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HEXOSAMINE BIOSYNTHESIS AND ACCUMULATION BY FUNGI IN LIQUID AND SOLID MEDIA*†

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ABSTRACT

Hexosamine biosynthesis and incorporation into polysaccharides and glycoproteins have been studied in four species of fungi—*Aspergillus niger*, *Penicillium citrinum*, *Cladosporium cladosporoides*, and *Fusarium moniliforme*. Hydrolytic, recovery, and analytical methods are described for the estimation of hexosamine accumulation in fungal growth. Optimum yields of hexosamine from matrix are achieved by using hydrolysis with strong acid (8M hydrochloric acid) over a period of 2–3 h. For all fungal-growth studies, hexosamine was quantitated, after hydrolysis, by an automated, amino acid analyzer programmed for the separation of amino sugars. Methods were also developed, using gas–liquid chromatography (nitrogen-selective alkaline flame-ionization detector and trimethylsilyl derivatives of hexosamines), and the modified Morgan–Elson reaction of *N*-acetylated hexosamines. Both the amino acid analyzer and gas-chromatographic method quantify nmol amounts of hexosamine per mg of dry-weight sample. In all phases of the growth cycle, a linear correlation was found for the four fungi between the amount of hexosamine per unit time, age of culture, and quantity of mycelial biomass in liquid medium. With solid corn-meal as a fungal growth-medium, samples also demonstrated a linear correlation, but only between hexosamine biosynthesis and age of culture, as biomass was not determinable. Autolysis of hexosamine occurs to only a limited extent during late stationary-phase in liquid medium and on corn.

INTRODUCTION

In a previous paper[2] concerned with nucleotide turnover during polysaccharide biosynthesis, it was noted that the hexosamine-containing nucleotide sugar, uridine 5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl diphosphate) (UDP-GlcNAc, **1**) is present in much larger quantity than any of the other nucleotides or "nucleotide"

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.
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sugars during the cell cycle (greater than half of the total nucleotides in the pool). As with other nucleotides in the pool, however, the "nucleotide sugars" are subject to several maxima and minima during the cell cycle, perhaps reflecting bioenergetic periodicity[2]. No exception to this nucleotide periodicity is noted for **1** in the fungi. Biosynthesis of **1** results in a direct precursor of the fungal cell-wall glycoproteins, chitinaceous mycelium, and extracellular glycoenzymes. In the fungi, total hexosamine signifies only 2-amino-2-deoxy-D-glucose (**2**), and 2-amino-2-deoxy-D-galactose (**3**). The present work was undertaken to examine carefully whether total hexosamine biosynthesis and accumulation in the total fungal system (nucleotide pool, mycelium, and extracellular glycoproteins) occurs continuously in a linear fashion, or with the same periodicity observed for **1** in the nucleotide pool[2].

A number of attempts have been made to correlate biosynthesis of fungal hexosamine with biomass accumulation[3-8]. These papers have been useful in the fermentation industry and in assessment of grain quality to obtain an early index of the degree of mold infection, by using mycelial hexosamine as one arbitrary unit of biomass content. A point not previously proven by careful chemical analysis, however, is whether the hexosamine in the fungal biomass accumulates linearly for each species, even though its relative quantity per unit of biomass differs from species to species. Also, the extent to which existing hexosamine is autolyzed by the growing network of multinucleated fungal chambers in the mycelium is not known. Four fungi were chosen for this study (see Experimental), representative of *fungi imperfecti*, to demonstrate the foregoing points among common field and storage molds. In addition to the fundamental biochemical questions addressed in this paper, it is also hoped that this research might reinforce other methods of fungal estimation in grain to give warning of potential mycotoxin sources. The relatively low concentrations of hexosamine in cereal grains makes the method more attractive because the quantity of fungal mycelium hexosamine is distinguishable from that of the grain.

In undertaking this work, it was apparent that adequate recovery of the hexosamine upon hydrolysis was of paramount importance. Therefore, a highly sensitive, automated hexosamine assay had to be developed to prove hydrolytic recoveries of hexosamine from grain polysaccharides as well as from mycelium. Because a rather expensive, automated, amino acid analyzer was chosen for this task, the cheaper and more-available gas-chromatographic and colorimetric methods for hexosamine analysis were also investigated to make the present results more available to other workers.

EXPERIMENTAL

Hexosamine-containing materials for hydrolytic-recovery studies. — Crude chitin (Aldrich Chemicals, Milwaukee, Wisconsin) was stirred at 10° for 48 h in 40% hydrochloric acid[9]. Cold water was added to dilute the suspension and precipitate the chitin. The suspension was centrifuged at 5000 r.p.m. for 15 min in a Sorvall centrifuge, Model RC2B. The supernatant was discarded and the residue was washed

repeatedly with double-distilled water until the supernatant was clear at pH 6.8. The residue was then freeze dried and stored at room temperature.

