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HEXOSAMINE METABOLISM DURING SPHERULE FORMATION IN *PHYSARUM POLYCEPHALUM*

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Summary

Hexosamine metabolism in relation to the spherule-wall synthesis in *Physarum polycephalum* was studied by the incorporation of labeled sugar into the wall and intermediary compounds in the biosynthesis of wall polysaccharide. The incorporation of [^{14}C]galactosamine into the wall material occurred after a lag period of about 10 h in an induction medium. Nucleotides and sugar phosphates in the acid-soluble fraction of spherulating plasmodia were analyzed by column chromatography on Dowex 1-X8 (formate). The primary labeled products formed in the spherulating plasmodia after incubation with [^{14}C]galactosamine were galactosamine 1-phosphate, UDP-galactosamine, *N*-acetylhexosamine 6-phosphate and UDP-*N*-acetylhexosamine. Spherulation was insensitive to polyoxin D, while it was completely blocked by cycloheximide. The activity of galactokinase and galactose-1-phosphate uridyltransferase increased 4—5-fold during the spherulation.

Introduction

The transfer of microplasmodia of the acellular slime mold, *Physarum polycephalum*, grown in a submerged culture to a non-nutrient salt solution leads to a formation of microsclerotia called spherules [1]. The spherulation is also induced by the addition of mannitol [2]. During the spherulation, the plasmodia cleaved into numerous portions of multi-nucleated cytoplasm and each

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1-4-nis(5-phenyloxazolyl-2)benzene.

of them produces a hard wall on their surface. The chemical composition of the wall has been studied by several workers and found to be very unusual [3—5]. The spherules contain galactosamine polymers as a major component of the wall, which has rarely been found among microbial species. In this investigation, we have studied some features of galactosamine metabolism during the spherulation. The process of galactosamine deposition onto the wall was followed by the determination of galactosamine content and the incorporation of labeled precursor. Changes were also observed in the chromatographic pattern of cytoplasmic galactosamine derivatives in spherulating plasmodia.

Materials and Methods

Organism and cultivation. Microplasmodia of *P. polycephalum* were grown at 26°C in a semi-synthetic medium containing yeast extract and tryptone [6]. The cultures were maintained in 100 ml of the medium in 300-ml Erlenmeyer flasks, which were agitated on a reciprocal shaker at 90 strokes/min. Spherulation was induced either by starvation of plasmodia in a non-nutrient salt medium [1] or by transfer to a nutrient medium containing 0.5 M mannitol [2]. In either case, spherulation was completed after about 48 h.

Chemicals. [$^{14}C_1$]Galactosamine (50 mCi/mmol) was purchased from New England Nuclear, Boston. [U-^{14}C]Glucose (272 mCi/mmol) and [$^{3}H_6$]uracil (15 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. Non-radioactive nucleotide sugars, sugar phosphates and enzyme were purchased from Boehringer, Mannheim. GalN-1-*P* was prepared enzymatically from galactosamine and ATP as described by Carlson and Roseman [7]. The products was isolated from the reaction mixture by the chromatography on a Dowex 1-X8 (formate) column with 1 N formic acid and lyophilized. Polyoxin D was a gift from Dr. S. Suzuki of the Institute for Physical and Chemical Research, Japan and also from Kaken Chemical Co., Japan.

Spherule wall preparation. Spherules were collected by centrifugation, washed with water and disrupted by sonication (Tomy UR-200p, Tomy Seiko Ltd., Tokyo) with intermittent cooling for the total of 30 min. The homogenate was centrifuged at $3000 \times g$ for 15 min and the pellet was washed 5-times with water and 7-times with 1 M NaCl. Galactosaminoglycan-rich fraction of spherule wall was obtained by repeated washings of the wall preparation with 0.1% sodium lauryl sulfate and 1 M NaCl as described by McCormick et al. [3], then defatted by ethanol. In some experiments, the wall preparations were further fractionated into hot alkali- and hot acid-soluble fractions. Alkali extraction was carried out at 100°C for 30 min with 1 N NaOH and the residue was extracted either with 1 N acetic acid or 3 N HCl at 100°C for 30 min.

