4N HCl. The mucopolysaccharide sample (5 mg) was applied to the column and noncationic molecules eluted with 4N HCl. Cationic molecules were eluted from the resin by washing with 0.5M NH_4OH. Aliquots of each sample fraction were tested for the presence of mucopolysaccharide via Wagner's colorimetric assay (41) and for activity in causing L-arginine efflux from reagent cells as described in the efflux bioassay of this text.

D. Enzymatic digestion of purified GAG-MP

The GAG-MP was digested individually by four enzymes as follows:

1) One unit (0.105 mg in 1 ml glass distilled water) of N-acetylglucosaminidase was added to 1 ml of 200 μg of GAG-MP/ml of solution (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 (Type H-1 from Helix pomatia) were added to 1 ml of 200 μg/ml of solution (pH 5.0). The solution was incubated for 1 h at 37 C.

3) One unit (5 mg in 1 ml glass distilled water) of Ribonuclease A (Type 1A, 5X, crystalized) was added to 1 ml of 200 μg of GAG-MP/ml of solution (pH 7.5). The solution was incubated at 37 C for 1 h.

4) One unit (0.1 mg in 1 ml glass distilled water) of Protease (Type VIII from B. subtilis) was added to 1 ml of 200 μg of GAG-MP/ml of solution (pH 7.5). The solution was incubated at 37 C for 1 h.

After the incubation period, 10% TCA (final concentration) was added to precipitate the protein (enzymes). GAG-MP was found to be soluble in 10% TCA (15). The mixtures were then centrifuged (3,000 rpm for 20 min) and the supernatant was dialyzed exhaustively against glass distilled water, lyophilized and resuspended in 1 ml of water. Each sample of digested GAG-MP was then tested for efflux activity as described in the efflux bioassay of this text.

E. Gel permeation chromatography

The size of the mucopolysaccharide was determined using a Sephadex G-100 gel filtration column (1.5 x 60 cm) equilibrated with 0.01M Tris HCl buffer (pH 8.0) and pumped with a Pharmacia P-3 pump to achieve a flow rate of 0.4 ml/min. The column was calibrated using Blue Dextran (2×10^6 daltons) to determine the void volume, αphosphorylase (94,000 daltons), bovine serum albumen (67,000 daltons), RNase (13,700 daltons), tryptophan (204 daltons) and galactosamine·HCl (215 daltons) as molecular weight markers, with the latter two indicating the inclusion volume of the column. A 5 mg sample of mucopolysaccharide in 1 ml of buffer was applied to the column and followed by a continuous flow of buffer. Eluents from the column were monitored with an ISCO UV/Visible

monitor/recorder at 254 nm and 5 ml fractions were collected using an ISCO fraction collector. The fractions were then lyophilized, resuspended in 2 ml glass distilled water and dialyzed exhaustively against glass distilled water in Spectrapore III dialysis tubing to rid the sample of small molecular weight molecules (3,000 m.w.). Aliquots of each sample fraction were tested for the presence of hexosamines (41) and for activity in causing L-arginine efflux as described in the efflux bioassay of this text.

F. Acetylation of the mucopolysaccharide

Acetic anhydride was used in the N-acetylation procedure described by Wheat (42) where aqueous solubilized mucopolysaccharide (200 µg/4 ml H_2O) was treated with 0.4 ml of saturated $NaHCO_3$ and 0.4 ml of 2% acetic anhydride (in acetone) at 25 C for 2 to 5 min. After deionization by treatment with Dowex 50 H+ form resin to remove sodium ions and unacetylated GAG-MP, the mucopolysaccharide was precipitated with 3 volumes of 95% ethanol, and stored in the cold (4-10 C) overnight. The precipitate was resuspended in glass distilled water, dialyzed exhaustively against glass distilled water in Spectrapore III dialysis tubing and tested for L-arginine efflux activity as described in the efflux bioassay of this text.

Physiological Effect of GAG-MP on Neurospora crassa

A. Preparation of reagent cells

Reagent cells were wild type conidial cells preloaded with uniformly labeled L-(^{14}C)-arginine. These cells were used to test the ability of the mucopolysaccharide to cause membrane permeability changes in N. crassa cells with subsequent loss of L-(^{14}C)-arginine

from the cell. It has been shown (29) that once L-arginine has been transported into a Neurospora cell it does not normally efflux yet remains soluble and free in pools within the cell. L-arginine, therefore, represents an ideal amino acid to use in measuring the ability of various molecules to alter the membrane permeability of Neurospora.

Reagent cells were prepared as follows: conidia from 7 day old cultures of wild type N. crassa were aseptically transferred 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. A dry weight/ml of conidial cells in the suspension was determined by filtering an aliquot of cells onto a tared glass fiber filter, drying and reweighing. A volume of conidial cell suspension sufficient to give a 0.2 mg dry weight of cells/ml, final concentration, was added to a sterile solution of 1% glucose and 1X Vogels salts (pH 5.8) in glass distilled water. The mixture was incubated for 3 h at 37 C with shaking. Cycloheximide (0.01 mg/ml final concentration) and L-(^{14}C)-arginine (0.01 uCi/0.1 mole/ml, final concentration) were then added and this mixture was incubated for 1 additional h 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 (DeBusk, personal communication).