Mycelium used in control experiments was *Aspergillus niger* grown in 3% yeast-extract broth for 4 days and then harvested by filtering and washing with distilled water. The resulting mat of mycelium was dried under vacuum to constant weight and stored under refrigeration until needed. Pericarp to be hydrolyzed to afford baseline levels of hexosamine in corn was hand-dissected from a hybrid (B37TMSCH84) (ON43RFXA619) obtained from the University of Illinois experital farm, as well as from 16 different commercial strains for comparison[10]. Corn pericarp was ground through either a 40- or 60-mesh screen on a Wiley mill, extracted with hexane, and then dried under vacuum.

Optimization of hydrolysis conditions with 8M hydrochloric acid. — Up to 10 mg of 2-amino-2-deoxy-D-glucose or 2-amino-2-deoxy-D-glucose-containing polysaccharides or mycelia in a 5-ml Reacti-Vial® (Pierce Chemical, Rockford, Illinois) were hydrolyzed[11] in 0.5 ml of 8M hydrochloric acid for various times from zero up to 6 h at 95°. The conditions established by Swann and Balazs[11] were adapted in this work to give maximum recovery of hexosamine. After heating, the hydrolyzates were cooled in ice, filtered through Celite to remove solid residues (if necessary), and then evaporated to dryness *in vacuo* (0.5 mm) over solid potassium hydroxide in a desiccator to remove acid. To expedite removal of acid under vacuum and protect against bumping, the Reacti-Vial® was fitted with a glass tube (10 × 150 mm) through the hole in the vial cover and sealed with a 10-mm i.d. Viton "O" ring over the tube under the screw cap. By first cooling to −60° and laying the vials with the tubes on a slight angle in a large desiccator, the acid was evaporated off very quickly (45 min) and any bubbles were caught in the tube. The contents of many as 50 such vials may be evaporated at once. Tygon® tubing was avoided as the "O" ring material, because it introduces phthalates into g.l.c. mixtures. The residue was then redissolved in 0.5M sodium citrate buffer (pH 2.2, 2 ml) for amino sugar analysis with the amino acid analyzer[12]. Hydrolyzates were analyzed on a Beckman, automated, amino acid analyzer[13], Model 121M equipped with a special amino sugar program written for this instrument and featuring automated changes of buffer[14]. In this program, **2** has a retention time of 63 min and **3**, 72 min.

For study on the recovery of **2**, 0.2 ml of aqueous solution of **2** (60 mg/ml) was pipetted into each of 30 vials. To this solution was added 0.4 ml of 12M hydrochloric acid at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 h; 3 vials at each time-period. The vials were placed into the oven at various times, and all withdrawn at the same time and cooled in ice.

Mixtures containing various combinations of **2**, chitin, mycelium, and corn were hydrolyzed in 8M hydrochloric acid for 2 h at 95° as just described. The weights of the various components were kept equal with respect to each other in each vial; for example, in a three-component mixture, the ratio of weights of components would have been 1:1:1. The ratio of weight of material with amount of acid was maintained in all vials.

Fungal growth curves. — Fungal cultures were acquired from the collection of the Northern Regional Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Peoria, Illinois, and are identified by the culture numbers assigned in that collection: *Aspergillus niger*, NRRL 6009; *Penicillium citrinum*, NRRL 6010; *Cladosporium cladosporoides*, NRRL 6078; and *Fusarium moniliforme*, NRRL 3197. Stock cultures were maintained either on Sabouraud Dextrose Agar from Difco Laboratories (Detroit, Michigan) or on corn meal (obtained from a local grocery store) milled by Big Spring Mill, Inc., Elliston, Virginia. Liquid medium used for growth studies was yeast-extract broth obtained from Difco Laboratories. Growth studies on solid media utilized either the white corn meal milled by Big Spring Mill, Inc., or corn kernels already described as pericarp source.

Growth curves of fungal species in liquid medium. — To 100 ml of 5% yeast-extract broth was added 1 ml of spore-water inoculum prepared by washing spores from a 2-week agar slant with 5 ml of sterile water. Cultures were rotated at ~125 r.p.m. on an Eberbach culture-shaker. Duplicate flasks were harvested every 24 h for 5 days by filtering through a Millipore® filter (3 μm pore size, Millipore, Inc., Bedford, Massachusetts), and washing the mycelia 3 times with double-distilled water. When the filtrate appeared clear, the mycelia were dried *in vacuo* overnight (16 h). The dried weight of the harvested material was recorded. Approximately 10 mg of dried material was weighed and hydrolyzed in 0.5 ml 8M hydrochloric acid for 2 h at 95°, and then immediately cooled in ice. After the samples had been evaporated to dryness *in vacuo* as described, the residue was redissolved in 1.5 ml of citrate buffer (0.5M, pH 2.2) for analysis of amino sugars on the automated, amino acid analyzer equipped with an Autolab System AA Integrator.