Chromatographic separation of labeled intermediary metabolites. Cultures (25 ml) of vegetative plasmodia in growth medium or developing spherules in non-nutrient medium were incubated with [^{14}C]glucose (1 μCi/ml). In a double-labeling experiment, spherulating plasmodia were fed with [^{14}C]galactosamine (1.6 μCi/ml) and [^{3}H]uracil (32 μCi/ml). The extraction of acid soluble nucleotides and sugar phosphates was done according to Bersier and Braun [8]. After incubation in a radioactive medium for 20 h, the plasmodia were collected by centrifugation, suspended in 10 vol. ice-cold 7.5% perchloric

acid in 40% ethanol and disrupted by two 60-s exposures to sonication. The homogenate was centrifuged at 10 000 × *g* for 30 min and the supernatant solution was shaken with an equal volume of chloroform/isoamylalcohol (24 : 1, v/v). The aqueous layer was taken out and neutralized with 5 N KOH, and the precipitate was removed by centrifugation.

The column chromatography was carried out on formate type Dowex 1-X8 column (200—400 mesh, 1.2 × 14 cm). Nucleotides and sugar phosphates were eluted by a step-wise increase of the concentration of formic acid and ammonium formate as described by Brumm et al. [9]. The flow rate was adjusted at 0.4 ml/min and fractions of 3.5 ml were collected. Ultraviolet absorption and radioactivity were measured in aliquots from the fractions, and peak fractions were pooled and lyophilized.

For the identification of individual metabolites, the lyophilized materials were rechromatographed on a Dowex 1-X8 (formate) column with authentic samples. Sugar phosphates and nucleotide sugars were hydrolyzed with dilute HCl or acid phosphatase. Sugar moiety was identified by paper chromatography with butanol/benzene/pyridine/water (5 : 1 : 3 : 3, v/v). Nucleotides were chromatographed on polyethyleneimine-cellulose plates according to Bersier and Braun [8]. Sugars were detected by spraying *p*-anisidine and amino sugars by ninhydrin. For the detection of labeled spots, the chromatograms were dissected into strips of 5 mm length and the radioactive materials were eluted with 0.25 ml water. The eluates were mixed with a scintillation fluid (PPO/POPOP/toluene/methylcellosolve, 4 : 0.5 : 500 : 500, v/v) and the radioactivity was counted by a scintillation spectrometer (Aloka LSC-653, Nihon Musen Ltd., Tokyo).

Analytical procedures. Hexosamine contents in acid soluble and wall fractions were determined by the method of Elson and Morgan according to Dische [10]. Before the assay, the acid soluble material was hydrolyzed in 2 N HCl at 100°C for 3 h and the wall fraction in 4 N HCl at 100°C for 18 h in a sealed tube. *N*-Acetylhexosamine was assayed by the procedure of Reissig et al. [11]. Protein content was determined by the method of Lowry et al. [12].

Enzyme assay. Galactokinase (EC. 2.7.1.6) assay was essentially the same as described by Carlson and Roseman [7]. Plasmodial homogenate (0.03 ml) was mixed to the following assay solution (final volume, 0.16 ml); 20 mM potassium phosphate buffer (pH 7.8)/2 μmol $MgCl_2$/4 μmol ATP/4 μmol galactosamine. The reaction was terminated after 15 min at 30°C by heating at 100°C for 2 min. Excess substrate was reduced to sugar alcohol by the addition of $NaBH_4$. The enzyme product was assayed by the method of Reissig et al. [11] after acetylation with acetic anhydride and acid hydrolysis to remove phosphate moiety [7]. 1 enzyme unit was defined as nmol GalN-1-*P* produced per min at 30°C.

