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Mucopolysaccharide Which Regulates Growth in *Neurospora*

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Neurospora produces a mucopolysaccharide (called MP) which inhibits its growth, causes vacuolation and agglutination of its cells, and precipitates its purified membrane protein. Cultures of a colonial strain display a phase of slow growth; the induction of this phase is traced to the production of MP by the mold. Stationary-phase cultures of wild type also produce MP. MP is a polymer of galactosamine, its amino groups only partially acetylated, probably containing other minor components. MP molecular weight is approximately 10^6. Complete acetylation abolishes the biological activities of MP. It is suggested that the regulatory effect of MP is mediated by its interaction with the protoplasmic membrane.

Studies on genetic regulatory mechanisms, for which the work of Jacob and Monod (7) can be taken as a starting point, have led to a comprehensive understanding of the regulation of the individual units of transcription (operons). It is now timely to ask whether the overall regulation of the major processes in living cells, for which Luria (10) has aptly coined the expression "macroregulatory phenomena," is controlled by a hierarchical network of such operons, or whether there exists a distinctly different type of macroregulatory mechanism. Although any attempt at a comprehensive answer would be premature, various lines of evidence (10, 15, 21) have implicated alternatively the protoplasmic membrane, ribonucleic acid polymerase subunits, and cyclic adenosine monophosphate as propagators of macroregulatory signals in diverse biological systems ranging from bacteriophages to mammalian cells. The present study deals with yet another system and identifies a mucopolysaccharide as a crucial link in the signaling circuit, thus contributing additional facts to the macroregulatory puzzle. This system involves the regulation of growth in a colonial mutant of the mold *Neurospora crassa*.

MATERIALS AND METHODS

Materials. *N. crassa* strain 74 (St. Lawrence 74A) is a wild type; 3821 (ED38-21a, from the collection of J.L.R.) bears the mutant *cot* (C102t, from M. Mitchell); 327 (FGSC327, from the Fungal Genetics Stock Center, Humboldt State College) is called slime and lacks cell wall (3). Sodium polyphosphate (average chain length, 59) was a gift of the Monsanto Chemical Co.; blue dextran (average molecular weight, 2×10^6) and D-galactosamine-hydrochloride were purchased from Sigma Chemical Co. The purity of the latter was ascertained by total N determination (Schwarzkopf Microanalytical Laboratory) and paper chromatography.

Cultures. Unless otherwise specified, 100 ml of Vogel's N medium (20) in 250-ml cotton-stoppered Erlenmeyer flasks were inoculated with 10^7 conidia of strain 3821 (1 to 3 weeks old). The flasks were shaken at 180 circular strokes per minute, 1 inch in diameter, in a well aerated atmosphere at 35 C. Stocks were kept on slants of solid Vogel's N medium. For strain 327, soluble starch was substituted for sucrose, and supplements of 0.75% yeast extract, 0.75% nutrient broth, and 0.02% arginine were added.

MP preparation. The mucopolysaccharide which is the object of this study (MP) was usually prepared from the medium of 5-day-old cultures. The mycelium was filtered off using a nylon stocking, and 2 volumes of cold 95% ethanol were added to the medium. After standing overnight at 5 to 10 C, the precipitate was collected by centrifugation and taken up in 1 M NaCl (40 ml per liter of medium). The next steps were as indicated by Marmur (13) for the isolation of deoxyribonucleic acid after his first ethanol precipitation, except that the precipitates before ribonuclease treatment were resuspended in 1 M NaCl, and only one isopropyl alcohol precipitation was performed by using 1.3 volumes of alcohol. The final precipitate was dissolved in water, which demanded patient heating and agitating. When required, a portion was dried over P_2O_5 to calculate dry weight. The rest was frozen, or autoclaved and stored cold. The yield was approximately 1 mg per 100 ml of medium.

MP extraction from the mycelium. Mycelium amounting to 50 to 150 mg (dry weight) was harvested by filtration through nylon fabric, rinsed with water, squeezed free of liquid, suspended in 5 ml of 1% trichloroacetic acid (diluted fresh from a 50% stock solution), agitated with a vibro-mixer for 0.5 min, and

shaken in a reciprocating shaker (140 2-cm strokes per min) for 1 hr at room temperature. Filtration through nylon fabric followed by squeezing yielded a residue and a turbid solution. The residue was washed in ethanol, dried, and weighed as reference to the mycelial dry weight. The solution was supplemented with 3 volumes of cold 95% ethanol. After standing overnight at 5 C, the precipitate was collected by centrifugation.