Growth curves of fungal species in solid medium. — To 40 g of commercial corn meal was added 1.5 ml of a 66 p.p.m. solution of tetracycline hydrochloride (Parke–Davis, Detroit, Michigan) in double-distilled water. This mixture was autoclaved and cooled. Duplicate flasks were inoculated with 1 ml each of a spore suspension of one species. The eight flasks were then shaken thoroughly to mix inoculum and growth at room temperature (about 26°) on a shaking apparatus at 125 r.p.m. Spatulas for sampling were sterilized with 70% ethanol and flamed as a precaution against contamination before being dipped into the flask aseptically. After solid had been removed and placed in small sample-vials, it was immediately evaporated to dryness *in vacuo* and frozen at 4° for storage if not assayed immediately. Hydrolysis was performed by using 15 mg of dried material in 0.6 ml of 8M hydrochloric acid for 2 h at 95°. Data for amino sugar analysis are expressed as μg of hexosamine per mg dry weight of sample harvested. Total hexosamine in each sample was the sum of the quantity of 2 and 3 present.

Gas-chromatographic analysis. — G.l.c. analyses were performed on a Varian–Aerograph series 2400 dual-column instrument equipped either with two flame-ionization detectors or with one flame-ionization and one alkaline flame-ionization detector for specific nitrogen detection. A Microtec (Tracor) Model 220 gas chro-

matograph equipped with a Coulson conductivity detector was used to check specific nitrogen detection. A Spectra Physics Autolab System I integrator was used to compute peak-areas and concentrations according to Spectra Physics method[15] 2C involving use of a known volume of internal standard in each sample. Mass spectra of the per(trimethylsilyl)ated 2-amino-2-deoxy-D-glucose (4) were taken on a Varian-MAT Model 112 gas chromatograph-mass spectrometer.

N,N-Methyldioctylamine (Eastman Kodak Company, Rochester, New York) was used as internal standard in determining 4. All reagents used in the trimethylsilylation mixture [bis(trimethylsilyl)trifluoroacetamide(BSTFA)], Tri-Sil Z®, chlorotrimethylsilane, and dry pyridine) were obtained from Pierce Chemical Company (Rockford, Illinois).

Trimethylsilylation. — The hydrolyzate of hexosamine as described was filtered through a Celite pad to remove debris. The filter was then washed with a small amount of water, and the eluents were combined and freeze-dried in the small vials. The residue was silylated in the Reacti-Vial® with a 3:2:2:2 mixture (1 ml) of BSTFA, Tri-Sil Z, chlorotrimethylsilane, and pyridine. The silylation mixture was then heated for 20 min at 90° in the sealed vial and then cooled in ice. The sample could then be injected directly into a gas chromatograph equipped with a flame-ionization detector, or the trimethylsilyl ethers could be extracted into chloroform by the method of Partridge and Weiss[16], with a few modifications to stabilize the derivative and remove interfering salts. The silylation mixture was quantitatively transferred to a screw-top, conical centrifuge-tube equipped with Teflon®-lined cover using 1 ml of chloroform. Hydrochloric acid (3M, 3 ml) was added and the mixture was shaken, and centrifuged to clarify layers. The acid layer was withdrawn with a disposable pipette, and the chloroform layer extracted with 1 ml of ion-free water, 1 ml of 0.5M sodium hydrogencarbonate, and then 1 ml of ion-free water. The resulting chloroform layer was dried with about 2 mg of anhydrous sodium sulfate and transferred to another screw-top vial for storage. For use with the nitrogen-specific detectors (alkaline flame-ionization or Coulson conductivity), chloroform is incompatible as a solvent and the sample had to be transferred into hexane. The chloroform was evaporated under a stream of nitrogen and the sample was redissolved in a known amount of "nanograde" pure-grade hexane containing the internal standard methyldioctylamine. Samples could be injected into a gas chromatograph equipped with flame-ionization detector, alkaline flame-ionization detector[17], or Coulson conductivity-detector. The column used was 5.5 m × 2 mm (i.d.) of 3 percent SP-2100 on 80–100 mesh Supelcon AW-DMCS. Peaks of 4 appeared at 28 and 30.5 min after injection. However, pertrimethylsilylated 3 could not be determined simultaneously with 4 by this method. Appropriate peak–area response calibration-factors for 4 were calculated and used in the Spectra Physics Autolab System I integrator.

Hexosamine by colorimetric assay. — In the complex mixture from whole mycelial hydrolyzate, the method of Elson and Morgan[18] in all of its modifications did not give reliable results. However, use of the filtered hydrolyzate as already described, and the procedure of Benson[19] for hexosamine determination in the

presence of primary amines, gave excellent reproducibility and correlation with results of amino acid analyses. For routine screening of fungal samples for hexosamine where extreme sensitivity was not a problem, it was considered that Benson's modification of the Morgan–Elson reaction[20] should be followed exactly, with the limitations set forth by the author[19]. However, a quantity of fungal samples 5–10 times that used for the methods described in this work should be employed to ensure adequate sensitivity.