B. Efflux bioassay

To determine the relative amounts of L-(^{14}C)-arginine remaining in the reagent cells after treatment with various test solutions, i.e., the GAG-MP, 5 ml of reagent cell suspension were first filtered onto a nitrocellulose filter (2 μm pore size) using vacuum suction, and washed

with glass distilled water. The filter and cells were transferred into a flask containing 5 ml of test solution and the cells were re-suspended after which 1 ml aliquots were removed at 2, 4, 8, and 16 min. Each aliquot was filtered through a nitrocellulose filter and washed with glass distilled water, placed into a scintillation vial and dried in an oven (60 C) overnight. Toluene based scintillation fluid (Omnimix, ICN) was added to the vials, each of which was 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 (15). The isolation procedure was designed to separate proteins and nucleic acids from carbohydrates that were secreted into the growth media (20). The procedure therefore allowed for maximal purification of the medial GAG-MP (Table 1). The repeated deproteination treatment with chloroform/isoamyl alcohol both before and after the RNase digestion allowed for the maximum removal of proteins. Ribonuclease and isopropanol treatments removed the remaining RNA and DNA, respectively, leaving medial polysaccharides in the remaining solvent (20). In the final supernatant, following lyophilization, the yield of purified GAG-MP averaged 5 mg of a dry powdery material per liter of media.

Chemical and Physical Characterization of Purified GAG-MP

A. Acid hydrolysis and thin layer chromatography of products

In an effort to resolve the question of chemical composition, an attempt was made to acid hydrolyze the purified GAG-MP. The hydrolyzed material was redissolved in a minimal volume of water and subjected to thin layer chromatography.

The thin layer chromatographic analyses of the hydrolysis products were sensitive to greater than 5 ng concentration of amino sugars and

Table 1. Chemical Analysis of Purified Medial GAG-MP[1] from the cot Mutant of Neurospora crassa.

Compound	Percent Detected
Galactosamine[2]	81.0
Unidentified Material	15.6
Amino Acids/Proteins[3]	3.4
Glucosamine[4]	0.0
Neutral Sugar[5]	0.0

[1]The purification procedure consisted of an initial precipitation of the mucopolysaccharide with 2 volumes of cold 95% ethanol, deproteination with chloroform/isoamyl alcohol and a final separation of the mucopolysaccharide from contaminating DNA with the addition of 1.3 volumes of isopropanol which will precipitate the DNA. The isopropanol is drawn off and the remaining liquid is dialyzed exhaustively against glass distilled water, lyophilized and frozen.

[2]Determined by Wagner's (41) colorimetric assay.

[3]Detected by Lowrey's protein assay using bovine serum albumen (BSA) standard.

[4]Thin layer chromatographic methods for the detection of amino sugars were used to assay for glucosamine and galactosamine content of the acid hydrolysis products.

[5]Thin layer chromatographic methods were used for the detection of neutral sugars.

10 ng concentration of 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 (Table 1).

Because of extensive degradation of the galactosamine by the hydrolytic procedures employed, it could not be quantitatively analyzed in the hydrolysis products. When the acid hydrolysis products were tested, only 55% of the expected galactosamine could be detected using Wagner's colorimetric assay (41).

B. Hexosamine determination

The galactosamine-glucosamine differential assay of Wagner (41) allowed for the detection of these amino sugars using a colorimetric process that measures absorbance of the reacted amino sugars at 530 nm where a linear relationship between absorbance and amino sugars exists. Since glucosamine could not be detected by thin layer chromatographic analysis in the purified GAG-MP, this assay was used to quantitate the amount of galactosamine present (Table 1). Using galactosamine·HCl as a standard, it was found that purified GAG-MP was composed of approximately 81%, by weight, galactosamine. It is possible that 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.

C. Ion exchange chromatography

If, as suggested (15, 28), the purified GAG-MP is a polymer of amino sugars, it should behave very much as a polycationic molecule. To determine if this was so, a sample of purified GAG-MP was dissolved in

glass distilled water, applied to a cation exchange resin (Dowex-50W) column and eluted with a strongly acidic solution (4N HCl) which passed the neutral and anionic molecules of the sample through the column as the first eluent, leaving the cationic molecules bound to the resin. The resin was stripped of the cationic molecules with the addition of a strongly basic solution (0.5M NH_4OH). The initial eluent of the ion exchange chromatographic analysis was totally devoid of both hexosamines and efflux activity, whereas the NH_4OH eluent contained both hexosamines and efflux activity. This observation would be expected if purified GAG-MP were a polymeric hexosamine.

D. Enzymatic digestion of purified GAG-MP

There exists a potential for a small amount of contaminating protein or nucleic acid to be mediating the efflux of L-(^{14}C)-arginine. Therefore, I digested samples of GAG-MP with a variety of hydrolytic enzymes to determine the effect of enzymatic digestion on efflux activity of GAG-MP (Table 2). Enzymes which hydrolyze carbohydrate linkages, N-acetylglucosaminidase, and β-glucuronidase, completely destroyed the biological activity of the purified GAG-MP. The ribonuclease and protease digestion of GAG-MP caused a detectable, but not complete loss of efflux activity.