UDPglucose-hexose-1-phosphate uridylyltransferase (EC 2.7.7.12) was assayed as described by Mayes and Hansen [13]. The reaction mixture contained in a total volume of 1 ml, 70 μmol glycine buffer (pH 8.7)/0.4 μmol UDPglucose/0.4 μmol Gal-1-*P*/0.4 μmol NADP/0.4 μmol $MgCl_2$/1.0 unit phosphoglucomutase/0.25 units of glucose-6-phosphate dehydrogenase. The procedure consisted of the determination of Glc-1-*P*, the enzyme product, by coupling the reaction with phosphoglucomutase and glucose-6-phosphate

dehydrogenase. The rate of increase in absorbance at 340 nm as a result of NADPH formation was measured spectrophotometrically. 1 enzyme unit was defined as nmol reduced NADP produced per min at 30°C.

Results

Amino sugar content. Cell wall material prepared according to McCormick et al. [3] consisted of about 75% galactosamine and 25% protein. The extent of *N*-acetylation in the galactosamine wall was determined after Filer et al. [14]. The maximum amount of free *N*-acetylgalactosamine released by acid hydrolysis (0.05 N HCl, 105°C for 4 h) corresponded to only a few percent of total amino sugar.

The amino sugar contents of acid-soluble and acid-insoluble fractions in spherulating plasmodia are listed in Table I. A small amount of hexosamine was detected in acid insoluble fraction from growing plasmodia. On initiating spherule formation by transfer to a mannitol medium, hexosamine content in wall fraction began to increase. After 48 h, its content had increased about 6-fold which accounts for about 2.5% of total dry weight. Amino sugar content in acid soluble fraction was measured after acid hydrolysis (2 N HCl, 100°C for 3 h). Concentrations of cytoplasmic hexosamine compounds as a whole did not show significant change during the spherulation but tended to decrease at the end of the developmental process.

Incorporation of [^{14}C]galactosamine into spherule wall. The rate of incorporation of [^{14}C]galactosamine into the spherule wall was relatively low during the initial phase of development (Fig. 1). The maximum rate of incorporation was reached after about 20 h of induction and lasted for about 24 h. Most of the galactosamino-glycan was soluble in hot dilute alkali or acid. The alkali-insoluble fraction of the wall increased slightly after 40 h. Similar results were obtained when the spherules were labeled with [^{14}C]glucose.

Ion-exchange chromatography of labeled intermediarty metabolites. Acid-soluble material was extracted from spherulating plasmodia after 20 h induction in a non-nutrient medium containing [^{14}C]galactosamine and [^{3}H]uracil and analyzed by anion exchange chromatography on Dowex 1-X8 (formate) column. Typical elution pattern is shown in Fig. 2. Region A contained a compound which coincided with enzymatically prepared GalN-1-*P*. The compound was easily dephosphorylated by a mild acid-hydrolysis (0.1 N HCl, 100°C for

TABLE I

AMINO SUGAR CONTENT IN SPHERULATING PLASMODIA

Spherulation was induced with 0.5 M mannitol. Each figure represents the mean of two determinations.

Hours in induction medium	Hexosamine content (μg/mg dry weight)	
	Acid-soluble fraction	Acid-insoluble fraction
0	1.7	4.3
10	1.5	5.0
20	1.9	12.6
48	1.2	25.5

