Chitosan Synthesis by the Tandem Action of Chitin Synthetase and Chitin Deacetylase from *Mucor rouxii*†

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ABSTRACT: The biosynthesis of chitosan was studied with cell-free extracts from the fungus *Mucor rouxii*. The possibility that chitosan was formed by incorporation of glucosamine from uridine diphosphate D-glucosamine or from another glucosamine nucleotide was ruled out. The only amino sugar nucleotide detected in the cytosol of *M. rouxii* was uridine diphosphate *N*-acetyl-D-glucosamine (UDP-GlcNAc). Chitosan was synthesized in vitro from UDP-GlcNAc with a mixture of chitin synthetase and chitin deacetylase. Neither enzyme alone catalyzed significant chitosan formation. The two enzymes appear to function in tandem. The properties of this binary enzyme system closely paralleled the properties of one of its components, chitin synthetase. The chitosan synthesizing system had a pH optimum of 6.5 and an absolute requirement for either Mg^{2+} or Mn^{2+}; it was stimulated by free *N*-acetyl-D-glucosamine and by proteases (rennilase or trypsin); it was sharply inhibited by polyoxin D (K_i = 0.99 μM). The product formed from UDP-GlcNAc by these enzymes was identified as chitosan by its (1) paper chromatographic immobility, (2) solubility in dilute acetic acid and insolubility in alkali, (3) release of glucosamine upon hydrolysis with HCl, (4) degradation by nitrous acid, and (5) digestion by chitosanase but not by chitinase. Biosynthesized chitosan contained a high proportion (26–29%) of acetylated residues. Although chitinase did not digest biosynthesized chitosan, its addition to the chitosan synthesizing mixtures substantially reduced chitosan formation. This finding provided a strong indication that deacetylation occurs after a chitin chain is formed; i.e., chitin is a precursor of chitosan. The physical state of chitin was crucial. Chitin deacetylase had very low activity on preformed chitin, but it readily deacetylated *nascent* chitin. The latter is probably the natural substrate for chitin deacetylase.

The vegetative cell wall of the dimorphic fungus *Mucor rouxii* contains three major polysaccharides: chitosan, chitin, and the acidic polysaccharide(s) mucoran/mucoric acid (Bartnicki-Garcia & Nickerson, 1962; Bartnicki-Garcia & Reyes, 1968). Chitosan, the β-1,4-linked polymer of glucosamine (GlcN),[1] makes up about 30% of the vegetative cell wall of *M. rouxii* whereas chitin, the β-1,4-linked polymer of GlcNAc, constitutes only about 10% of the cell wall.

Glaser & Brown (1957) first demonstrated that UDP-GlcNAc is a precursor for chitin synthesis by cell-free extracts of *Neurospora crassa*. Since that time, UDP-GlcNAc has been confirmed as the glycosyl donor for chitin synthesis in cell-free extracts from many other fungi [see review by Gooday & Trinci (1980)], including *M. rouxii* (McMurrough et al., 1971). The transfer of GlcNAc from UDP-GlcNAc into chitin is catalyzed by chitin synthetase.

Studies on the biosynthesis of chitosan are more limited. An enzyme that deacetylates glycol chitin, a soluble derivative of chitin, was found in cell-free extracts of *M. rouxii*, strain AHU 6019, by Araki & Ito (1974, 1975). About 14% of the chitin deacetylase was associated with particulate cell fractions, 49% was associated with a soluble 20000*g* supernatant fraction, and about 37% was extracellular. Chitin deacetylase also catalyzed the deacetylation of *N*-acetyl chitooligomers; it had essentially no activity toward the monomer or dimer of GlcNAc but showed increasing activity as the chain length increased from trimer to pentamer. The enzyme was inactive toward bacterial cell wall peptidoglycan, N-acetylated heparin, and a fungal polymer of *N*-acetylgalactosamine. On the basis of this evidence, Araki and Ito advanced the hypothesis that deacetylation of chitin accounts for chitosan synthesis in vivo. This was somewhat premature since their enzyme had low activity toward genuine chitin, tested in colloidal or powdered form, and thus they were unable to synthesize chitosan in vitro. We embarked on a study of the biosynthesis of chitosan to evaluate the findings of Araki and Ito and to explore the possibility of a pathway for chitosan synthesis independent of chitin.

In the present study, we describe the conditions necessary for the synthesis of chitosan in vitro and the factors that affect the combined operation of chitin synthetase and chitin deacetylase in the biosynthesis of chitosan.