These findings suggest that the efflux activity of the GAG-MP is due primarily to the carbohydrate components of the molecule. Proteins and nucleic acids associated with the molecule may play a secondary role in maintaining the biologically active GAG-MP by contributing to secondary or tertiary structure of the galactosamine polymer, or by playing an active part in the electrostatic interaction of the macromolecule with the conidial plasma membrane. This finding would be

Table 2. Sensitivity of Biologically Active Purified GAG-MP to Enzymatic Hydrolysis in Isolates of the *cot* Mutant of *Neurospora crassa*.

Enzyme	Percent L-Arginine Efflux From Preloaded Conidial Cells[2]
None	95.0
Ribonuclease A Type 1A (from Bovine Pancreas)	56.0
Protease Type VIII (from *B. subtilis*)	25.0
N-acetylglucosaminidase	0.0
B-glucuronidase Type HI (from *Helix pomatia*)	0.0

[1]100 µg of purified GAG-MP was reacted with at least 1 unit of each enzyme for 1 h at 37 C at the proper pH (See Materials and Methods section).

[2]The present efflux of radiolabeled arginine from reagent cells was measured as the amount of radioactivity remaining in the cells after 16 min at 37 C. Glass distilled water was used as a control, i.e., no efflux.

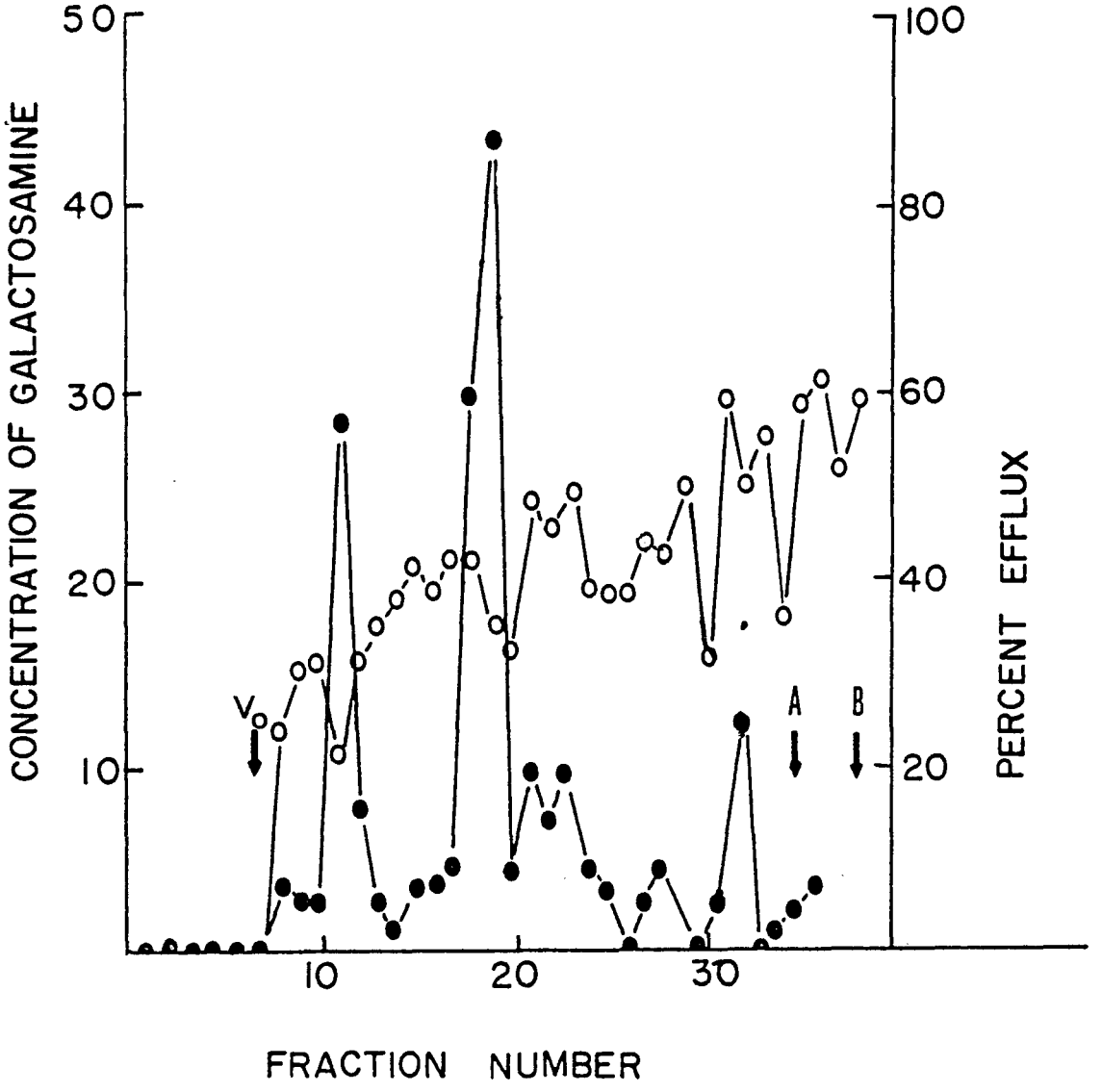
expected if some protein was covalently linked to the GAG-MP polymer.