Chemical assays. MP was hydrolyzed for 6 hr in 6 N HCl at 100 C before galactosamine assay. The product was dried over NaOH under vacuum. MP preparations purified from the medium as indicated earlier could be assayed for galactosamine without further ado. When using impure MP, it was necessary to free it of sugars which interfere with the assay. The procedure is described here in reference to the trichloroacetic acid mycelium extract. The alcohol precipitate was hydrolyzed, dissolved in 1 ml of water, and passed through a column [Dowex 50 by 8–200 (H^+)] with a bed volume of 1 ml. The column was washed with 4 ml of water and eluted with 8 ml of 0.5 N HCl. The acid eluate was evaporated to dryness as indicated above and assayed for galactosamine.

Galactosamine was assayed by the procedure of Ludowieg and Benmaman (9), reducing the volumes fivefold for greater sensitivity; the "hexosamine method" was used whenever glucosamine could be present, but the "total hexosamine method" was used at other times.

Protein was assayed by the method of Lowry et al. (8), after heating in 0.5 N NaOH for 10 min at 100 C. The ninhydrin method was performed by the method of Hirs (6), on a manual scale. Acetyl groups were determined by the method of Ludowieg and Dorfman (18).

Chromatography. To determine the composition of MP, samples were hydrolyzed in vacuo for 6 to 72 hr in 2 or 6 N HCl at 100 C. After drying over NaOH, they were taken up in water and subjected to descending chromatography on Whatman no. 1 paper, using *n*-butanol-acetic acid-water (120:30:50) as solvent. Sugars were located with the silver reagent, and amino acids were located with ninhydrin (22). For the identification of galactosamine, the solvent was butanol-pyridine-water (6:4:3), and the paper was used as such or treated with 0.1 M $BaCl_2$ (22).

Gel filtration of MP was performed by using BioRad A15 and A150 gels, equilibrated with 0.1 N NH_4OH. The sample (with 0.02% blue dextran as a marker) and the eluant also contained 0.1 N NH_4OH. The ammonia from the fractions was eliminated over H_2SO_4 under vacuum.

Acetylation of MP. Acetylation was performed with acetic anhydride in acetone-water-bicarbonate (22). After 20 min, acetyl MP was precipitated by the addition of 3 volumes of ethanol, stored overnight in the cold, and centrifuged. The precipitate was suspended in water and dialyzed.

Biological assays. For the measurement of inhibitory activity, the unknown sample was autoclaved for 10 min at 121 C. A 0.5-ml amount was placed in a sterile steel-capped Wasserman tube, and 0.5 ml of N medium containing 10^4 conidia of strain 3821 was added. A series of such tubes was prepared containing twofold serial dilutions of the unknown, up to a dilution having no inhibitory effect. Growth was scored after 2 days. The tube with the lowest concentration preventing growth contains, by definition, one unit of inhibitory activity.

For the agglutination assay, 0.1 ml of a suspension in saline of protoplasts from 327 was placed in a 0.25-dram vial. An equal volume of the unknown was added in 0.01- to 0.02-ml portions, shaking by hand after each addition. Agglutination was easily detected with the naked eye by the formation of large clumps and the clearing of the suspension. The agglutination of conidia was studied under similar conditions.

For the vacuolation assay, 2 μliters of the unknown were mixed on a cover glass with 1 μliter of water containing 10^4 conidia of 3821. One microliter of 0.02% neutral red was often added. After mixing, the cover slip was inverted over a depression slide and examined microscopically for up to 30 min. A control, without MP, was run in parallel. A positive test was indicated by the formation of vacuoles and the diffusion of the dye throughout the conidia in the majority of the population.