RESULTS AND DISCUSSION

Hexosamine release from mycelial matrices. — A review of the literature on dissolution of biological materials and subsequent determination of hexosamine revealed that some investigators, primarily interested in the determination of hexosamine, had used concentrated acids and shorter heating-times with lower temperatures[7,11]. To determine if their approach might be applicable to the present problem, compound **2**, chitin, mycelium, and mixtures of these components were hydrolyzed in 8M hydrochloric and 4M sulfuric acids at 95°. Results shown in Table I indicate that the weight-percent yields of **2** obtained with 8M hydrochloric acid followed by direct evaporation of acid were considerably higher than those with 4M sulfuric acid and neutralization by basic ion-exchange. Loss of hexosamine was assumed to be due to adsorption to the polystyrene resin-matrix. In Table II, the yield of **2** from the **2**-chitin mixture indicates little influence on the recovery of **2** from admixture. Yields of **2** from mycelia mixed with corn meal appear much lower. This lower amount of **2** might be accounted for, in part, by reactions of hydrolytic products of corn meal with those of **2** in Maillard, Schiff base, Amadori rearrangement and subsequent, reverse aldol reactions.

The release of **2** as a function of time from polysaccharides containing **2** was determined by hydrolyzing chitin alone (acid swollen as described in the Experimental section), and chitin plus mycelium mixtures as graphed in Fig. 1. For chitin alone, there was a sharp rise in release of **2** within one h followed by a small decrease and

TABLE I

COMPARISON OF PERCENT RELEASE OF **2** BY USING 8M HYDROCHLORIC AND 4M SULFURIC ACIDS

Material	*Release (%)*	
	8M *HCl*	4M H_2SO_4
2	75.5	40.8
Chitin	38.9	14.8
2 + Chitin[a]	57.2	12.6
Mycelium[b]	0.9	0.1

[a]Ratio of 1:1 on weight basis. [b]Percent wt. of **2** detected from hydrolysis mixture.

TABLE II

COMPARISON OF RELEASE OF 2 FROM POLYMERS WITH M SULFURIC AND 8M HYDROCHLORIC ACIDS

Fungus	*Acid used*	*Time of hydrolysis (h)*	*Amount of hexosamine*[a]
Aspergillus niger			
mycelium	M H_2SO_4	6	22.6 μg/mg
	8M HCl	1.5	40.8 μg/mg
corn meal + mycelium[b]	M H_2SO_4	10	407 ng/mg
	8M HCl	2	488 ng/mg
Penicillium citrinum			
mycelium	M H_2SO_4	6	23.5 μg/mg
	8M HCl	1.5	84.9 μg/mg
corn meal + mycelium[b]	M H_2SO_4	10	886 ng/mg
	8M HCl	2	1119 ng/mg
Cladosporium cladosporoides			
mycelium	M H_2SO_4	6	13.7 μg/mg
	8M HCl	1.5	77.8 μg/mg
corn meal + mycelium[b]	M H_2SO_4	10	243 ng/mg
	8M HCl	2	374 ng/mg
Fusarium moniliforme			
mycelium	M H_2SO_4	6	37.1 μg/mg
	8M HCl	1.5	113.5 μg/mg
corn meal + mycelium[b]	N H_2SO_4	10	169 ng/mg
	8M HCl	2	197 ng/mg

[a]Values expressed at time of maximum release. [b]Ratio of 1:1 on weight basis.

then an increase again at 3 h. The maximum yield at 3 h was followed by a gradual decrease. The presence of two maxima probably represent two different regions in chitin–those acetal linkages that are readily hydrated and those that are more resistant. The steady decrease after the maxima is probably due to degradation of 2 in the acid mixture. In the chitin–mycelium mixture, there occurred after a period of 1 h a balance between release and decomposition of 2. After 4 h, a steady increase was observed in the release of 2 from the mixture. In the chitin–mycelium experiment, the yields were even much lower, again indicating loss of 2 by reaction with mycelial products. Increase after 4 h is probably due to preponderant quantities of 2 free of further reaction with diminished quantities of neutral sugars from the mycelium.

As shown in Table II, there is less degradation of 2 with 8M hydrochloric acid during the hydrolytic period than when M sulfuric or 2M hydrochloric acids were used for 10 h. At 2 h, for example, about 75% of starting material is recovered when 8M hydrochloric acid is used.

To optimize the conditions for release of 2 from fungal material in 8M hydrochloric acid, an experiment was performed involving hydrolysis of samples of the four fungal species of interest for various times, evaporating to dryness *in vacuo*, and assaying for hexosamine levels with the amino acid analyzer. Results in Fig. 2 show a rapid release of hexosamine within 0.5 h; each species releases a characteristic, and

