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HIGH-PRESSURE LIQUID-CHROMATOGRAPHIC ASSAY OF NUCLEOTIDE-POOL CONCENTRATIONS DURING POLYSACCHARIDE BIOSYNTHESIS IN FOUR ASCOMYCETES*

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ABSTRACT

High-resolution liquid-chromatographic methods developed for analyzing nucleotide pools at the nanogram level in four representative species of ascomycetes (*Penicillium citrinum*, *Aspergillus niger*, *Fusarium moniliforme*, and *Cladosporium herbarum*) were used to study polysaccharide biosynthesis Nucleotides extracted from the mycelial mat were preseparated from interfering polysaccharides, glycoproteins, and nucleic acids on a column of Biogel P-2 Resolution of 18 nucleotides from each fungal species was accomplished on AS-Pellionex-SAX, pellicular anion-exchanger by using a high-pressure liquid chromatograph Nucleotides were identified by comparing peak retention-times, by differential u v absorption with two detectors in series at selected wavelengths, and by acid or enzymic hydrolysis with product identification by liquid chromatography Pyrimidine bases exceeded purines by at least three fold, and uridine nucleotides often constituted 60–80 mole percent of the total nucleotides, extractable cytidine nucleotides were negligible Uridine 5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl disphosphate) is the preponderant nucleotide throughout the growth cycles of all four species, amounting to 30–60% of all nucleotides present For all four fungal species, a burst of nucleotide formation was observed after the first 48 h (15–30 μmol/g tissue), with fluctuations that eventually fell to 0 1 μmol/g on the tenth day

INTRODUCTION

Upon initiating a detailed study of the fungal biosynthesis of amino sugars and the polysaccharides containing them, it became apparent that few quantitative data exist concerning the cellular-pool concentrations of "sugar nucleotides" and their precursors, the purine and pyrimidine nucleotides[2] Salvage of the bases, as well as

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bioenergetic utilization of the pyrophosphates in nucleotide pools for activation of glycosyl groups in acetal-linkage reactions, dictates that there be a dynamic system of feedback from this pool to all other aspects of carbohydrate metabolism[3,4] Fungi constitute an interesting study of these reactions, as the bulk of their bioenergetic and synthetic abilities is invested in polysaccharide biosynthesis

Although several laboratories have attempted to quantitate nucleotide pools during fungal growth[6-12], more-advanced methodology for resolution and quantitation of nucleotides is now available with the development of high-pressure liquid chromatography and high-resolution, pellicular anion-exchange resins[13-15] and conventional resins[16] These rigid, uniform-size cationic beads, combined with low dead-volume fittings and sensitive flow-through u v detectors, having internal volumes of 10 μl, now permit rapid, reproducible assay of nucleotides at nanogram levels[17,18]

The present work applies these recent advances extensively to establish quantitative values for nucleotide-pool concentrations in four ascomycetes, namely, *Penicillium citrinum*, *Cladosporium herbarum*, *Aspergillus niger*, and *Fusarium moniliforme* These four representatives are morphologically different, and synthesize hexosamine-containing polysaccharides in different amounts[1] Data presented here are, therefore, intended to guide future research on biosynthesis of fungal polysaccharides Although the literature[13-18] presents apparently straightforward methods for nucleotide analysis, the application of these methods to fungi is difficult because their large, vesicular, mycelial mats contain many extractable glycoproteins, polysaccharides, proteins, nucleic acids, and a host of other secondary metabolites from which the nucleotides must be freed before quantitation This separation was, therefore, also undertaken to develop methodology applicable to nucleotide analysis in other complex biological systems

EXPERIMENTAL

Extraction of nucleotides from fungal cultures — Four pure isolates of ascomycetes were obtained from the fungal-culture collection of the Northern Regional Research Laboratory, Peoria, Illinois *Aspergillus niger* (NRRL 6009), *Penicillium citrinum* (NRRL 6010), *Cladosporium herbarum* (NRRL 6078), and *Fusarium moniliforme* (NRRL 3197) These organisms were cultured in autoclaved, aqueous yeast extract (0 5% wt/vol, Difco, Detroit, Michigan) under uniform shaking, with each inoculated flask containing 150 ml of medium Inoculation was effected with a wire transfer-loop from spores of mycelia grown on slants of Sabouraud's dextrose agar (Difco, Detroit, Michigan) At appropriate intervals during the growth cycle, the cultured biomass was isolated by suction through coarse filter-paper and a stainless-steel Buchner funnel equipped with a metal screen (Millipore, Inc , Bedford, Massachusetts) The mycelial mat was washed on the filter with water, and weighed immediately (3–10 g of damp biomass per sample) The filtrate was not preserved for analysis in this study Growth curves for the four fungi were carefully estimated from

total accumulation of biomass A stationary phase was reached on the fifth or sixth day of growth in this medium for each organism, and nucleotide levels were monitored until the tenth day

The sample of mycelium was transferred to a Potter–Elvehjem tissue homogenizer and extracted with cold, aqueous trichloroacetic acid (10% wt/vol, 10–20 ml) The homogenate was quantitatively transferred to polypropylene tubes (50 ml) and centrifuged in a refrigerated centrifuge at 800 *g* The extraction with trichloroacetic acid was repeated twice more on the pellet in the homogenizer and the aqueous layers were combined The mycelial pellet was dried in a desiccator over potassium hydroxide under diminished pressure to obtain the dry-residue weight of sample The combined aqueous layers were extracted 5 times with ethyl ether (presaturated with water) to remove trichloroacetic acid The aqueous layer (pH 2–3) was then adjusted to pH ~6 8 with potassium hydroxide, freeze dried, and the residue reconstituted with a known volume (3–4 ml) of water (refer to *Calculations* for the relative quantities) before analysis Solutions were frozen in vials until the next separation step