RESULTS

Growth pattern of the cot strain. The *cot* (for temperature-sensitive colonial) mutant of *Neurospora* grows like wild type at temperatures below 30 C, but forms button-like colonies on plates at higher temperatures (14, 19). Two different factors contribute to colonial morphology: branching, which slows down the rate of advance of the growing front while increasing the density of the mycelial mat, and growth inhibition. The timing of growth inhibition determines colony size. Thus, although *cot* colonies are small, modifier "gulliver" mutations at any one of six *gul* loci lead to large colony size (19). The above observations refer to cultures on solid media. For a more quantitative description of colonial growth, it was necessary to turn to liquid cultures.

Figure 1 shows the growth of *cot* at 35 C. The various curves correspond to cultures inoculated with different numbers of conidia. Notice that after a short lag (indicated by a gap in the curves) growth proceeds at a fast (unrestricted) rate for 10 to 12 hr, with a protein doubling time of 2.5 hr. In the following hours, the rate slows down, finally reaching a doubling time of about 14 hr. This restricted phase proceeds at a steady rate until the stationary phase. The various cultures reach the stationary phase at different times, as would be expected if this phase results from the exhaustion of nutrients or oxygen. The converse is true for the restricted phase, which starts independently of culture density—as if timed endogenously. This paper is largely devoted to the identification of the inducer of the restricted phase.

Substance isolated from cot inducing restricted growth. A substance inducing restricted growth

was detected in restricted and in stationary *cot* cultures (17). It was routinely isolated from the latter cultures because it could easily be purified from the medium by precipitation with ethanol and isopropanol, and deproteinization with chloroform-isoamyl alcohol. As will be seen in later sections, this substance is a mucopolysaccharide. This biologically active mucopolysaccharide is referred to in this paper as MP.

Addition of MP to unrestricted cultures slowed them down to an extent independent of the carbon source (Fig. 2) or the MP concentration over at least an eightfold range (Table 1), increasing the doubling time from 2.5 to about 8 hr. This is less than the 14-hr doubling time noted for Fig. 1. However, when cultures in the restricted phase were diluted into fresh medium, their growth rate was stabilized at 9 to 10 hr of doubling time, which is not significantly different from the rate induced by exogenous MP.

Table 2 shows that no further growth inhibition was caused by MP added during the restricted phase of growth, while the same dose of MP had the usual inhibitory effect on a younger unrestricted culture of comparable mass.

The foregoing results can be most simply interpreted if the inhibition produced by exogenous MP is the same phenomenon described as restricted growth in unsupplemented cultures.

Additional effects of MP: inhibition at 25 C. At 25 C the growth inhibition produced by MP is virtually complete. The levels of MP required

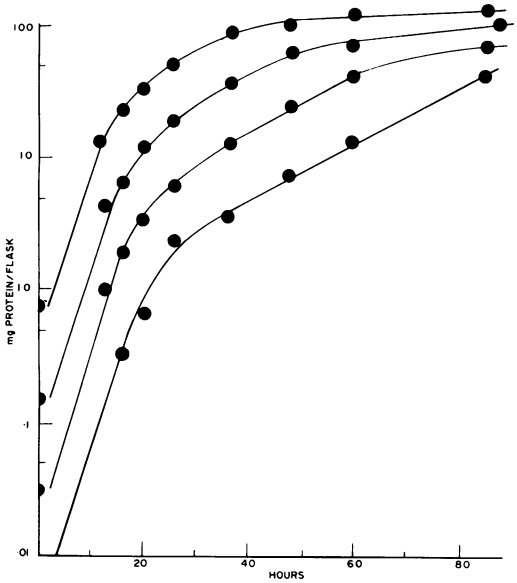

FIG. 1. *Growth of N. crassa cot under standard culture conditions, but with various inocula; from bottom up: 4 × 10⁵, 2 × 10⁶, 10⁷, and 5 × 10⁷ conidia per flask.*

TABLE 1. *Plateau in the inhibition of growth of N. crassa caused by MP at 35 C*[a]

Expt[b]	MP added (μg/ml)	Growth after 60 hr (mg of protein/flask)
1	0	10.0
	16	5.6
	32	5.6
	65	4.3
	130	5.3
2	0	0.38
	16	0.14
	32	0.12
	65	0.14
	130	0.09

[a] Cultures in 50-ml Erlenmeyer flasks with 10 ml of media.