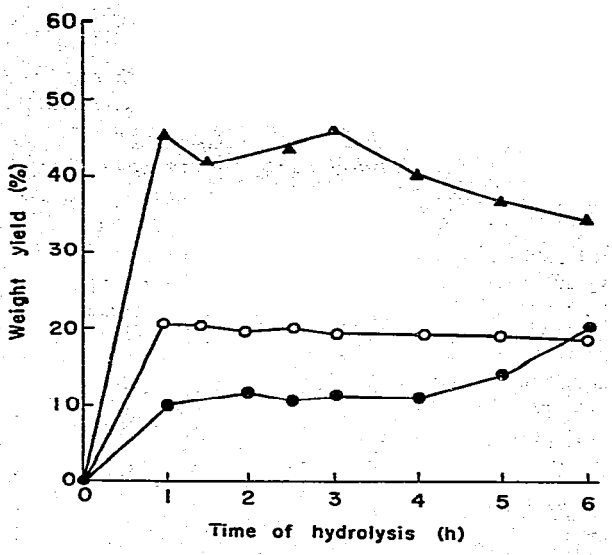

Fig. 1. Hydrolysis of chitin and of chitin plus mycelium by 8M hydrochloric acid. Chitin (▲–▲) and a mixture of chitin and mycelium (1 : 1 by weight; *Aspergillus niger* grown in yeast extract) (●–●) were hydrolyzed for various times in 8M hydrochloric acid at 95°, and then analyzed for hexosamine on the amino acid analyzer. The quantity of **2** recovered was expressed as the weight-percent yield of **2** as compared to the initial weight of material placed in the vial. The line given with open circles (○–○) shows the expected weight-percent yield for the mixture, as calculated from values obtained when the components were hydrolyzed individually.

perhaps chemotaxonomic, quantity of hexosamine. The release of hexosamine then oscillates with time; that is, there is a steady or fluctuating level of hexosamine from the mycelium. It would seem from this experiment that a 2-h hydrolysis of fungal mycelium would release a high proportion of the hexosamine present. Further hydrolysis would serve only to increase the degradation of previously released material and would not release appreciably more.

Earlier studies of corn components established that most of the **2** contained in the corn kernel is present in the outer layer, the pericarp[10]. In the pericarp, **2** has been shown to be a component of a glycosaminoglycan[10]. To establish the highest possible background levels of **2** that could be encountered in the screening of corn samples for fungal contamination, pericarps from 16 varieties of hybrid corn were ground and analyzed for **2**. Values of **2** in pericarp ranged between 100 and 202 p.p.m. of hydrolyzed sample, as contrasted to 20–40 p.p.m. in whole corn. There is a notable absence of **3** in corn.

To test these optimized hydrolytic conditions, a series of fungus-infected corn samples collected in the field or in storage were hydrolyzed to determine the hexosamine content. All samples had been found by agar culture to be infected with fungus and also by microscopic examination of dissected corn kernels before grinding;

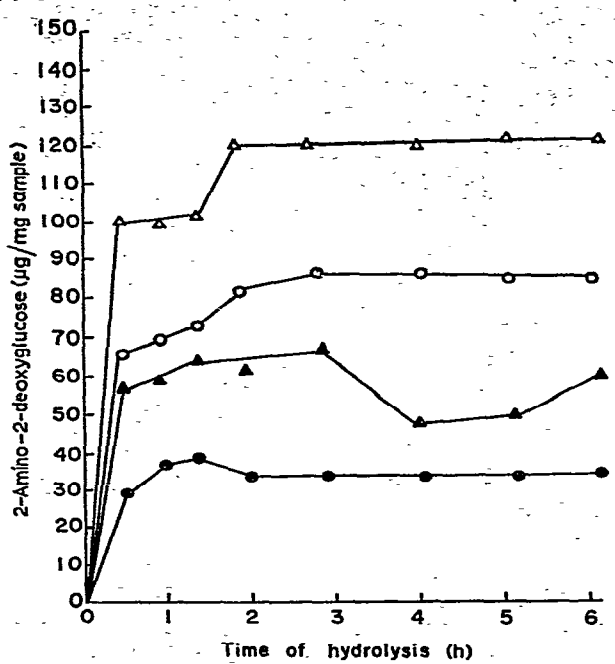

Fig. 2. Release of 2 and 3 from mycelia of four fungal species upon hydrolysis with 8M hydrochloric acid. Samples of mycelia from *Aspergillus niger* (●–●), *Penicillium citrinum* (△–△), *Cladosporium cladosporoides* (▲–▲), and *Fusarium moniliforme* (○–○) grown in yeast-extract broth were hydrolyzed in 8M hydrochloric acid at 95° for various times. Triplicate samples were analyzed for hexosamine by the amino acid analyzer. Only traces of 3 were found in this experiment. Points denote the sum of 2 and 3.

some had greater degrees of infection than others*. Results were compared to the release of 2 by M sulfuric acid for 10 h at 100°. In almost every case, values of hexosamine release were higher for 8M hydrochloric acid hydrolyzates. Some values for infected corn were lower, however, than control values on uninfected corn. These results demonstrate the problems encountered in obtaining a representative sample. There is also the problem of diluting the hexosamine produced by the fungus with those parts of the corn kernel that are not infected. This experiment shows the importance of grinding the sample as finely as possible and thoroughly mixing the ground sample. It must also be noted that this method has greater scatter below 100 p.p.m. of hexosamine because of the matrix problems. To overcome the problem of accuracy at the lower confidence level, use of increased amounts of sample and acid should be explored before routine use of this method is made to monitor fungal fermentations on solid medium.

Hexosamine biosynthesis as a monitor of fungal growth. — The question remaining to be answered in this study concerns whether the accumulation of hexosamine

*Agar culture was performed at the Northern Regional Research Laboratory, A.R.S., U.S.D.A., Peoria, Illinois (by personal communication, Dr. C. W. Hesseltine).