Prepurification of fungal nucleotides by chromatography on Biogel P-2 with water elution — Polymers required separation from the nucleotides prior to high-resolution, anion-exchange chromatography without accompanying accumulation of large amounts of buffer salts in the nucleotide concentrate Earlier methods employing borate complexes of the nucleotide[19], or a volatile buffer[20] on polyacrylamide-gel columns, were unacceptable The procedure devised used simple elution from a Biogel P-2 column by water, separating the nucleotides from the bulk of protein glycoprotein, polysaccharide, and nucleic acid by what appears to be partition chromatography Biogel P-2 (200–400 mesh, Bio-Rad Laboratories, Richmond California) was swollen in double-distilled water and packed into three columns fitted with fine-mesh end-filters (2 5 × 50 cm, Pharmacia, Piscataway, N J) These columns were connected in series to the 19-μliter, ISCO flow-through detector system described later for use with a Bausch and Lomb Spectronic 700 spectrophotometer set at 254 nm Washing and elution of the column was effected with double-distilled water at 160 ml/h by using a Milton–Roy Minipump® (Milton–Roy, Inc Philadelphia, Pennsylvania) Eluate from the monitor was collected mechanically in 5-ml fractions Samples of the trichloroacetic acid extract in a few ml of water were applied to the column by means of a hypodermic syringe and a four-way valve port A typical separation is shown in Fig 1, the nucleotides emerged principally in fractions 1 and 2 at the time-intervals stated The fractions were pooled as in Fig 1, lyophilized, and the lyophilizate taken up in 1 ml of double-distilled water for injection into the high-pressure liquid chromatograph

High-pressure liquid chromatography of nucleotides — A Varian–Aerograph LCS 1000 nucleotide analyzer was used as the basic system Instead of the conventional long, narrow, anion-exchange column (3 3 m × 1 mm, 1 d), a shorter and wider, stainless-steel tube (1 m × 2 1 mm, 1 d) was dry-packed with AS-Pellionex™-SAX, strong anion-exchanger (Reeve Angel, Clifton, N J) This packing is composed of pellicular, hard-core glass beads (37–57 μm diameter) having a 1–2 μm-skin of

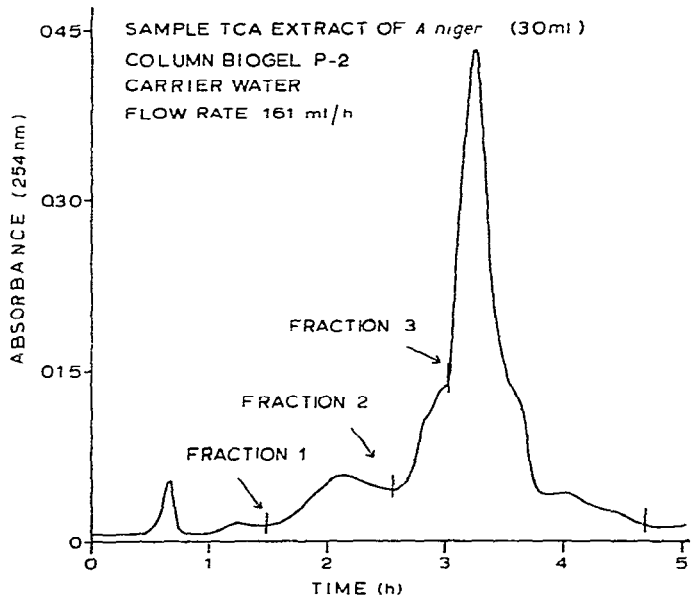

Fig 1 Example of preseparation on polyacrylamide gel (Biogel P-2) of nucleotides extracted from fungi (*A niger*) Other species gave similar results

anion-exchange groups To pack the column, the stainless-steel tube was fitted with a 2 1-mm, stainless-steel fritted disc having a 15-μm pore size (Reeve Angel, Clifton, N J) Small amounts of resin were added, and the column was tapped on the floor for 45 sec between additions of resin until the column was filled A 10 cm × 1 mm (i d) precolumn was connected to the column by a 1 59-mm to 3 175-mm stainless-steel union The precolumn was then packed by the same method A 3 175-mm stainless-steel end-plug was drilled through with a 1 59-mm bit A flat-ended, 3 175-mm drill was used to clear the inner wall of the end plug so that the column could be inserted completely into the port leading to the detector, so as to minimize any dead volume that could cause turbulent mixing The 1-m column was coiled in one loop to fit into a thermostated oven

Nucleotide analysis was effected by use of a linear buffer-gradient The concentrated buffer was M potassium dihydrogenphosphate (J T Baker, Co , reagent grade), pH 4 3, and 0 25M potassium chloride The dilute buffer was 2 5mM potassium dihydrogenphosphate, pH 3 6 Both buffers were passed through an 0 8-μm Millipore® filter (Millipore Corp , Bedford, Massachusetts) before use, to remove dust and bacteria The column flow-rate was 24 ml/h, the column pressure, 500 lb in^{-2} (gauge), and the column temperature, 70° Primary detection employed a u v detector monitoring at 254 nm

For consecutive monitoring of the same nucleotide peaks at two wavelengths, the exit port of the primary detector was connected in series by low dead-volume, stainless-steel capillary tubing (1 59 × 0 229 mm, i d) and low dead-volume unions to

a 19-μl, 10-mm light-path, flow-through cell (rated 200 lb in^{-2}, Instrumentation Specialties Corporation, Lincoln, Nebraska) The cell was fitted into the sample compartment of a Bausch and Lomb Spectronic 700 single beam, variable wavelength, u v –visible spectrometer (generally set at 280 nm) To monitor fluorescent peaks, an Aminco Fluoro-colorimeter having a flow-through cell (volume 40 μl) was similarly connected, in series with the effluent of the cell from the Spectronic 700 instrument All three detectors were connected to separate recorders Peak-area molar response-factors were calibrated for each separate detector