[b] Experiment 1: 2 × 10⁶ conidia per flask, MP added at time zero. Experiment 2: 2 × 10⁴ conidia per flask, MP added after 18 hr of growth (culture density ca. 0.02 mg of protein/flask.)

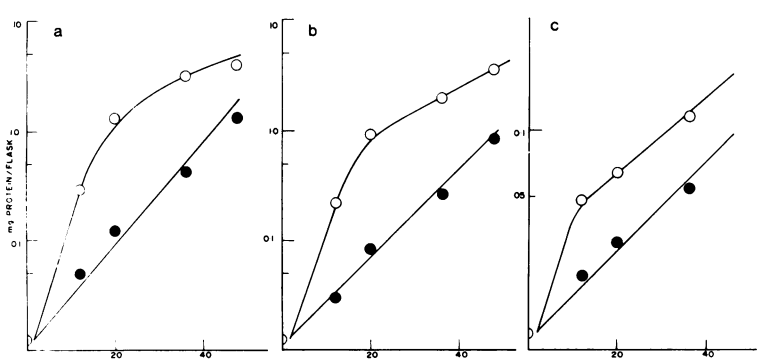

FIG. 2. *MP as inhibitor of growth of N. crassa. Standard culture conditions were used, except that volumes were scaled down 10-fold and C sources were as follows: a, 2% sucrose; b, 1% glucose; c, 2% glycerol. Symbols: ○, no addition; ●, 260 μg of MP added at time zero.*

to inhibit at 25 C were of the same order of magnitude as for 35 C: 16 μg of MP in 1 ml prevented the growth of 10^5 conidia, but 4 μg did not affect it. Using 10^6 conidia for 16 μg of MP, no growth was observed in the first day, but the culture escaped inhibition on the second day.

Agglutination. When 1 to 2 μg of MP was mixed with a suspension of *Neurospora* protoplasts (i.e., cells from the slime mutant) containing approximately 0.1 mg of protein, the suspension agglutinated with the formation of large clumps and consequent clearing of the supernatant fluid. The same was true for conidia previously grown in liquid media at 25 C for 7 hr with gentle agitation. Ungerminated conidia, or conidia cultured for 4 hr or less, have consistently given negative results in the standard agglutination test, even if suspended in the media where the 7-hr cultures had grown. To assess the significance of this negative finding, a pitfall and a limitation of the method used should be discussed. The pitfall stems from the fact that excess of either MP or conidia prevents visible agglutination. The concentration of MP in the stock solution is also important: 0.1 volume of 10× MP may fail to give a test, where 1 volume of 1× MP gives it. This pitfall was avoided by testing a wide range of MP concentrations. The limitation results from our definition of a positive test as one giving massive agglutination. If this criterion is relaxed, and ungerminated conidia are extensively washed, a weak agglutinating reaction can be detected in the presence of MP (J. E. Glasgow, *unpublished data*).

Interaction with membrane protein(s). Plasma membrane protein, purified by the method of Woodward (Val Woodward, *personal communication*) precipitated in the presence of an equal weight of MP (5 to 25 μg of each per ml). The precipitate dissolved in excess of either ingredient.

Vacuolation. Addition of 16 μg of MP to 10^6 conidia in 1 ml of water caused the formation of large single vacuoles in most conidia. The addition of neutral red (to 0.005%) makes it easier to see the MP effect. Higher concentrations of MP led to the formation of multiple vacuoles and abnormally shaped conidia. Vacuoles did not form in 0.2 M NaCl and, if previously elicited by MP in water, they disappeared when in 0.2 M salt. It is as if MP increases the permeability of the cell membrane to water (and to neutral red as well), allowing the cells to behave as osmometers.