E. 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). From the 5 mg sample of GAG-MP applied to the column, only 18.3% galactosamine polymers were recovered with 10% of the efflux activity expected for that concentration of material. Analysis of aliquots of each fraction revealed only two sizes of polygalactosamine polymers capable of causing efflux of L-arginine from reagent cells (Fig. 1). The two sizes of active polymers were approximately 85,000 daltons and 4,800 daltons as determined by comparative column runs with known molecular weight markers.

The methodology used to estimate molecular weights by Sephadex column chromatography yielded figures that can be considered only rough values for the fractions analyzed. Tryptophan (M.W. of 204.2) and galactosamine·HCl (M.W. of 215) had similar molecular weights yet galactosamine·HCl eluted 5 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 weights for the polygalactosamines may be low). The column run was terminated with the fraction which corresponded to that fraction in which galactosamine·HCl was eluted. This indicated the inclusion volume of the column for amino sugars. It is reasonable to assume that the next few fractions not tested would contain no galactosamine and sugsequently, no peaks of activity associated with the presence of this molecule. In addition, it was noted that GAG-MP appeared to have a deleterious effect on Sephadex

Figure 1. Analysis of size distribution of polygalactosamine in μg/5 ml concentrations of purified, medial GAG-MP from the cot mutant of Neurospora crassa and efflux activity after 16 min incubation versus size. Concentration of GAG-MP (O-O), percent efflux activity (●-●). The samples in each fraction were first dialyzed against glass distilled water before being tested for efflux activity. Blue Dextran (2×10^6 daltons) was used to measure the V_o (void volume). Tryptophan (215 M.W.), indicated as A, and galactosamine·HCl (204 M.W.), indicated as B, were used to measure the V_e (inclusion volume).


50
40
30
20
10
100
80
60
40
20
V
A
B
10
20
30
CONCENTRATION OF GALACTOSAMINE
PERCENT EFFLUX
FRACTION NUMBER

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 reuse of such column materials is suggested.

Despite such problems there are clearly two size ranges of poly-galactosamine (GAG-MP) that have both an adequate molecular weight (size) and concentration of material to effect membrane permeability of Neurospora 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

A. 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 days when maintained at 0 C. In addition, the radiolabeled arginine preloaded in the reagent cells represented greater than 95% of the total extractable radiolabel.

The effects of increased concentrations of GAG-MP on the reagent cells were tested (Fig. 2), monitoring the amount of L-(^{14}C)-arginine left in the cells after 2, 4, 8 and 16 min of incubation. Incubation of reagent cells with increasing concentrations of GAG-MP, from 1 μg/ml to 20 μg/ml, caused an increasingly rapid loss of radiolabel from the cells. The efflux of radiolabel appeared to consist of two steps, 1) a rapid initial loss of radiolabel followed by 2) a second slower loss of radio-label, thus two aspects of efflux, the initial rate (Fig. 3) and the percent of total efflux were examined. The apparent two step efflux could be caused by different sensitivities of conidial types (macroconidia

Figure 2. Effects of increasing concentrations of GAG-MP on efflux of L-(^{14}C)-arginine from reagent cells of Neurospora crassa. One μg/ml GAG-MP (□-□), 2 μg/ml GAG-MP (Δ-Δ), 5 μg/ml GAG-MP (▲-▲), 10 μg/ml GAG-MP (+-+), 20 μg/ml GAG-MP (●-●), H_2O control (0-0).