Identification of nucleotides, calculation of sample quantities from detector responses, and recovery of standard nucleotides after extraction with trichloroacetic acid — In both the standard mixtures (high-purity nucleotides obtained from Sigma Chemical Co , St Louis, Missouri, and P–L Biochemicals, Inc , Milwaukee, Wisconsin) and the biological extracts, nucleotides were identified by their retention times, by "spiking" the standards, and comparing the ratio of peak areas at two different wavelengths (usually 254 and 280 nm) Retention times of both standards and fungal nucleotides are shown in Figs 2–5 Furthermore, sugar nucleotides and other nucleotides were identified by enzymic hydrolysis with snake-venom nucleotidase II (pyrophosphatase, Sigma Chemical Co , St Louis, Missouri) To 50 μl of the nucleotide preparation were added 10 μl of M magnesium chloride, and approximately one μg of snake-venom nucleotidase II The hydrolyzed nucleotides were collected reinjected, and products identified UDP-hexosamine peaks were also identified by acid hydrolysis (8M hydrochloric acid for 2 h at 95°), evaporation of the acid under vacuum, dissolution of the residue in pH 2 3 citrate buffer and quantitation of 2-amino-2-deoxy-glucose or -galactose by means of a Beckman amino acid analyzer[21], with the computer integration and amino sugar program prepared for this instrument[22] Fluorescence detection was valuable for monitoring the nucleotide chromatograms to rule out the possibility of fluorescent impurities Purines and pyrimidines were determined by cation-exchange chromatography after hydrolysis in 88% formic acid and elution with 0 01M ammonium dihydrogenphosphate, pH 5 56

Recovery of standard nucleotides from the trichloroacetic acid extract was performed by the same procedure described The nucleotide standards were prepared gravimetrically The standards were also passed through a prepurification step on Biogel P-2 (see later for details) Recoveries were calculated as an average, percentage recovery after anion-exchange resolution of the mixture and calculation of peak-area ratios for individual components (in order of elution as in Fig 4), affording the following results,

CMP	104 2%	UMP	97 8%	AMP	101 4%	GMP	100 1%
CDP	103 5	UDP	103 7	ADP	99 4	GDP	98 8
CTP	102 9	UTP	100 0	ATP	98 8	GTP	99 9

These values averaged 100 9% for all nucleotides, indicating that there is complete recovery of nucleotides and that none were decomposed during the extraction procedure

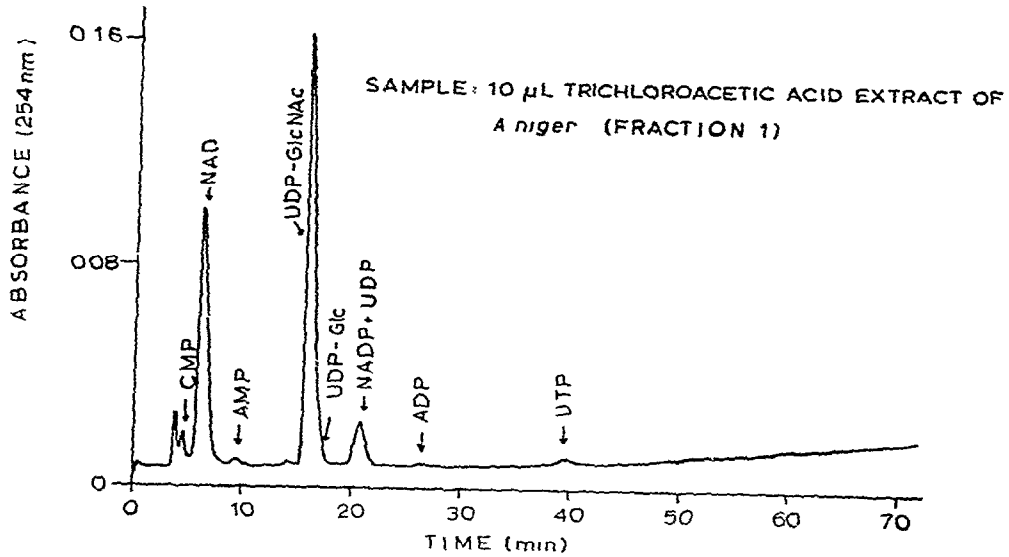

Fig 2 Chromatogram monitored at 254 nm for nucleotides in fraction 1, Fig 1.