Biological activities of MP preparations reside in a galactosamine-containing macromolecule. Galactosamine was identified as the major component of MP preparations, by paper chromatography of the hydrolyzate. Its R_F was clearly distinguishable from those of glucosamine and mannosamine standards. Confirmatory evidence was established by the ninhydrin degradation of Wheat (22) and by using his no. 5 solvent for chromatography of the resulting pentoses. The degradation product of MP-hexosamine migrated as lyxose (obtained from a known galactosamine sample by the same treatment), but differently from arabinose, xylose, and ribose standards. In the colorimetric assay (9), the product of MP hydrolysis gave a "total hexosamine" to "hexosamine" ratio of 1.6; the same ratio observed with galactosamine. The hexosamine recovered in this assay accounted for about 50% of the dry weight of the MP preparations. Part of the shortage may be due to destruction and incomplete hydrolysis; however, paper chromatography also reveals a number of amino acids and neutral sugars in MP hydrolyzates as described previously (17). A key question is whether the various sugars and amino acids are part of the same macromolecule as galactosamine. This appears likely to be so, since MP purified by ion-exchange chromatography still yielded a set of minor components on hydrolysis.

During gel filtration in A15 and A150 columns, the bulk of MP, assayed as hydrolyzable hexosamine, migrated slightly behind blue dextran. From these results, the average molecular weight of MP was estimated to be approximately 10^6 daltons.

Most of the amino groups of galactosamine are unacetylated in MP, as indicated by (i) high affinity of MP for Dowex 50 (Fig. 3); (ii) unhy-

TABLE 2. *Lack of inhibition by MP during the restricted phase*[a]

Cultures at time of MP addition		MP added (μg/ml)	Doubling time after MP addition (hr)[b]
Age (hr)	Density (mg of protein/flask)		
6	0.05	0	2.6
		16	6.0
		32	7.5
33	0.08	0	12
		16	14
		32	17
		64	12

[a] Cultures in 50-ml Erlenmeyer flasks with 10 ml of media; inocula: 10^6 conidia for the 6-hr series and 2×10^4 for the 33-hr series.

[b] Determined from the plot of milligrams of protein versus age for the period immediately after the time of MP addition.

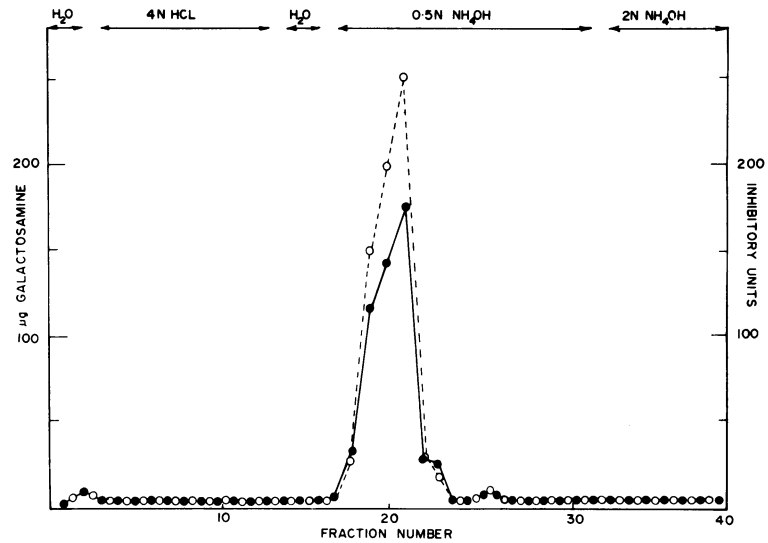

FIG. 3. *Chromatography of MP on Dowex 50 (H^+). A column (1.1 by 9 cm) was charged with ca. 4 mg of MP and eluted as indicated. Fraction volume: 4 ml. Recovery as hexosamine: 60%. Symbols: ●, micrograms of hexosamine per fraction; ○, units of inhibitory activity per fraction. The error in the latter values is ca. 50%.*