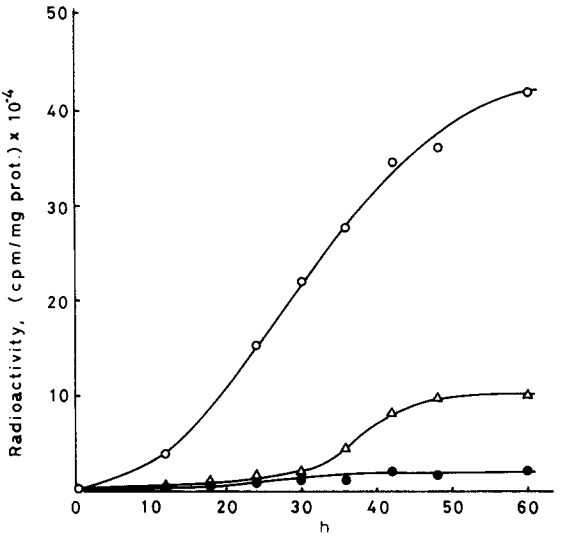

Fig. 1. Incorporation of [^{14}C]galactosamine into spherule wall. Growing plasmodia (3-day-old culture) were transferred to a non-nutrient medium containing [^{14}C]galactosamine (0.5 μCi/ml). At timed intervals, 5-ml portions were removed and mixed with unlabeled spherules as carrier. Wall preparations obtained by the method of McCormick et al. [3] were further fractionated into hot alkali-soluble (○———○), hot acid-soluble (●———●) and insoluble (△———△) fractions. The extraction was carried out at 100°C with 1 N NaOH for 30 min, then with 1 N acetic acid for 30 min.

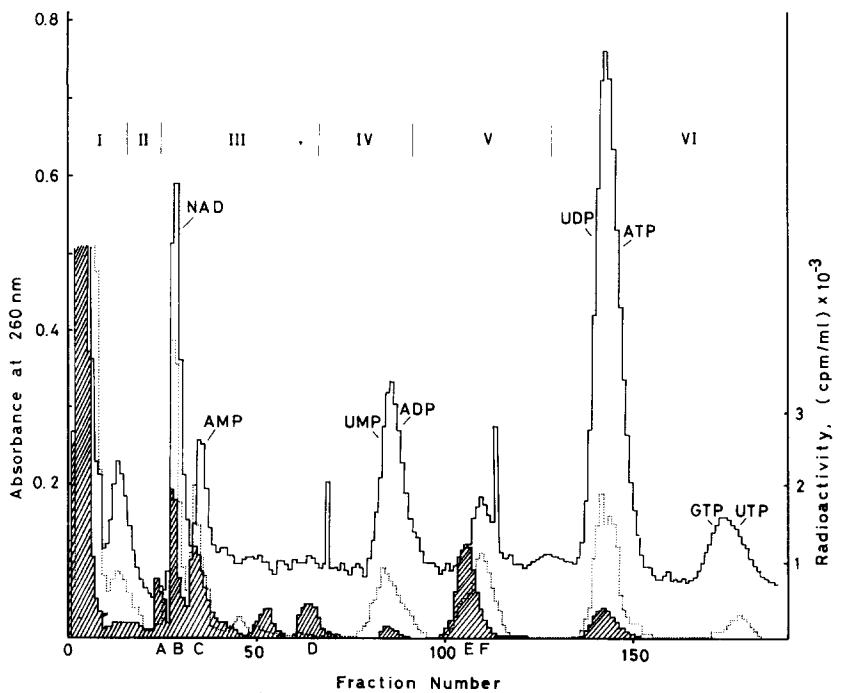

Fig. 2. Chromatography of acid-soluble compounds from spherulating plasmodia. Spherule formation was induced by transfer of plasmodia to a non-nutrient medium containing [^{14}C]galactosamine (1.6 μCi/ml)/[^{3}H]uracil (32 μCi/ml). An acid-soluble fraction was obtained after 20 h induction. The steps in the elution media were: I, H_2O; II, 1 M HCOOH; III, 4 M HCOOH; IV 4 M HCOOH + 0.2 M $HCOONH_4$; V, 4 M HCOOH + 0.4 M $HCOONH_4$; VI, 4 M HCOOH + 0.8 M $HCOONH_4$. Solid line, absorbance at 260 nm. Dotted line, radioactivity of ^{3}H-labeled compounds. Shaded area, radioactivity of ^{14}C-labeled compounds.