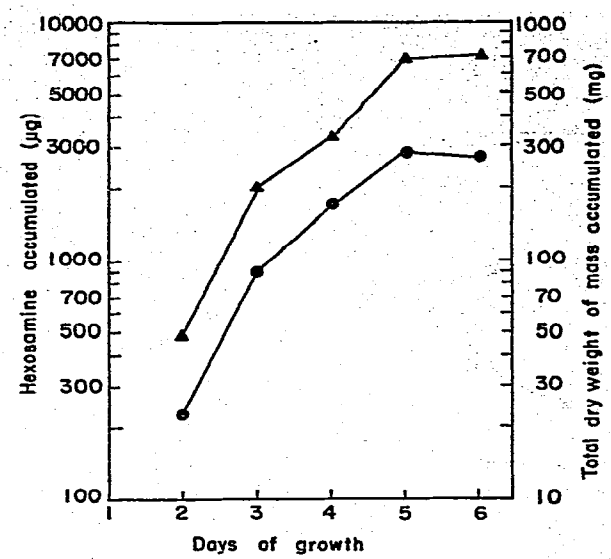

Fig. 3. Growth curve of *Aspergillus niger* in liquid culture. *Aspergillus niger* was grown in yeast-extract broth. Duplicate flasks were harvested at 24-h intervals by filtration, dried, and then weighed. A portion of the resulting, dried, mycelial mat was hydrolyzed and analyzed by using an amino acid analyzer for hexosamine. Values for 2 are the average of 4 determinations.

during growth follows an exponential growth pattern with biomass, as with other growth parameters of the organism. To verify that growth was indeed being measured, growth curves in liquid medium were monitored by the accumulation of biomass weight and of hexosamine. Fig. 3, typifying all four *fungi imperfecti* studied in liquid medium, shows that as the weight increased, hexosamine levels increased concomitantly. The two parameters were linear (within experimental error) on log scale for all four fungi.

A decrease in weight of fungal biomass after reaching stationary-culture phase has been noted by other workers[21,22]. After a carbon source had been exhausted, or other starvation conditions of some nutrient ensued, the fungus began to recycle cellular components of older cells for energy purposes. During the first day of starvation, cellular levels of nucleotides, lipids, and amino acids begin to decrease. During the second day of exposure to these conditions, carbohydrate levels decrease. These workers found, however, that hexosamine, once incorporated into cell-wall chitin, remains metabolically inert[21,22]. This same phenomenon was observed in this work when using species of *Aspergillus*, *Penicillium*, and *Cladosporium*. *Fusarium moniliforme* seems to deviate somewhat from these observations, showing a definite decrease (~10%) in hexosamine released in hydrolyzates from the period of stationary growth. This decrease could be attributed to an actual decrease in the amounts of hexosamine present, or could be attributed to a reduced extractability of the chitin and other polysaccharides in the cell wall. Upon aging, fungal cell-wall components become

deposited in multiple layers surrounded with lipid, melanin, lignin-like material, and protein.

Studies were extended to cultures of the four species grown on solid medium. Corn meal was inoculated with spores suspended in a minimal amount of water (one ml). Cultures were grown on a shaker to prevent sporulation[23], sampled every 3 days for 3 weeks, and once every 7 days for an additional 4 weeks. After the first 3 days, it was necessary to add additional moisture (5 ml of sterile distilled water) to support growth. Microscopic examination of material from the first harvest showed that the spores had germinated and mycelia were found. As early as 5 days after inoculation, the cultures of *Penicillium citrinum*, *Cladosporium cladosporoides*, and *Fusarium moniliforme* were becoming pigmented from the fungal growth: sun-yellow, dull orange, and bright orange, respectively. After two weeks of growth, the fungi had formed a matrix, holding corn meal in clumps and preventing efficient shaking; sporulation began at this time in all cultures except *Aspergillus niger*, in which sporulation occurred within nine days of inoculation.

The accumulation of hexosamine in these cultures on solid medium (on a μg per mg basis) is shown in Fig. 4 for *Aspergillus niger*, which is representative of all four species on corn. Each line represents a separate culture; each point, the average of two determinations. Harvested material was placed in a small vial, dried for at least 6 h *in vacuo*, and ground with a spatula to obtain a homogeneous sample. However, even with these precautions, the acquisition of a representative sample became a problem after the first 2 weeks. At that time, formation of the matrix of