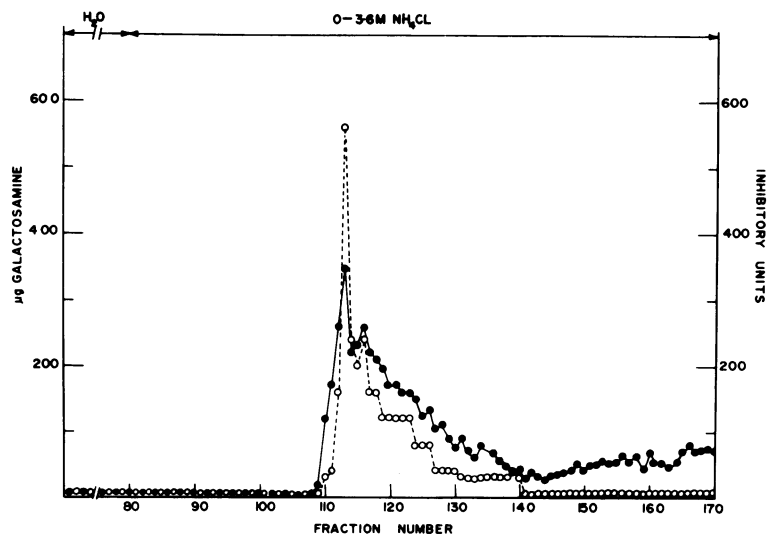

FIG. 4. *Chromatography of MP on CM-cellulose (NH_4^+). A column (2.7 by 56 cm) was charged with 70 mg of MP and eluted with the indicated linear gradient. Fraction volume: 20 ml. Recovery as hexosamine: 16%. Symbols: ●, micrograms of hexosamine per fraction; ○, units of inhibitory activity per fraction. The error in the latter values is ca. 50%.*

drolyzed MP preparations giving a high ninhydrin test, which increased only 2.4-fold during hydrolysis for 6 hr in 6 N HCl; (iii) determination of acetyl groups giving a value 25% of that expected if all of the galactosamine was *N*-acetylated and no *O*-acetyls were present.

The following experiments were performed to verify whether the three biological activities described earlier (inhibition, agglutination, and vacuolation) are properties of the galactosamine-containing macromolecule or are due to contaminating substances. (i) Hydrolysis for 0.5 hr in 2 N HCl or for 3 hr in 1 N NaOH at 100 C, followed by dialysis, eliminated hexosamine and abolished all three activities. (ii) Coincident peaks of hexosamine and inhibitory activity were obtained by stepwise elution of MP from a Dowex 50 column (Fig. 3). The agglutinating and vacuolizing activ-

ities were detected only in fractions 19 to 21. Similar results were obtained by chromatography on CM-cellulose, using gradient elution (Fig. 4). (iii) Harold (4) observed that inorganic polyphosphate firmly binds to a galactosamine polymer found in the cell walls of *Neurospora*. This suggested testing polyphosphate for anti-MP activity. It was found that roughly equivalent concentrations (w/v) of polyphosphate abolished all three activities of MP. (iv) MP was acetylated by a mild procedure known to *N*-acetylate hexosamines. The product failed to give the ninhydrin test, developing a brown color instead, and lost all three biological activities.

Taken as a whole, the above evidence strongly supports the contention that the active compound in MP preparations is MP itself, meaning by this a galactosamine polymer probably containing also neutral sugars and amino acids as minor components.

Physiological role of MP. If, as argued above, endogenous MP restricts growth in *cot* strains, why then the 0.5-day lag in the onset of the restricted phase? Since there is no such lag in sensitivity to added MP (Fig. 2), the obvious thing to do was to investigate the timing of MP production. Assay of the MP content of culture media showed this to be an unpromising parameter: MP accumulated only in media of cultures 3 days old or older, much after the onset of the restricted phase. Furthermore, as argued in reference to Fig. 1, the onset of restricted growth is not traceable to the condition of the medium. Extraction of MP from the mycelium was thus attempted. Highest and most reproducible yields were obtained by extraction with 1% trichloroacetic acid. Table 3 shows that in the period from 8 to 24 hr the MP content increases 10-fold. The increment may be greater if it should turn out that the traces of galactosamine assayed at 8 hr are not due to MP. The 24-hr material, on the other hand, appears to be true MP insofar as it is not dialyzable and has the usual inhibitory activity.

TABLE 3. *Lag in the production of MP by N. crassa cot cultures*

Age (hr)	Trichloroacetic acid-extractable MP (μg of galactosamine/mg of residue)[a]	Dry weight (mg) per flask
8	0.09	3.8
24	0.85	67.5
32	0.96	104.5

[a] Cultures were grown under usual conditions, but with 4×10^7 conidia per flask. Before extraction, the mycelium was pooled from enough flasks to get about 100 mg (dry weight) for each age.