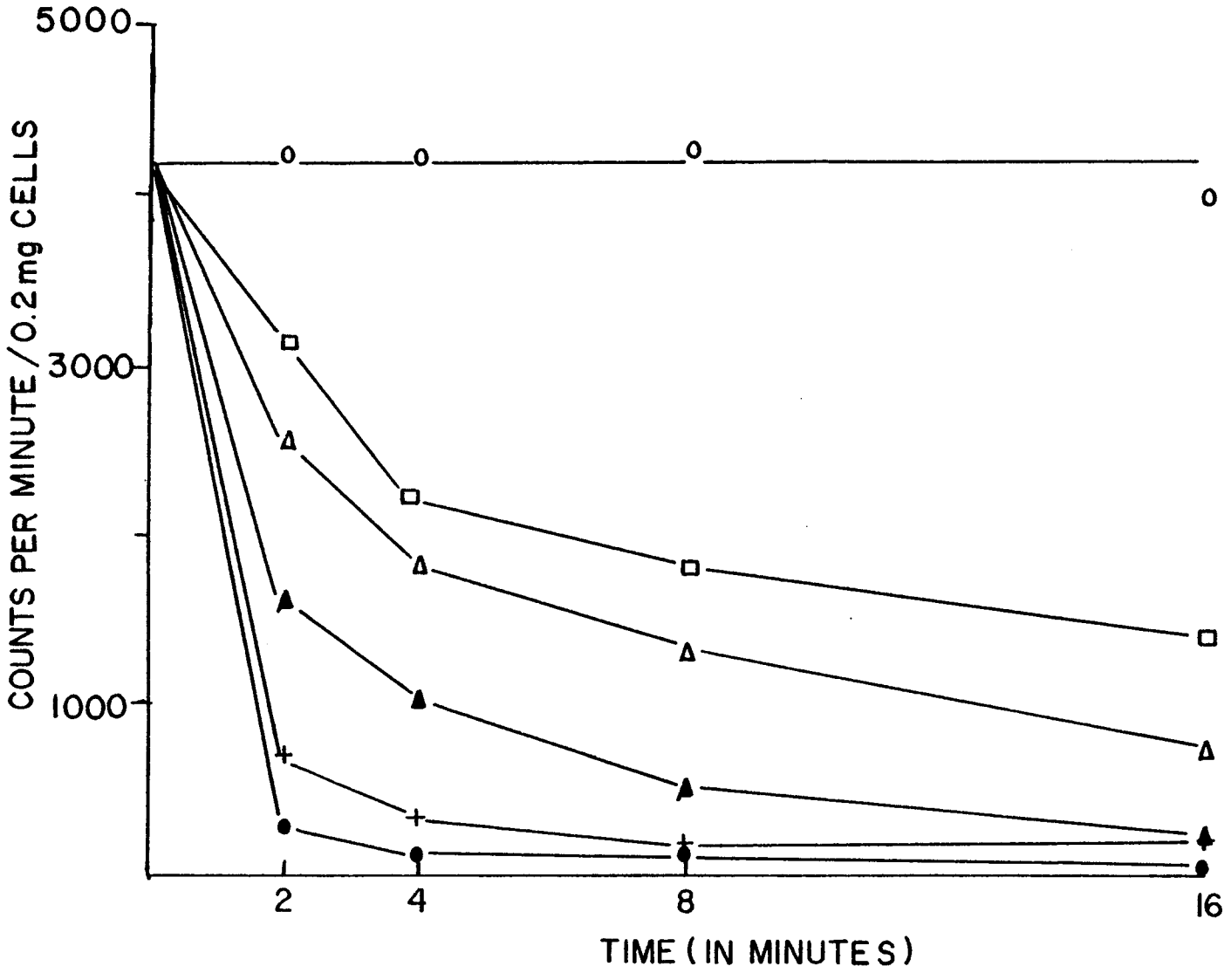
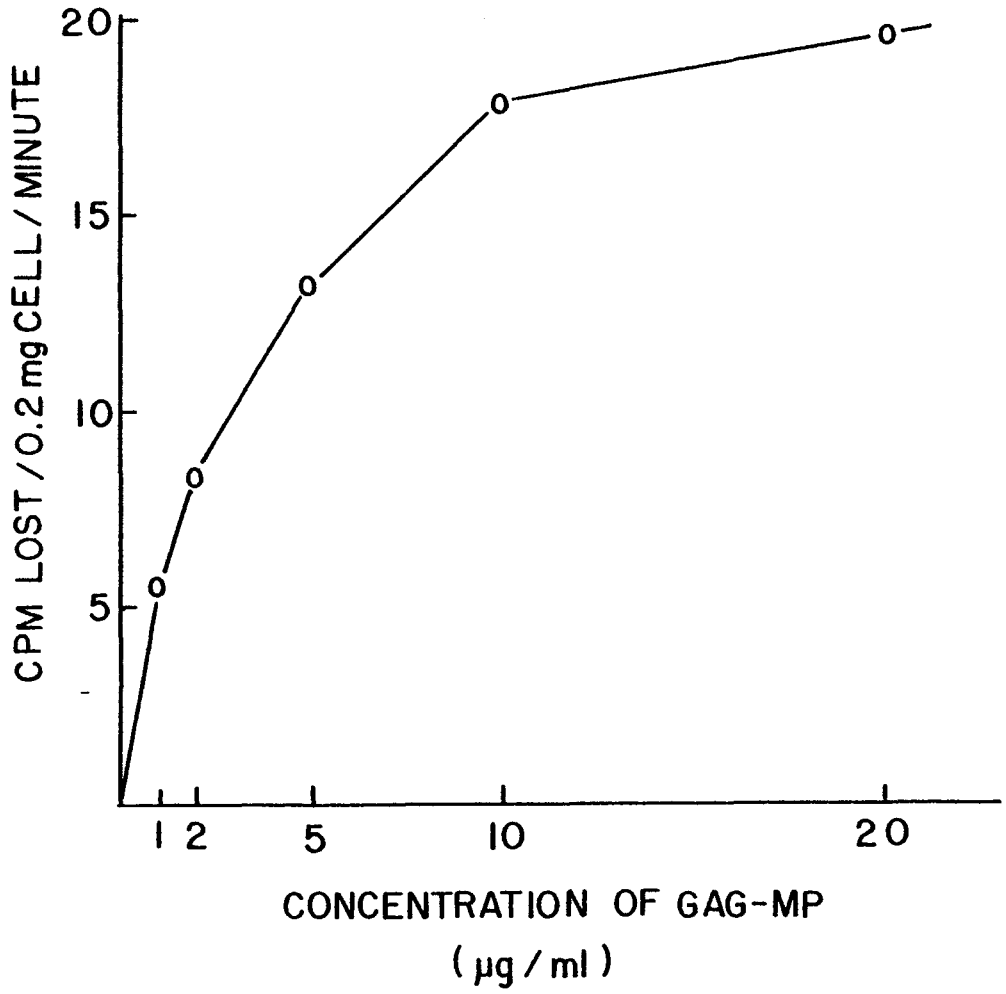