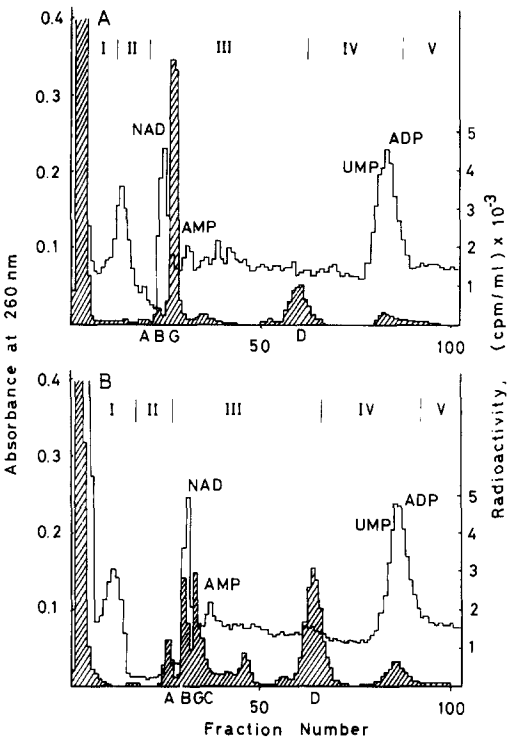

Fig. 3. Chromatography of acid-soluble compounds from growing (A) and spherulating (B) plasmodia. Acid-soluble fractions were obtained after 20 h incubation with [^{14}C]glucose (1 μCi/ml). Other experimental conditions were the same as in Fig. 2.

15 min) and yielded galactosamine. Region B contained a radioactive substance which appears to be UDP-galactosamine. The sugar moiety was easily liberated by a mild acid-hydrolysis (0.1 N HCl, 100°C for 15 min) and the paper chromatographic analysis revealed that this fraction contained galactosamine and a small amount of glucosamine. Uridylic acid was also identified as the breakdown product of the radioactive substance in region B. The radioactive peak in region D contained what appeared to be *N*-acetylhexosamine phosphate. After hydrolysis with acid phosphatase (Tris-maleate buffer, pH 6.0, 2 h at 37°C), nearly equal amounts of *N*-acetylglucosamine and *N*-acetylgalactosamine were detected by paper chromatography. The phosphate group was not released by heating to boiling temperature in 1 N HCl for 10 min. Radioactivities in regions E and F coincided with authentic samples of UDP-*N*-acetylglucosamine and UDP-glucose, respectively. Paper chromatography after acid hydrolysis (0.1 N HCl, 100°C for 15 min), however, revealed that both regions contained galactose derivatives as well as those of glucose.

A similar chromatographic pattern was obtained when [^{14}C]glucose was used as a labeled precursor (Fig. 3). In this cas, however, the accumulation of a large amount of radioactive lactate was noted (region G), which was not obvious in the plasmodia labeled with galactosamine. When the elution pattern of the metabolites extracted from spherulating plasmodia was compared with

TABLE II

EFFECT OF POLYOXIN D ON THE INCORPORATION OF [^{14}C]GALACTOSAMINE INTO SPHERULE WALL

Spherulation was induced in a medium containing 0.5 M mannitol and 0.3 μCi/ml [^{14}C]galactosamine. Concentration of polyoxin D was 200 μg/ml.

Hours in induction medium	Radioactivity in wall fraction (cpm/ml culture)	
	Control	+ Polyoxin D
24	146.0	131.5
48	402.2	372.1
60	451.6	451.4

that of growing plasmodia (Figs. 3A and B), the most notable differences were found in regions A, B and D. In growing plasmodia, radioactivities in regions A and B were very low, whereas in spherulating plasmodia the accumulation of radioactivity in these fractions obviously increased. The radioactivity in region D in [^{14}C]glucose-fed plasmodia was found to be partly due to Glu-6-*P*.