Further evidence on the physiological role of endogenous MP was sought by studying the effect of polyphosphate on *cot* cultures. Since polyphosphate antagonizes the effect of exogenous MP, it was argued that it might also act against endogenous MP. Unfortunately, polyphosphate is, itself, a growth inhibitor at concentrations approaching 1 mg/ml. At lower concentrations (50 to 400 μg/ml), polyphosphate stimulated the growth of *cot* (Fig. 5). Notice that the slopes of the restricted and unrestricted phases remain unchanged. The twofold increment in mycelial yield in the presence of polyphosphate results, rather, from a slower transition from one phase to the other. This effect of polyphosphate, while not so dramatic as might have been expected, points in the right direction should MP be the effector for the transition from unrestricted to restricted growth.

The standard *cot* cultures show signs of agglutination after some 8 hr of growth, both to the naked eye and the microscope. This process could be quantified by plating on solid media portions from cultures of different ages and by scoring the growing colonies after incubation at 35 C for 3 days. The number of colonies decreased 100-fold during the first 24 hr of growth in liquid. On the other hand, when the media were supplemented with polyphosphate (100

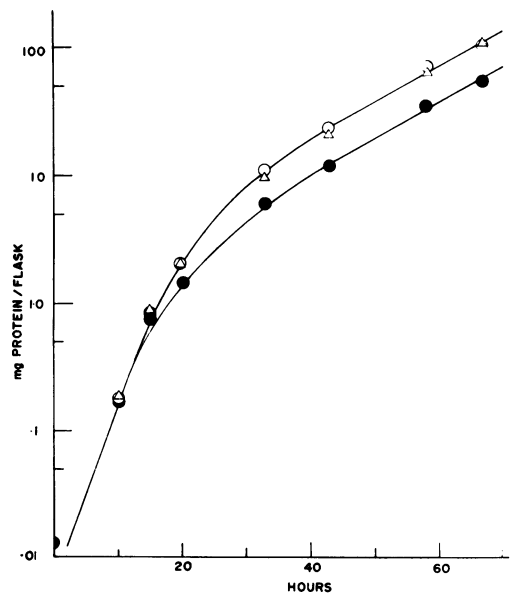

FIG. 5. *Effect of polyphosphate on the growth of N. crassa cot. Standard culture conditions were used but with an inoculum of 10^6 conidia/flask. Symbols: ●, no addition; ○, 0.1 mg of sodium polyphosphate/ml; △, 0.3 mg of sodium polyphosphate/ml.*

μg/ml), this number dropped no further than twofold, and other evidences of agglutination were absent. These results are most easily understandable if endogenous MP is the agent causing agglutination, and polyphosphate prevents it by competing for MP. This raised the possibility that the effect of MP on growth rate might be a byproduct of its agglutinating activity. A "minimal" hypothesis could, in fact, account for the two effects of polyphosphate, namely, that MP causes agglutination, agglutination causes restricted growth as a mere consequence of the large size attained by colonies at an earlier time, and polyphosphate antagonizes restricted growth via its anti-agglutination effect. This hypothesis could be tested because it predicts that the size of the colonies at the onset of restricted growth should be the same in the presence and absence of polyphosphate. The number of colonies in the cultures used for Fig. 5 was estimated by mixing portions from cultures of different ages with warm semisolid agar, diluting where needed in the same suspending media, plating, and counting without further incubation. Only colonies large enough to contribute significantly to the culture mass were estimated by this method. Counts were feasible only in cultures 33 hr old or older. For each series, the number of colonies per flask remained constant with age: 0.5×10^3 for the unsupplemented cultures and 20×10^3 to 60×10^3 for the polyphosphate cultures. By using these values and extrapolating the restricted and unrestricted growth curves of Fig. 5 to the point of intersection to determine the ideal phase transition point in a synchronized population, it was calculated that the unsupplemented colonies undergo phase transition at the average mass of 5 μg of protein, whereas in the presence of polyphosphate the corresponding mass is 0.1 μg. There is, therefore, no agreement with the "minimal" hypothesis. Rather, it would seem that polyphosphate acts both against agglutination (very effectively) and against growth restriction (less effectively). Consequently, insofar as polyphosphate antagonism is an indicator of MP activity, endogenous MP would be responsible both for restricted growth and for spontaneous agglutination in *cot*.