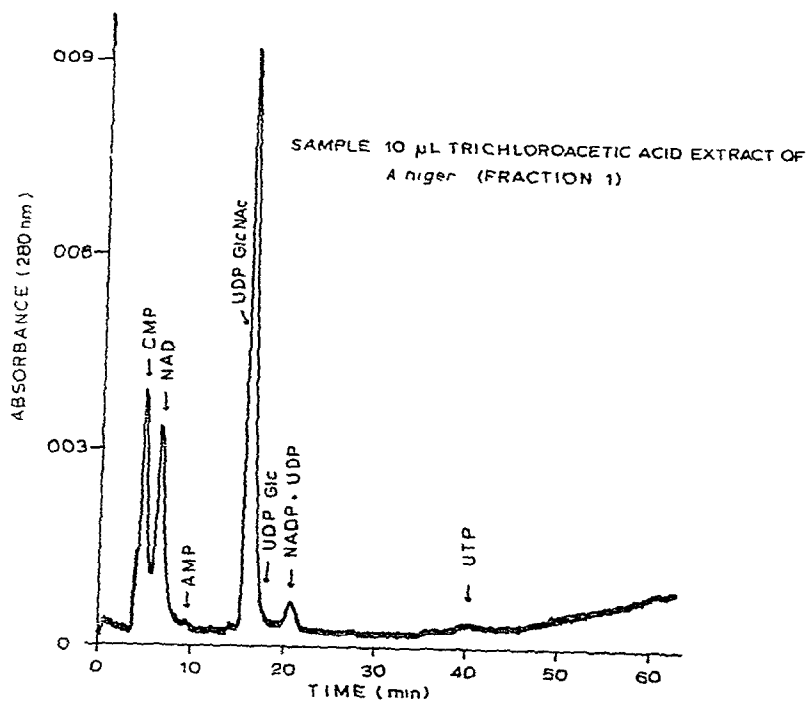

Fig 3 Chromatogram monitored at 280 nm for nucleotides in fraction 1, Fig 1

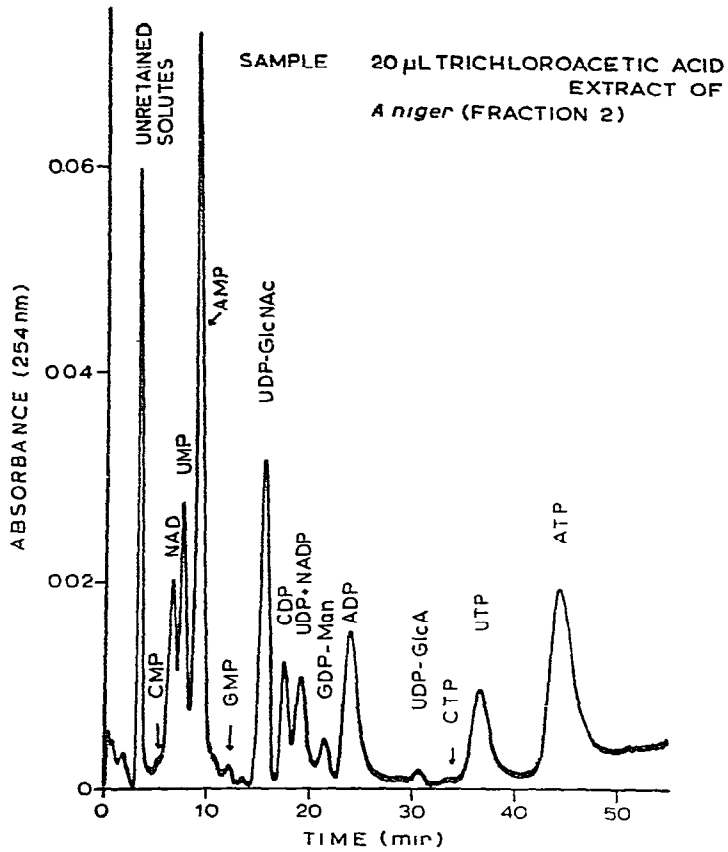

Fig 4 Chromatogram monitored at 254 nm for nucleotides in fraction 2, Fig 1

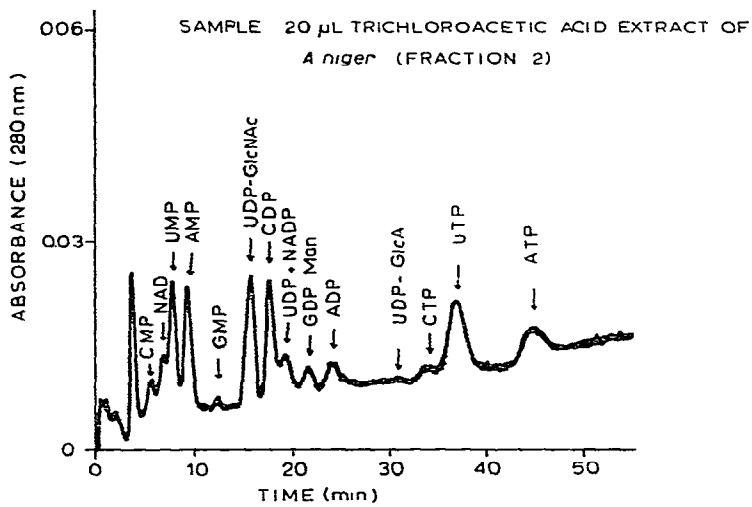

Fig 5 Chromatogram monitored at 280 nm for nucleotides in fraction 2, Fig 1

For each nucleotide injected, the peak area (peak height × peak width at half height, mm) was plotted against the amount injected (nmol) The slope of this plot is the peak-area response-factor for each nucleotide The slope calculated in the range of linear response on the plot was used for quantitation

Peak-area response-factors (picomol/mm^2) for nucleotides determined at 254 nm were as follows

CMP	20 31	UMP	16 78	AMP	9 00	GMP	4 21
CDP	124 0	UDP	36 52	ADP	21 00	GDP	19 06
CTP	99 35	UTP	32 72	ATP	22 72	GTP	24 93
NAD^+	14 73	UDP-Glc	26 49	UDP-GlcA	21 83	GDP-GlcNAc	24 58
GDP-Glc	14 27	GDP-Man	17 96				

By using the foregoing information, the formula for calculating the quantity of each nucleotide in the fungal biomass is as follows

$$\frac{\text{moles of nucleotide}}{\text{gram of lyophilized mycelium}} = \frac{\left(\begin{array}{c}\text{peak-area}\\ \text{response-factor}\\ \text{in pmol/mm}^2\end{array}\right)\left(\begin{array}{c}\text{area of peak}\\ \text{on chromatogram}\\ \text{in mm}^2\end{array}\right)\left(\begin{array}{c}\text{total volume of}\\ \text{sample from}\\ \text{extract}\\ \text{volume injected}\end{array}\right)}{\text{lyophilized weight, in grams, of mycelium extracted}}$$