Effect of antibiotics. The incorporation of galactosamine into the spherule wall was not sensitive to polyoxin D (Table II), which has been shown to be an inhibitor for chitin synthesis [15]. Spherulation was somewhat delayed in the presence of the antibiotic (200 μg/ml) but the wall composition was the same as in the control culture. Cycloheximide at a concentration above 30 μg/ml completely inhibited the spherule formation when added at the start of the induction. Data in Table III show that the incorporation of [^{14}C]galactosamine into hot alkali-soluble material, a major fraction of the spherule wall, was markedly inhibited. When the same amount of cycloheximide was added to the culture 10 h after the induction, some spherule-like structures were occasionally found in the culture medium. However, these spherules were irregular in their shape and did not germinate when transferred to a nutrient medium.

Changes in the activities of galactokinase and galactose-1-phosphate uridyltransferase. Figs. 4 and 5 show the changes in the specific activity of galactokinase and galactose-1-phosphate uridyltransferase after the induction of sphe-

TABLE III

EFFECT OF CYCLOHEXIMIDE ON THE INCORPORATION OF [^{14}C]GALACTOSAMINE INTO SPHERULE WALL

Spherulation was induced in a medium containing 0.5 M mannitol and 0.3 μCi/ml [^{14}C]galactosamine. Concentration of cycloheximide (CH) was 30 μg/ml. Wall materials were extracted at 100°C for 30 min, first with 1 N NaOH then with 3 N HCl.

Hours in induction medium	Radioactivity (cpm/ml culture)					
	NaOH-soluble		HCl-soluble		Total	
	Control	+ CH	Control	+ CH	Control	+ CH
20	93.8	16.4	4.9	7.9	99.7	24.3
40	405.0	63.8	37.9	45.7	442.9	109.5
60	564.0	53.4	53.9	58.0	617.9	111.4

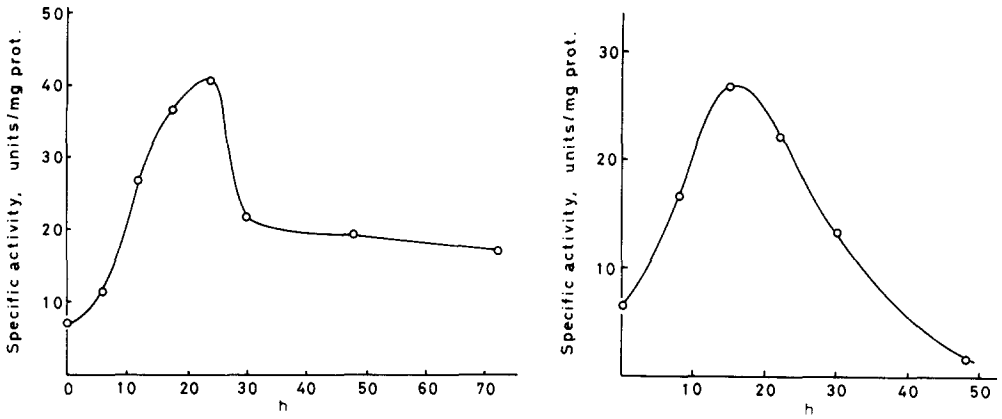

Fig. 4. Change in galactokinase activity during spherule formation. Spherulation was induced with 0.5 M mannitol.

Fig. 5. Change in galactose-1-phosphate uridyltransferase activity during spherule formation. Spherulation was induced with 0.5 M mannitol.

rulation by the addition of mannitol. These enzymes may open the pathway to the biosynthesis of GalN-1-*P* and UDP-galactosamine. In either case, about a 4—5-fold increase in the enzyme activity occurred during the early phase of spherule development. After the initial rise, however, the activity of both enzymes decreased sharply.

Discussion

Data in Table I and Fig. 1 show that the synthesis of spherule wall became active after 10—20 h in induction medium. At this stage of development, most of the plasmodia were found to be filled with numerous globules. These results coincide with the cytological observations of Guttes and Guttes [1] and of Höweling et al. [16], who have shown that the wall formation begins after the cytoplasmic cleavage and the rounding up of plasma pieces.