5000
3000
1000
COUNTS PER MINUTE / 0.2 mg CELLS
2
4
8
16
TIME (IN MINUTES)

Figure 3. Initial rates 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 Figure 2.


20
15
10
5
CPM LOST / 0.2 mg CELL / MINUTE
1 2
5
10
20
CONCENTRATION OF GAG-MP
(μg / ml)

versus microconidia) in the cell population, or different kinetics of action of the two sizes of active GAG-MP (Fig. 1) on all cells in the population. Alternatively, the slow down of efflux with time may be the result of the ability of conidia to correct the membrane permeability change induced by GAG-MP and thus reduce the rate of efflux or begin retransporting the effluxed L-arginine.

Figure 3 shows how the initial rate of efflux varies with concentration of GAG-MP. The rate of efflux appeared to become independent of GAG-MP concentration at concentrations greater than 20 μg/ml. A double reciprical plot of Figure 3 gave two values: 1) a maximal attainable rate of efflux at infinitely high concentrations of purified GAG-MP (670 ng of L-(^{14}C)-arginine/mg of conidial cells/min*) and 2) a concentration of GAG-MP that provided for an efflux rate one-half of the maximal rate attainable (2.86 μg/ml). Based on the number of cells in the assay (3-4 x 10^6cells) and the estimated molecular weight of the purified, biologically active GAG-MP (4,800 daltons and 85,000 daltons), 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. Two numbers resulted, depending on which molecular weight was chosen. To achieve one-half the maximum attainable efflux rate would require 1.0 x 10^8 molecules of 4,800 molecular weight per conidial cell or 5.7 x 10^6 molecules of 85,000 molecular weight per conidial cell. Further interpretation of these values is not possible

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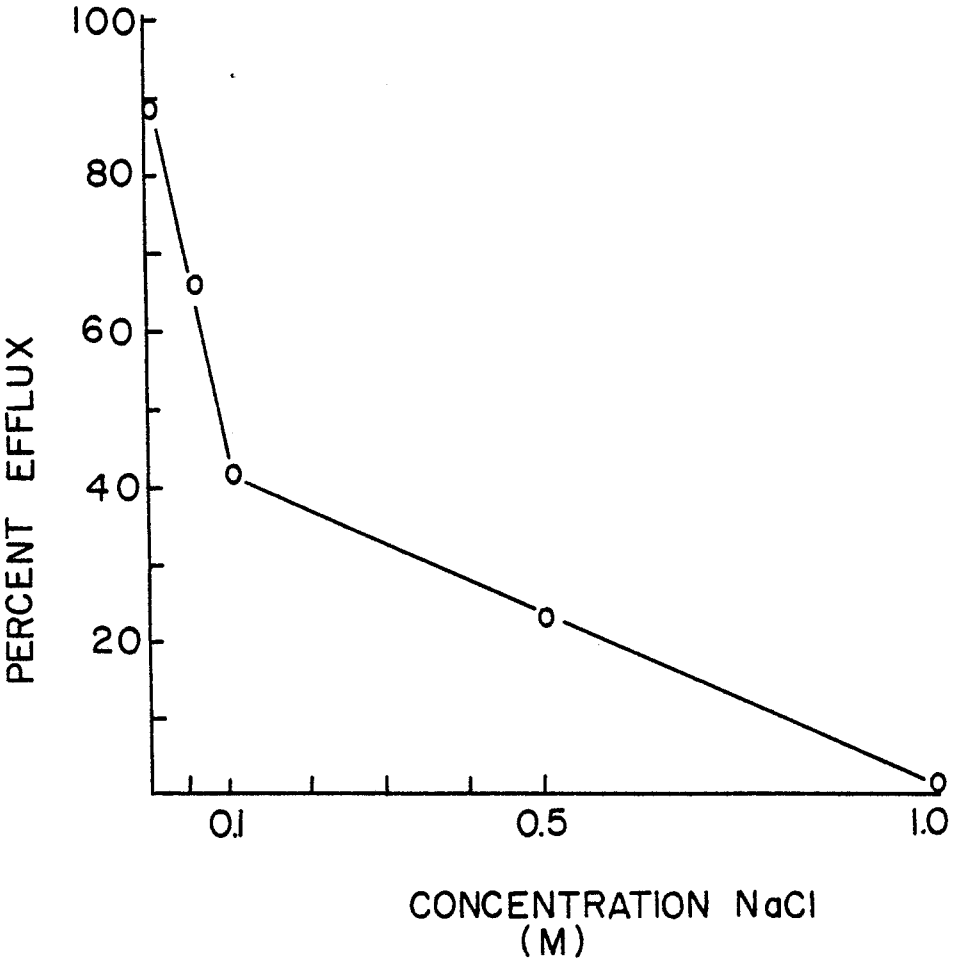
*This value was based on the calculation of the amount of L-arginine accumulated and did not take into account the dilution of the specific activity of radiolabel by endogenously synthesized L-arginine.