The total volume of each fungal-nucleotide sample from the extract was usually 1–2 ml, and the volume injected was 5 or 10 μl The weight of lyophilized mycelium ranged from 0 3–1 0 g Results for all four fungi reported in Tables I–IV were calculated in this way Blanks in certain columns of Tables I–IV indicate that the corresponding nucleotide was not detected within the limits of this instrument during the analysis Duplicate samples taken from the sample culture were pooled for each point in the growth curve and had an average standard-deviation of $\pm 3\%$

RESULTS AND DISCUSSION

Well-resolved separations of nucleotides on the dry-packed, pellicular anion exchanger (such as Fig 4) were reproducible during several years However, the columns were changed at six-monthly intervals to prevent loss of efficiency through to possible degradation of the resin The nucleotides themselves, in standards or extracts, were stable and showed degradation only after several cycles of thawing and refreezing The basic chromatographic-system described here is a practical tool of great stability, proved in hundreds of nucleotide assays to perform in a manner that affords accurate determinations of the biological medium Column stability, performance, and response factors were monitored by periodic injection of standard nucleotides for comparison with earlier analyses

TABLE I

QUANTITATIVE RELATIONSHIPS OF TOTAL NUCLEOTIDES DURING GROWTH CYCLE OF *Aspergillus niger*

Nucleotide	*Growth time (h)*								
	45	*51*	*71*	*120*	*142*	*165*	*189*	*231*	*237*
CMP		58[a] 0.38[b]		80 1.00	174 1.21	26 0.28		108 0.69	1,164 11.67
UMP	3,958 18.21	492 3.25	155 1.21		71 0.49	184 1.96	453 4.43	238 1.52	1,851 18.56
AMP	1,650 7.59	255 1.68	1,903 14.86	27 0.34	34 0.24	144 1.54	95 0.93	413 2.64	1,363 13.67
GMP	62 0.28	796 5.26	37 0.29	13 0.16	12 0.08	25 0.27	129 1.26		32 0.32
UDP-GlcNAc	4,808 22.12	6,673 44.11	3,749 29.28	5,434 67.83	8,987 62.65	5,131 54.79	3,333 32.59	5,653 36.13	1,112 11.15
CDP	883 4.06							1,024 6.54	1,919 19.24
UDP+NADP	2,136 9.83	482 3.18	6,611 20.39	1,198 14.95	1,574 10.97	1,537 16.41	2,611 25.53	1,643 10.50	683 6.85
ADP	3,057 14.06	2,809 18.57	1,244 9.72	75 0.94	999 6.96	551 5.88	756 7.39	384 2.45	452 4.53
GDP	181 0.83	68 0.45	176 1.37				95 0.03		

(Table continued on p. 462)

TABLE I (*continued*)

Nucleotide	*Growth time (h)*								
	45	*51*	*71*	*120*	*142*	*165*	*189*	*231*	*237*
CTP	177 0.81		600 4.69	44 0.55					1,397 14.01
UTP	1,384 6.37	100 0.66	1,262 9.86	364 4.54	359 2.50	415 4.43	807 7.78	763 4.88	
ATP	2,526 11.62	270 1.78	339 2.65	27 0.34	1,453 10.13	546 5.83	579 5.57	881 5.63	
GTP			96 0.78						
UDP-Gal+ UDPGlc	817 3.76	1,079 7.13	253 0.75	643 8.03	63 4.41	772 8.24	1,308 12.79	420 2.68	
UDP-GlcA								55 0.38	
NAD		2,047 13.53	378 2.95					3,913 25.01	
GDPGlc				23					
GDP-Man	95 0.44			83 1.04	49 0.34	33 0.35	71 0.69	151 0.96	
Total[c]	21,734	15,129	12,803	8,011	14,344	9,364	10,228	15,646	9,973

[a]nmol/g of lyophilized mycelium. [b]Mole% of all nucleotides present. [c]Refers to nmoles of all nucleotides per gram of lyophilized medium.

TABLE II

QUANTITATIVE RELATIONSHIPS OF TOTAL NUCLEOTIDES DURING GROWTH CYCLE OF *Penicillium citrinum*

Nucleotide	*Growth time (h)*							
	20	*40*	*64*	*90*	*114*	*137*	*165*	*185*
UMP[a]	7,218[b]	2,295	829	1,834	3,563	1,521	3,100	61
	49 45[c]	31 81	16 85	18 08	17 05	27 47	35 62	42 36
AMP	1,378	114	551	128		231	395	
	9 44	1 58	11 20	1 26		4 17	4 54	
GMP	101		25			14		
	0 69		0 51			0 25		
UDP-GlcNAc	793	1,395	2,313	2,672	8,157	2,824	4,110	83
	5 43	19 34	47 00	26 34	39 33	51 00	47 23	57 64
CDP		1,286			1,281			
		17 83			6 18			
UDP+NADP	158	960	484	407	1,341	98	493	
	1 08	13 31	9 84	4 01	6 47	1 77	5 67	
ADP	3,350	293	168	502	487	114	240	
	22 95	4 06	3 41	4 95	2 35	2 06	2 76	
GDP			50		368		147	
			1 02		1 77		1 69	
CTP			174		1,680			
			3 54		8 10			
UTP	306	509	153	4,071	806	148		
	2 10	7 06	3 11	40 13	3 89	2 67		
ATP	799	118	173	147	549	276		
	5 47	1 64	3 52	1 45	2 65	4 98		
GTP					151			
					0 73			
UDP-Gal+UDPGlc	393	149		372	2 046	88	194	
	2 69	2 07		3 67	9 86	1 59	2 23	
GDPGlc		12						
		0 17						
GDP-Man	100	83	1	11	340	223	23	
	0 69	1 15	0 02	0 11	1 64	4 03	0 26	
Total[d]	14,596	7,217	4,921	10,144	20,742	5,537	8,702	144