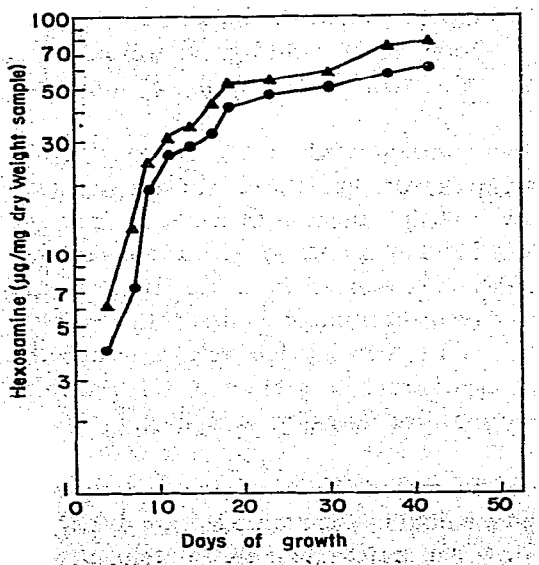

Fig. 4. Growth of *Aspergillus niger* in solid medium. Duplicate cultures of *Aspergillus niger* (autoclaved corn meal moistened with 5 ml of sterile distilled water 3 days after inoculation) were sampled at intervals and analyzed for hexosamines by using an amino acid analyzer. Each point given is the average of two determinations of 2 and 3; each line represents one culture flask.

TABLE III

HEXOSAMINE ANALYSIS OF FUNGUS-INFECTED CORN KERNELS[a]

	21 days (total kernel)	*56 days (embryo)*	*105 days (embryo)* 2	3	*Total*
Aspergillus niger					
Outer region		11.91			
Embryo		17.56	11.65	0.95	12.60
Total Sample	2.28	29.47			12.60
Penicillium citrinum	4.95	11.63	17.14	1.14	18.28
Cladosporium herbarum	1.36	18.58	6.79	1.69	8.48
Fusarium moniliforme	0.87	7.73	1.50	1.33	2.83
Control	0.46				

[a]All values reported as nmol/mg of sample hydrolyzed.

mycelia and corn meal occurred with accompanying sporulation, further complicating the procedure of harvest. Plating of material after 4 weeks of growth showed no bacterial or exogenous fungal contamination present. In each culture, except those of *Penicillium citrinum*, there seemed to be two rates of growth, perhaps reflecting the availability of nutrient. A higher growth-rate when nutrient such as starch from corn was available was followed by a lower rate as some nutrients became rate-limiting. This growth limitation was not observed in *Penicillium citrinum*. In *P. citrinum*, however, growth limitation may be explained by formation of a complex matrix, and the fact that sporulation did not occur to a great extent with this species. *P. citrinum* also seems to attack small pieces of corn meal and to grow around these small portions, rather than form large areas of growth and consume all nutrients at once.

Fungal contamination in corn is often concentrated in the embryo region of the kernel. In experiments connected with the present study, surface-sterilized corn kernels were inoculated with spores by inserting them with a needle into the embryo region. These inoculated kernels were incubated and portions were sampled at 21, 56, and 105 days and ground in a Wiley mill. Results in Table III shown an increase in hexosamine content at 21 and 56 days over control values, followed by a decrease in three species and increase in one at the 105-day harvest. Although the experiment reported in Table III on whole corn kernels is preliminary, it indicates the usefulness of hexosamine accumulation to monitor fungal growth, even in whole-corn kernels.

Application of gas–liquid chromatography to analysis for 2 *in fungal hydrolyzates.* — The analysis for fungal hexosamine in solid medium by the amino acid analyzer[12,14] has proven a sensitive method of detection. When automated, this system may be run without the need of a technician's attention overnight and weekends. However, the amino acid analyzer method takes 7 h per sample from weighing of the dry, ground sample to printout of data by the integrator. The system itself is expensive

and would therefore not be practical for use in extended experiments on fungal hexosamine metabolism.

G.l.c. methods for hexosamines have already been studied by many workers[24]. Some derivatives of hexosamine are found to require long and tedious procedures for preparation, and do not represent any savings of time over the amino acid analyzer method. Among the derivatives reported for hexosamines are the alditol acetates[25,26], the 2,5-anhydroaldononitrile peracetates[27], 2,5-anhydroalditol peracetates[28], and the trimethylsilyl ethers[29,30]. Alditol acetates and aldononitrile aceates have proven excellent for neutral sugars, are very stable weeks after formation, and are eluted as a single peak. However, much less success is observed when hexosamines are derivatized by the foregoing methods. The pertrimethylsilyl ether (**4**) of **2** was found to take about 25 min to prepare (but decomposed upon being kept for a few h) and gave two peaks when chromatographed. An extraction procedure to stabilize trimethylsilyl ethers of sugars[16] was modified for this application; the derivative **4** proved to be stable for a period of days and made trimethylsilylation the method of choice in the present work. The procedure used for analysis of **2** in fungus-infected corn by g.l.c. is described in the experimental section. Gas chromatography–mass spectroscopy of both peaks in Figs. 5 for **4** have identical parent-ion masses at *m/e* 539. This molecular weight corresponds to an *N*-monotrimethylsilyl-1,3,4,6-tetra-*O*-trimethylsilylated derivative of 2-amino-2-deoxy-D-glucose for each peak in the