considering the data at hand 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.

B. Salt effect on GAG-MP's biological activity

Resig (27) and Jensen (15) have provided substantial evidence that interaction of GAG-MP with conidia occurs via electrostatic binding of GAG-MP molecules to anionic components at the cells surface. Indeed, the action of purified GAG-MP can be blocked with increasing concentrations of NaCl (Fig. 4). For example, the addition of NaCl (1M) to a suspension of reagent cells completely blocked the activity of GAG-MP at a concentration (20 μg/ml) sufficient to cause maximal efflux of L-arginine from these cells; however, the sensitivity of the GAG/MP/conidia reaction to increasing concentrations of NaCl was biphasic. Jensen (15) observed a similar situation in a concurrent study and proposed that the receptors for GAG-MP on the conidial surface may have different affinities for GAG-MP molecules, i.e., high and low affinity receptors. He proposed that the low affinity receptor 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 the findings in this study that two sizes of active GAG-MP polymers may exist, an alternative model is plausible. Both 4,800 dalton and 85,000 dalton polymers could bind to cells by ionic interactions and the binding constants may exhibit differential sensitivities to NaCl concentrations as dictated

Figure 4. The percent of radiolabeled L-arginine remaining in the reagent cells of Neurospora crassa after a 16 min incubation period with 10 μg/ml concentration of GAG-MP and increasing concentrations of NaCl. Reagent cells resuspended in glass distilled water only were used as a control as it was necessary to assess radioactivity remaining in cells where no efflux (0% efflux) occurred for comparative purposes.


100
80
60
40
20
PERCENT EFFLUX
0.1
0.5
1.0
CONCENTRATION NaCl
(M)

by their sizes and number of ionic determinants, i.e., primary amines.

The loss of efflux activity of a 20 μg/ml 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 2.5M, were tested with a 20 μg/ml concentration of GAG-MP. The result was a 95% efflux of intracellular L-(^{14}C)-arginine (Table 3), 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.

As controls, sucrose (0.05-2.5M) and NaCl (0.05-2.5M) were used by themselves to test for their ability to cause efflux of radiolabel from reagent cells and neither compound resulted in loss of radiolabeled L-arginine from reagent cells (Table 3). Thus, the efflux caused by GAG-MP was not due to an osmotic inbalance in the reagent cells and NaCl by itself causes no detectable loss of radiolabel from reagent cells.

C. Efflux activity of other compounds

Poly-lysine (M.W. 15,000-30,000), a cationic homopolymer of lysine, may be considered a molecular analog of GAG-MP. Both are large, positively charged molecules capable of electrostatic interaction. At very low concentrations (20 μg/ml to 50 μg/ml), poly-lysine exerts 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 (1M) also blocked this effect when incubated with the conidial cells prior to the introduction of poly-lysine (Table 3). Acetylation of poly-lysine and GAG-MP via Wheat's (42) procedure prevents both molecules causing efflux of intracellular L-(^{14}C)-arginine

Table 3. The Effect of Various Compounds on the Efflux of L-(^{14}C)-Arginine from Reagent Cells of Neurospora crassa.

Test Solution	Concentration	% L-Arginine Effluxed[a]
Water	---	0.0
GAG-MP	20 µg/ml	95.0
Acetylated GAG-MP	100 µg/ml	0.0
GAG-MP + 2.5M Sucrose	20 µg/ml	95.0
GAG-MP + 1M NaCl	20 µg/ml	0.0
Poly-lysine	50 µg/ml	95.6
Poly-lysine	20 µg/ml	91.5
Poly-lysine	10 µg/ml	64.4
Poly-lysine + .1M NaCl	20 µg/ml	37.6
Poly-lysine + 1M NaCl	20 µg/ml	11.1
Acetylated Poly-lysine	100 µg/ml	0.0
Sucrose	0.05-2.5M	0.0
NaCl	0.05-2.5M	0.0
Gramacidin D	1mM	90.1
Valinomycin	1mM	94.6
2,4-dinitrophenol	1mM	92.7

[a]The percent efflux of radiolabeled arginine from reagent cells was measured as 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.

(Table 3).