[a]No CMP was detectable [b]nmol/g of lyophilized mycelium [c]Mole % of all nucleotides present
[d]Refers to nmoles of all nucleotides per gram of lyophilized mycelium

TABLE III

QUANTITATIVE RELATIONSHIPS OF TOTAL NUCLEOTIDES DURING GROWTH CYCLE OF *Fusarium moniliforme*

Nucleotide	*Growth time (h)*							
	43	*67*	*99*	*121*	*143*	*167*	*191*	*215*
UMP[a]	1,786[b]	4,174	2,211	1,846	279	343	240	158
	5 24[c]	16 48	12 44	10 93	6 46	8 91	9 07	4 56
AMP	2,355	163	22	2,332	169	65		
	6 91	0 64	0 12	13 81	3 92	1 69		
GMP	24				12	5		
	0 07				0 28	0 13		
UDP-GlcNAc	10,791	13,502	9,998	6,940	1 948	2,215	655	1,686
	31 66	53 31	56 27	41 10	45 13	57 53	24 76	48 66
CDP	4,231							
	12 41							
UDP+NADP	1,406	1,076	1,259	4,130	149	190	463	140
	4 13	4 25	7 09	24 46	3 45	4 94	17 50	4 04
ADP	1,524	2,362	827	604	316	370	215	20
	4 47	9 33	4 65	3 58	7 32	9 61	8 13	0 58
GDP	312	130		46				
	0 92	0 51		0 27				
CTP	137		106	613				
	0 40		0 60	3 63				
UTP	2,070	966	621		144	132	93	33
	6 07	3 81	3 50		3 34	3 43	3 52	0 95
ATP	3,142	1,005	1,670	117	270		296	866
	9 22	3 97	9 40	0 69	6 26		11 19	24 99
GTP	435	191	69		142			
	1 28	0 75	0 39		3 29			
UDP-Gal+UDPGlc	2,071						86	
	6 08						3 25	
UDP-GlcA	1,590	1,567	564		887	479	564	537
	4 67	6 19	3 17		20 55	12 44	21 32	15 50
GDP-Man	2,206	191	420	258		51	33	25
	6 47	0 75	2 36	1 53		1 32	1 25	0 72
Total[d]	34,080	25,327	17,767	16,886	4,316	3,850	2,645	3,465

[a]No CMP was detectable [b]nmol/g of lyophilized mycelium [c]Mole% of all nucleotides present
[d]Refers to nmoles of all nucleotides per gram of lyophilized mycelium

TABLE IV

QUANTITATIVE RELATIONSHIPS OF TOTAL NUCLEOTIDES DURING GROWTH CYCLE OF

Cladosporium herbarum Nucleotide	*Growth time (h)*						
	44	*70*	*96*	*120*	*144*	*187*	*234*
CMP	97[a] 0 78[b]						
UMP	1,698 13 71	712 13 90	608 19 51	446 6 85	597 10 07	718 19 92	528 13 71
AMP	136 1 10	70 1 37	203 6 51	516 7 93	339 5 72	98 2 72	223 5 79
GMP	83 0 67	11 0 21	26 0 83	31 0 48		12 0 33	23 0 60
UDP-GlcNAc	6,301 50 87	1,477 28 82	568 18 23	1,363 20 93	1,612 27 18	632 17 53	947 24 58
UDP+NADP[c]	752 6 07	201 3,92	771 24 74	1,671 25 66	1,277 21 53	741 20 55	373 9 68
ADP	916 7 39	1,991 38 86	337 10 82	486 7 46	400 6 74	153 4 24	563 14 62
GDP	137 1 11		155 4 97	69 1 06	233 3 93	152 4 22	74 1 92
CTP	454 3 67						
UTP	299 2 41		107 3 43	351 5 39	432 7 28	83 2 30	132 3 43
ATP	1,330 10 74	329 6 42	293 9 40	999 15 34	787 13 27	279 7 74	503 13 06
GTP				297 4 56	190 3 20		23 0 60
UDP-Gal+UDPGlc		318 6 21				141 3 91	143 3 71
UDP-GlcA				176 2 70		356 9 88	150 3 98
GDPGlc							15 0 39
GDP-Man	184 1 49	15 0 29	48 1 54	106 1 63	63 1 06	240 6 66	155 4 02
Total[d]	12,387	5,124	3,116	6,511	5,930	3,605	3,852

[a]nmol/g of lyophilized mycelium [b]Mole% of all nucleotides present [c]No CDP was detectable
[d]Refers to nmoles of all nucleotides per gram of lyophilized mycelium

As a preparative tool, a column of Biogel P-2 was employed to prepurify and concentrate the nucleotides from the trichloroacetic acid extract Attempts to bypass this preseparation step resulted in unacceptable results through poor resolution and sensitivity of nucleotide detection, loss of linearity in absorbance, peak tailing, and other factors In Fig 1, the fractions 1 and 2 still contained some polysaccharide and protein, as determined by amino acid analysis and by g l c of their hydrolyzates However, fraction 3 in Fig 1 contained the bulk of these non-nucleotide materials, as determined by protein determination, phenol–sulfuric acid assay for carbohydrates, and purine–pyrimidine determination after hydrolysis in 88% formic acid It was not expected that the polysaccharides and other products would be retained so long on the column of Biogel P-2 In attempting the prepurification of nucleotides on Biogel P-2 with water elution, it was anticipated that the material of higher molecular weight would emerge in the void volume (with some nucleotides possibly complexed in their matrix) This expectation would be consistent with normal gel-filtration behavior However, we suggest that elution with water partitions the higher-molecular weight material through the primary amide hydrogen-bonds and residual free carboxylate groups of the acrylamide gels as a stationary phase, while at the same time some molecular sieving of the nucleotides takes place The present procedure on Biogel P-2 is comparable to that reported by Khym[19], in which he used borate complexes of the ribonucleosides on Biogel P-2 to effect separation of ribonucleotides (at the void volume), nucleosides, pyrimidines, and purines, in that order of elution