McCormick et al. [3] and Farr et al. [4] have reported that the spherule wall of *P. polycephalum* is composed mostly of polygalactosamine. Zaar et al. [5] have recently shown that the wall contains neutral sugars other than galactosamine, but the galactosamine polymers are still considered to be major fraction of the wall. From the solubility of the galactosamine polymers in alkali or acid, McCormick et al. [3] considered that the extent of *N*-acetylation was very low. In fact, we found that the acetylated amino sugar constituted only a few percent of the total galactosamine. These results indicate that the wall component of the spherule is unique since such a galactosaminoglycan is not commonly found in microbial walls. Distler and Roseman [17] have reported the occurrence of a galactosamine polymer in the culture filtrate of *Aspergillus parasiticus*. Later, Reissig and Glasgow [18] found a similar galactosamine polymer produced by *Neurospora crassa*, which inhibited hyphal growth and caused vacuolation. The polymer is only partially acetylated and the complete acetylation diminished the biological activity. Galactosamine polymers have also been

found in some fungi as a minor component of cell wall [19]. Although no information is available concerning the synthesis of these galactosamine polymers, several lines of evidence suggest that in *Neurospora*, UDP-*N*-acetylgalactosamine is the precursor for cell wall polygalactosamine [19,20]. Edson and Brody [20] have shown that UDP-*N*-acetylgalactosamine in this organism was synthesized via the epimerization of UDP-*N*-acetylglucosamine. Occurrence of *N*-acetylglucosamine-4-epimerase (EC 5.1.3.7) in spherulating plasmodia of *P. polycephalum* has been reported by Hiatt and Whitely [21]. The enzyme activity somewhat increased during the spherulation but the change was less marked as compared to the large increase reported for the myxospore coat synthesis in *Myxococcus xanthus* [22], in which the most of galactosamine residue in wall polysaccharide was found to be *N*-acetylated [14]. If the polygalactosamine in spherule wall is synthesized via UDP-*N*-acetylgalactosamine the most closely related example would be the synthesis of chitin. Polyoxin D, a known inhibitor for chitin synthetase (EC 2.4.1.16) [15], however, showed little effect on the formation of galactosamine-wall in the spherulating plasmodia.

In this experiment, we compared the chromatographic pattern of nucleotide sugars and sugar phosphates in spherulating plasmodia with that of growing plasmodia. However, the quantitative evaluation of the incorporation rate of radioactive sugar to individual intermediary compounds was difficult because the carbon source in spherulating plasmodia was mainly supplied by the degradation of reserve substances, such as glycogen. Total amount of hexosamine derivatives in cytoplasmic fraction was relatively unchanged during spherule formation (Table I), but qualitatively, obvious differences were observed in the chromatographic elution profile of acid soluble substances. It was found that the major change in the labeling pattern occurred in the regions of GalN-1-*P* and UDP-galactosamine. These compounds were found in the acid soluble fraction of rat liver after perfusion with [^{14}C]galactosamine and it is suggested they were synthesized by the pathway of galactose metabolism [23]. The enhanced synthesis of these galactosamine derivatives is also conceivable from the change in the activity of galactokinase and galactose-1-phosphate uridyltransferase. These observations suggest that a possibility of the direct involvement of these hexosamine compounds to the biosynthesis of spherule wall without *N*-acetylation cannot be excluded.

The addition of cycloheximide blocked the formation of spherule wall. Thus, there is an apparent requirement for protein synthesis for the incorporation of galactosamine into the wall polymer. Zaar et al. [5] found the existence of glycoprotein in the spherule wall. It is possible that the co-synthesis of wall protein is necessary for the galactosamine incorporation. Increase in some enzyme activities associated with the morophological change [21,24] may be, at least partly, due to de novo synthesis of protein.

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