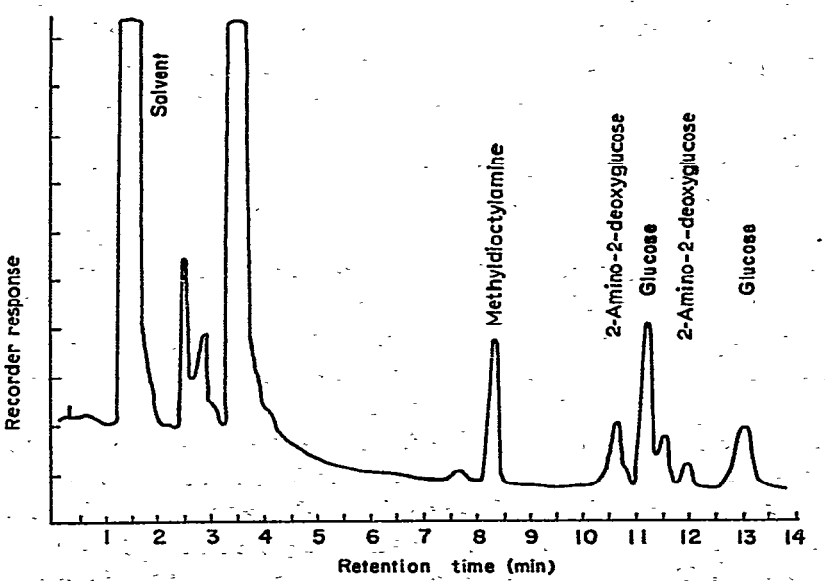

Fig. 5. Gas chromatogram of **4** in analysis of fungus-infected corn sample. Gas chromatogram obtained from analysis for **2** from *Pencillium citrinum* grown on solid-medium (corn meal) culture after 21 days of growth by the fungus. Chromatographic conditions used were: column packing, 3% SP-2100 on 80–100 mesh Supelcon SW-DMCS; column dimensions, 5.5 m × 2 mm (i.d.); temperature program, 160–230° at 2°/min; flow rates, helium (carrier gas) = 25 ml/min, hydrogen = 42 ml/min, air = 217 ml/min; alkaline flame-ionization detector; injector temperature = 255°, detector temperature = 242°.

chromatogram, assuming an α-, β-anomeric mixture. The pertrimethylsilylated **3** could not be determined by this method, because of poor resolution of its major peak from the second peak of **4**. This was not considered of great import in the present study, because the ratios of **2** to **3** were generally in the range of greater than 10 to 1.

The separation of **4** and per-*O*-(trimethylsilyl)-D-glucose from the foregoing fungal hydrolyzates was insufficient always to permit establishment of the concentration of 2-amino-2-deoxy-D-glucose. The high concentration of the glucose derivative masked out that of **4** (even in samples known to contain much **4**). Use of an alkaline flame-ionization detector, or a Coulson specific phosphorus–nitrogen conductivity detector, and a longer column essentially eliminated the difficulty. The nitrogen in **4** enhanced the signal in its peak response over that of non-nitrogen-containing sugars. The theoretical explanation of how the alkaline flame-ionization detector operates is still unknown. The detector consists of a rubidium sulfate salt-tip located at the anode. When an organic compound containing nitrogen or phosphorus passes through the flame and onto the cathode, the salt tip is thought to ionize. There can be a 10,000 to 1 response in favor of one molecule of nitrogen, as contrasted to the reference compound without nitrogen, when the temperature and symmetry of flame are finely tuned for nitrogen-containing organic compounds[17].

The linearity of response to **4** by this detector was demonstrated, with the peak area versus volume or amount injected being linear over a ten-fold range. Detector response to the chosen internal standard, methyldioctylamine, was equally as linear. The relationship of peak area of **4** as compared to peak area of the internal standard also was compared. As both relationships are linear, the response of the detector to the two compounds may be compared as a quantitatively equivalent ratio of the compounds.

As an illustration of the g.l.c. method, samples for growth curves on corn meal were hydrolyzed and analyzed by using both the amino acid analyzer method and the gas chromatographic method for **2**. Quantitative results are slightly higher for **2** by g.l.c. than those given by the amino acid analyzer method, but show the same upward trend of hexosamine accumulation during growth. This work would also recommend the alkaline flame-ionization detector for general analysis of **2** in the presence of neutral sugars, irrespective of source. The Coulson conductivity detector gives similar results but is much more difficult to operate.

Hexosamine could also be quantitated in the foregoing hydrolyzates by using a colorimetric method for 2-acetamido-2-deoxy-D-glucose, as published by Benson[19]. This method permits analysis of the *N*-acetylhexosamine in the presence of relatively high concentrations of primary amines (from amino acids, and the like that normally interfere). Both the amino acid analyzer and gas chromatographic analyses described here are much more sensitive (limits of hexosamine detection, 0.1 to 0.3 nmol/mg sample) than the Benson method[19], which in our hands was the most linear in the 15–50 nmol/ml range. However, the sample size and hydrolytic methods in this paper are readily scaled upward to the sensitivity range of this assay, in which case, Benson's method[19] is quite satisfactory for the purposes of monitoring fungal growth. When

applied to growth curves of the fungi, plots of accumulated 2 followed the exponential-growth curves with less scatter than with the conventional method[18].

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