Identification of the nucleotides by differential u v absorption through the use of detectors in series has proved in this work to provide an effective method of characterization By using relatively inexpensive, spectrophotometric equipment found in most laboratories, indications are given for subsequent steps in characterization of a peak Because the detectors were sensitive to as little as 50 nanograms of nucleotide in a peak, it was not always practical to collect the very smallest peaks for hydrolytic studies and product examination However, by virtue of the prepurification on Biogel P-2, the peak retention-times became very reproducible and, by "spiking" the column with standards, became quite reliable for the variety of nucleotides present in the fungal extracts

Fractions 1 and 2 from the Biogel P-2 column were monitored consecutively at two different wavelengths (254 and 280 nm, see Figs 2 and 3, and 4 and 5) Visual comparison of the relative peak-areas at the two wavelengths for any one nucleotide indicates the reproducibility of the absorbance ratio This ratio was determined for each nucleotide and used as a physical constant in its identification Advantage was taken of absorbance ratios for identifying sugar nucleotides before and after hydrolysis by pyrophosphatase The result was that, in Figs 2 and 3 or 4 and 5, the peaks for pyrophosphate-linked dinucleotides (NAD^+, $NADP^+$, UDP-Glc, UDP-GlcNAc, UDP-GlcA, and the like) disappeared after treatment with pyrophosphatase, with corresponding elevation in the mononucleotide peaks resulting from the cleavage

A distinct advantage in the use of liquid-chromatographic methods is the small amount of fungal mycelium needed to effect determination of nucleotides Thus, in

other experiments on these growth studies, even fungi grown on solid, damp, corn meal could be extracted, and a complete resolution of nucleotides performed with high recovery Application of this nucleotide method to many kinds of microorganisms and tissues seems feasible as a general method for comparative turnover of nucleotides during growth

In Tables I–IV, the results of monitoring nucleotide turnover during fungal growth are listed In the eukaryotic organism, there are many complications in interpreting the metabolic meaning of nucleotide concentrations at various times in the growth cycle[3–5] These problems with analysis of nucleotide turnover are brought about by action of nucleases on polynucleotides, by *de novo* synthesis of purine and pyrimidine nucleotides, and operation of salvage pathways for the nucleotides The present study emphasizes the biosynthesis of the polysaccharide precursors, the nucleoside 5′-(glycosyl diphosphates) Hassid has considered the bioenergetics of formation of the latter as reactive intermediates[2] The efficiency of quantitative, enzymic conversion of the nucleoside 5′-(glycosyl diphosphates) into polysaccharides *in vitro* is very high[2], and the equilibrium constants for the glycosyl-transferase reaction lie far to the right However, the dynamics of formation of nucleoside sugar diphosphates *in vivo* could be one source of regulation of polysaccharide biosynthesis

As no other strict quantitation of nucleotide levels on a mole-percent basis throughout the growth cycle of fungi has been reported, the data reported in Tables I–IV for nucleoside 5′-(glycosyl diphosphates) give some concept, in form of a profile of what these levels are in the pool Earlier work on analysis of nucleotides in fungi[7–12] was limited to very long periods of time for product resolution, with the result that the type of tabulation of products presented in Tables I–IV was impractical In this study, duplicate cultures of each species could be extracted, and the nucleotides resolved, peaks identified, and components quantitated during a short period of time This is no criticism of the excellent work reported in references 7–12, but rather an extension and confirmation of the painstaking work reported therein In fact, the nucleotides that were positively identified by high-pressure liquid chromatography are probably not the only nucleotides present, and as many as a dozen minor, unidentified peaks are present in chromatograms such as Fig 4 Ballio[8] identified 26 nucleotide-related compounds in an acid extract of *Fusarium* species Interestingly, Ballio's estimate of the nucleotides present was 1500 μmol/100 g dry-weight of mycelium[8], and the results for the four ascomycetes reported here are generally of a similar order of magnitude at the peak of nucleotide production (μmol/100 g) *A niger*, 2173, *P citrinum*, 1459, *F moniliforme*, 3408, and *C herbarum*, 1238 μmoles/100 g (Tables I–IV), the last seems to be lower in total nucleotides throughout the growth cycle

Total nucleotides in nmol/g of dry-weight tissue increase sharply for all four species during the first 40 h of growth (see totals, Tables I–IV) The washed mycelium could not have simply absorbed this quantity of nucleotide from the yeast-extract medium which has nucleotide concentrations that are relatively much lower (baseline values of 100 nmol/g) With rises and falls, evident throughout the cycle, all four species cease

growth with much lower nucleotide totals than after the first 40 h With depletion of a limiting metabolite as the probable cause for growth levels to have stopped where they did, under the same circumstance of growth, *P citrinum* not only had the shortest growth-time span, but also the lowest nucleotide level at the end of this period Analysis for total weight of mycelium and extent of hexosamine biosynthesis for these four organisms in separate experiments[23] indicated that both values increase sigmoidally almost to the end of the growth cycle of the organism, whereas the changes in nucleotide levels are more paraboloid All the curves for total nucleotides display more than one maximum, and the concentrations of individual nucleotides themselves oscillate through peaks Assays of the culture filtrates are not included in these studies However, it should be noted that all of the fungi studied do secrete nucleosides into the medium, but little phosphorylated nucleotide These extracellular nucleosides are probably important in terms of recovery of the pool size through reabsorption. Previous workers have found this same phenomenon with respect to nucleosides in the medium[11]