The mechanism by which GAG-MP's electrostatic interaction 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, gramacidin D and valinomycin (ionophores) and 2,4-dinitrophenol (a proton conductor), all known for their depolarizing effect on Neurospora plasma membranes (35, 39), were tested for their effects on L-arginine efflux from conidial cells. One mM concentrations of these depolarizing agents were used. In all three cases, over 90% of the intracellular L-(^{14}C)-arginine was effluxed from the cells within 16 min of incubation (Table 3). 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 (11, 18, 28) during late log phase and later excreted into the media of growing N. crassa 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 (15). They are highly positively charged molecules with a heterogeneous molecular size.

Reisig and Glasgow (28) recovered 50% of the dry weight of their purified, acid-hydrolyzed mucopolysaccharide as galactosamine. They stated that this shortage may be due to destruction and incomplete hydrolysis of the macromolecule. Jensen (15) detected 97.4% galactosamine, 0.71% amino acids, 0.068% neutral sugars, and 0.075% unknown ninhydrin positive material in his chemical determination of purified medial GAG-MP. In this study, medial 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 medial 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 concentration and molecular size of the galactosaminoglycan (GAG-MP). Two distinct molecular weights 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 cells surface or why particular molecular sizes cause permeability changes is uncertain. In their work on determining pore size in the Neurospora cell wall, Trevithick and Metzenberg (40) suggested that a molecule as large as 4,750 daltons was capable of penetrating the cell wall through pores. Hence, the smaller molecular weight GAG-MP molecule (4,800 M.W.) may be small enough to penetrate the cell wall and yet still be large enough to cause membrane leakiness. The larger molecular weight GAG-MP (85,000 M.W.) may be able to blanket a portion of the cell surface, affecting numerous sites where the plasma membrane comes in contact with 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 radiolabeled metabolites from conidial cells, may be blocked in 3 ways: 1) by acetylation, 2) by enzymatic digestion, and 3) 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 hydrolyze carbohydrate linkages caused complete loss of activity, whereas digestion with protease or ribonuclease diminished activity. These results suggested a major involvement of the carbohydrate portion of the molecule (a galactosamine polymer) in biological activity with a lesser involvement of proteins and/or nucleic acids.

The biphasic appearance of the effects of increasing concentrations of NaCl on blocking GAG-MP's ability 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 with 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 (4,800 M.W.) molecule, while much higher NaCl concentrations would be required to release the bound (85,000 M.W.) GAG-MP molecule. In a concurrent study, Jensen (15) observed a similar sensitivity to salts and proposed that two different GAG-MP "receptors" may have differing affinities for GAG-MP, i.e., "high affinity receptors" and "low affinity receptors". He suggested that low affinity receptors may release the bound GAG-MP readily in the presence of low concentrations of salts whereas the higher affinity receptors may require a higher ionic strength in order to release bound GAG-MP. He argued that a discrete number of binding sites for the GAG-MP might exist and that these sites ordinarily bind Ca^{++}. The conidia presumably incurred an initial, rapid and sublethal binding at low concentrations of GAG-MP (less than 3 μg GAG-MP/10^6 cells) and the conidia remain

viable. Large effluxes of radiolabeled metabolites were not evident, yet electrostatic binding of GAG-MP to the conidia was still apparent. That GAG-MP reaches a saturation in binding at higher concentrations is shown in this study 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 small metabolite leakage.

In previous studies, polycations were shown to be effective in causing ultrastructural changes (24) and membrane leakiness (43). This study has shown that high concentrations of the cationic polymer of galactosamine (GAG-MP) caused permeability changes in the Neurospora cell surface and Sadler (30) concluded that this efflux was specific for small molecular weight molecules.

Large transport fluxes of metalizable compounds occur across the N. crassa membrane as a result of depolarization of the membrane (22). The actions of valinomycin and gramacidin D, known ionophores, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, have long been recognized as depolarizing agents (39) and the data presented here indicated that the physiological activity of these compounds mimic the action of GAG-MP in causing membrane leakiness.

Those concentrations of GAG-MP used in this study represented an approximate 1000-fold increase in concentration from normal physiological concentrations of GAG-MP in Neurospora cultures. Only 5 mg of purified polysaccharide was obtained from 1 liter of cot media, and cot was an overproducer of the substance. However, by using these dramatic 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 (22) has shown that, in Neurospora, membrane depolarization produces an increase in cAMP levels by stimulating the membrane bound adenylate cyclase. It is suggested that additional cAMP may be produced in the cell in response to the GAG-MP plasma membrane interaction. The elevated levels of cAMP may activate genetic and enzymatic machinery now necessary for this phase of development in the fungus. Marked and transient increases in cAMP concentrations are found in wild type Neurospora cells in restricted phase metabolism (37) and Trevillyan and Pall (39) found that increases in cAMP decrease cell membrane permeability, increase cell wall thickness and affect 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. (37), it was found that cAMP 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 (31).

Although the biological role of the GAG-MP is as yet not clearly understood, a model may be postulated whereby GAG-MP acts in a hormonal fashion to stimulate a cell surface enzyme to produce endogenous cAMP in the restricted phase of growth. The cAMP in turn switches on the enzymatic and genetic machinery necessary for that developmental phase of metabolism.

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