MP in wild-type strains. Under the standard conditions used here, wild-type cultures went directly from the unrestricted phase (1.5- to 2-hr doubling time) to the stationary phase. This transition occurred about 20 hr after inoculation. MP could be isolated from the culture medium of 1-day-old cultures, as indicated both by the presence of undialyzable galactosamine in the ethanol precipitate and by the biological activity of this fraction. Furthermore, the concentrations of MP required for a positive inhibition or vacuolation test were found to be identical for wild type and *cot*. The two strains also showed a comparable temperature dependence of MP sensitivity. It was therefore of interest to test for the presence of MP in cultures of different ages by the trichloroacetic acid extraction method. The results (Table 4) show that there is no, or only traces of, MP in the fast growing younger cultures, but a significant level in stationary cultures. Thus, the presence of MP in wild-type is compatible with a role in growth regulation.

DISCUSSION

The occurrence of a galactosamine polymer in the culture filtrate of *Neurospora* was first mentioned by Distler and Roseman (2) while reporting in more detail the isolation of such a compound from *Aspergillus*. This latter compound was found to be 38% *N*-acetylated and appeared to contain no other sugars or amino acids. Later, Harold (4) and Mahadevan and Tatum (11) reported a galactosamine polymer in the cell wall fraction of *Neurospora*. It is reasonable to assume that these reports deal with the same compound referred to here as MP, although its biological activity was not investigated until recently (17).

The data presented here strongly support the hypothesis that MP is an essential link in the regulatory circuit eliciting restricted growth in *cot* strains. A cardinal observation is that, at 35 C and during the early growth of the culture, before MP is made, addition of MP induces restricted growth; however, at later times, when the cultures make MP and have entered the restricted phase, addition of MP has no effect. Further evidence bearing on the above hypothesis is being sought in studies involving the gulliver (*gul*) mutants (19) which modify the *cot* phenotype favoring unrestricted growth. Of particular interest is the case of *gul-6*, which makes an MP of lower molecular weight than its *gul-6*$^+$ counterpart (Reissig, *unpublished data*). Although the relationship between molecular weight and the

TABLE 4. *Lag in the production of MP by wild-type N. crassa*

Age (hr)	Trichloroacetic acid-extractable MP (μg of galactosamine/mg of residue)[a]	Dry weight (mg) per flask
8	0.09	2.0
24	3.01	239
36	3.08	475

[a] Same as footnote *a*, Table 3, except with 10^7 conidia per flask.

unrestricted phenotype is not yet clear, the implication of MP in growth restriction is strongly supported by this observation.

MP is not limited to *cot* strains, as evidenced by previous work (2, 4, 11) and the present paper. The situation in wild type is not so favorable for physiological studies as in *cot*. In the latter strain induction of MP occurs early in the unrestricted phase; in wild type it occurs so late that any regulatory effects would be obscured by the stationary situation. It would be interesting to know whether under different conditions it may be possible to evince in wild-type cultures a restricted phase separate from the stationary.

Evidence was presented which suggests two ways in which the activity of MP could be modulated: by acetylation or by complexing with polyphosphates. However, the evidence falls short of demonstrating that this actually happens physiologically. In fact, with respect to polyphosphates, the general rule is that they accumulate under stationary conditions (5), an unlikely time for an antagonist of inhibition to act.

The occurrence of MP in the cell wall fraction (4, 11, 12) is particularly interesting on two accounts. It shows that the cell wall is not just an inert casing, and it localizes a macroregulatory function in a component of the cell envelope. The target of MP action is also likely to be on the surface, considering MP's large molecular weight and its agglutinating ability.

The mechanism of action of MP is of no small interest. Several lines of evidence point towards an involvement of membranes: the vacuolizing effect of MP, which indicates altered permeability; the agglutination of protoplasts; and its direct interaction with purified membrane protein(s). The idea that membranes function as receptors and propagators of macroregulatory signals has found experimental support in other systems and served as basis for promising speculations (1, 10, 15, 21). It is also appealing with reference to the system under consideration.

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