In the present work, the findings of Bezborodov *et al* [11 12] and others[6–10] are confirmed that, regardless of the age of the fungal culture, or cultivation conditions for ascomycetes, the preponderant nucleotides in the fungi studied are uridylic acid derivatives (60–80% of all nucleotides) Such dominance by the pyrimidine pathway is contrasted to the very low content of cytidylic acid derivatives (Tables I–IV) One explanation could be a rapid turnover of cytosine, and salvage through its deaminase after utilization to recover uridine of the pool In general, the bases were, in decreasing order of concentration uridine $\geq$ adenine $>$ guanine $\geq$ cytosine (Tables I–IV)

Although it is assumed that nucleoside sugar diphosphates are principally utilized in polysaccharide biosynthesis (Tables I–IV), of all the sugar nucleotides, uridine 5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl diphosphate) (UDP-GlcNAc) preponderates, even surpassing other nucleotides in the four species studied Hexosamine-containing polysaccharides and glycoproteins represent only a lesser part of the total glycan of these organisms[23], with (1→3)-β-D-glucan being the principal structural polysaccharide Other workers[6–12] have reported high levels of UDP-GlcNAc in fungi, but these earlier studies were not on as quantitative a basis as the present work The culture medium contains little hexosamine (2-amino-2-deoxy-D-glucose or -galactose) at the outset UDP-GlcNAc and UDP-GalNAc were not resolved from each other on the anion-exchange column Hydrolysis and analysis of hexosamines in the collected UDP-GlcNAc peaks by use of an amino acid analyzer showed that $<1\%$ of the hexosamine in the UDP-hexosamine is 2-amino-2-deoxy-D-galactose, although other work in this laboratory[23] has confirmed that both of the foregoing hexosamines are present in the fungal glycosaminoglycans produced, in the ratio of about 4 parts 2-amino-2-deoxy-D-glucose to 1 part 2-amino-2-deoxy-D-galactose UDP-Glc and UDP-Gal are only moderately resolved from the UDP-NAc hexosamines, although the resolution was adequate for quantitation of each

Atkinson[24–27] defined the energy charge of intracellular nucleotides as

$E = (ATP + 1/2ADP)/(ATP + ADP + AMP)$ After extensive literature survey, very few examples of nucleotide analyses during growth of ascomycetes could be found Smith and Valenzuela-Perez have published data from which the energy charge of the ascomycete *A niger* could be calculated[28] The value of E thus calculated from their data for fungi ranges from a ratio of 0 35 to 0 57 (Table V) The average E value calculated from Tables I–IV is 0 60 for *A niger*, 0 63 for *C herbarum*, 0 60 for *F moniliforme*, and 0 45 for *P citrinum* (Table VI) Although E has been shown[24–27] to fall within a narrow range of 0 85 to 0 90 for a wide variety of organisms (not including ascomycetes), values calculated from the data by Smith and Valenzuela-Perez[28] and in Table VI show that E is possibly smaller when calculated on the basis of total biomass of fungi Values determined in this study lie very close to the range published by Smith and Valenzuela-Perez These data are reinforced by a recent report by Subramanian[29], who, on careful analysis of the energy charge of *A flavus*, found E to fall in the range of 0 31 to 0 51 during normal growth leading to biosynthesis of aflatoxin Given the biological fluctuations made apparent by plotting values in Tables I–IV *vs* time, an additional explanation of these low energy-charges

TABLE V

ENERGY CHARGE OF *A niger* CALCULATED FROM LITERATURE VALUES[28]

	Sporing mycelium[a]				*Non-sporing mycelium*[a]			
Growth time (h)	24	48	72	96	24	48	72	96
E_{calc}	0 40	0 41	0 57	0 53	0 35	0 50	0 50	0 46
E_{av}	0 48				0 45			

[a] *A niger* in liquid medium Calculations made from values for AMP, ADP, and ATP reported by the authors

TABLE VI

ENERGY CHARGES CALCULATED FROM TABLES I–IV FOR FUNGI DURING GROWTH CYCLES

A niger										
Time (h)	45	51	71	120	142	165	189			
E	0 56	0 50	0 28	0 79	0 78	0 66	0 67		E_{av}	0 60
C herbarum										
Time (h)	44	70	96	120	144	187	234			
E	0 75	0 53	0 56	0 62	0 65	0 67	0 61		E_{av}	0 63
F moniliforme										
Time (h)	43	67	99	121	143	167	191	215		
E	0 56	0 62	0 83	0 12	0 58	0 42	0 78	0 98	E_{av}	0 60
P citrinum										
Time (h)	20	40	64	90	114	137	165			
E	0 45	0 50	0 29	0 51	0 67	0 54	0 19		E_{av}	0 45

could lie in the multinucleated chambers of the living cells of fungal mycelium Some of these cells are newly divided, whereas others are old or dying The E value found for these fungi probably reflects an average for healthy and old cells Values for other species of microorganisms studied by Chapman, Fall, and Atkinson[30] and for nutritional and anaerobic stress-conditions of *S cerevisiae* by Ball and Atkinson[31], show explicable deviation from the value of 0 8–0 9 for E in healthy cells The present study indicates that further work is needed to explain anomalous energy–charge states of ascomycetes

In conclusion, therefore, the methodology and results of this study on fungal-nucleotide analysis and turnover are a starting point to further investigations of perturbants of biosynthesis of fungal polysaccharides The practical value of fungal polysaccharides warrants continued careful study of the metabolic regulation leading to promotion of their biosynthesis, or repression of undesirable products The present assay method for fungal nucleoside sugar diphosphates will permit such a study of polysaccharide biosynthesis at the metabolic-precursor level

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