Experimental Procedures

Culture Conditions and Cell-Free Extract Preparation. *Mucor rouxii*, strain IM-80 (ATCC 24905), was maintained on solid YPG medium (0.3% yeast extract, 1% peptone, 2% glucose, and 2.5% agar, pH 4.5) (Bartnicki-Garcia & Nickerson, 1962). Spores were harvested in sterile distilled water and were used to inoculate three 2-L Erlenmeyer flasks, each containing 600 mL of liquid YPG medium, to a final concentration of about 5×10^5 spores/mL. A gas mixture of 70% N_2–30% CO_2 was bubbled through the cultures to achieve the yeast form of *M. rouxii*. Cultures were incubated in a reciprocating shaker bath at 28 °C and were harvested after 13 h by filtration through a Millipore SM 5-μm membrane. The cells were resuspended in about 20 mL of 50 mM KH_2PO_4–NaOH buffer, pH 6.5, containing 10 mM $MgCl_2$ (phosphate–magnesium buffer), mixed with an equal volume of glass beads (0.45 mm in diameter), and broken in a Braun MSK cell homogenizer (Bronwill Scientific, Rochester, NY)

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CDP, cytidine 5′-diphosphate; GDP, guanosine 5′-diphosphate; GlcN, glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; TDP, thymidine 5′-diphosphate; UDP, uridine 5′-diphosphate; UDP-GlcN, uridine diphosphate D-glucosamine; UDP-GlcNAc, uridine diphosphate *N*-acetyl-D-glucosamine; UMP, uridine 5′-phosphate.

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for 30 s while the vessel was cooled with liquid CO_2. Cell walls were removed by centrifugation at 1000*g* for 10 min.

To grow the mycelial form of *M. rouxii*, filtered air was bubbled through cultures prepared as above. The cultures were incubated in a reciprocating shaker bath at 22 °C and were harvested after 13.5 h by filtration through a fine nylon net. Large batches (10 L) of *M. rouxii* mycelium were grown in a 14-L Microferm fermentor (New Brunswick Scientific Co., Edison, NJ) under similar conditions. The cells were resuspended in phosphate–magnesium buffer, mixed with glass beads (0.45 mm in diameter), and broken in a Braun MSK cell homogenizer in three cycles of 15 s each. Cell walls were removed by centrifugation as described above.

Extraction and Analysis of Sugar Nucleotides. Six-hour-old cultures of *M. rouxii* mycelium were labeled by adding [*GlcN*-^{14}C]GlcNAc (9 Ci/mol) to a final concentration of 10^4 dpm/mL. After 30 min of labeling, the mycelium was harvested by filtration through a 5-μm Millipore SM membrane, and a cell-free extract was prepared, as described above. An equal volume of absolute ethanol was added to the crude cell-free extract, and the mixture was placed in a boiling water bath for 30 min. The mixture was centrifuged at 20000*g* for 30 min, and the pellet was reextracted with 50% ethanol as described above. The two ethanol extracts were pooled and dried in a rotary evaporator. The nucleotide sample was resuspended in a small volume of 50% ethanol and analyzed by high-performance liquid chromatography. Samples of 100 μL were injected into a Varian AX-10 anion-exchange column (300 × 4 mm) in a Varian Model 5000 liquid chromatograph (Varian Instrument Group, Palo Alto, CA). The column was eluted at a rate of 2 mL/min with a linear gradient consisting of 40 mL of 5 mM ammonium phosphate buffer (NH_4OH + H_3PO_4), pH 2.85, in the mixing chamber and 40 mL of 0.75 M ammonium phosphate buffer, pH 4.5, in the reservoir (Wehr, 1980). The UV absorbance of the eluate was monitored continuously at 260 nm. The eluate was collected in 0.4-mL fractions, and radioactivity was determined by liquid scintillation.

Preparation of Chitin Deacetylase. Solid ammonium sulfate was gradually added to yeast cell-free extracts (1000*g* supernatant) to get 60% saturation. After 24 h at 4 °C, the slurry was centrifuged at 20000*g* for 30 min. The supernatant was then adjusted to 85% ammonium sulfate saturation. After 24 h at 4 °C, the slurry was centrifuged as above. The pellet was dissolved in and dialyzed against phosphate–magnesium buffer, followed by centrifugation in a Beckman 30 rotor at 54000*g* (R_{av}) for 3.5 h. The supernatant was used as crude chitin deacetylase.

Preparation of Chitin Synthetase. Mycelial walls (1000*g* pellet) were washed 3 times with phosphate–magnesium buffer, suspended in an equal volume of 1% digitonin in phosphate–magnesium buffer, and incubated for 30 min at 22 °C, followed by centrifugation at 48000*g* for 15 min. The supernatant was lyophilized and stored at −12 °C. Samples of the lyophilized digitonin extract of mycelial cell walls were redissolved in water before use.

Preparation of [*Ac*-^{3}H]*GlcNAc-Labeled Cell Walls.* Six-hour-old mycelial cultures of *M. rouxii* were amended with [*Ac*-^{3}H]GlcNAc (500 Ci/mol) to a final concentration of 4.9×10^4 dpm/mL. After 30 min of incubation, the mycelium was harvested by filtration through a 5-μm Millipore SM membrane. The filtered mycelium was washed with about 1 L of cold distilled water. The cells were suspended in water, mixed with an equal volume of 0.45-mm-diameter glass beads, and broken in a Braun MSK homogenizer for two consecutive cycles of 10 and 5 s. Cell walls were sedimented by centrifugation at 1000*g* for 10 min. The walls were resuspended in cold distilled water and were sonicated in a Bransonic 220 sonicator (Branson Cleaning Equipment Co., Shelton, CT) for 3–4 min at maximum output to homogenize the suspension. Additional cold distilled water was added, and the suspension was centrifuged at 1000*g* for 10 min. The supernatant was removed, and the process of homogenization and centrifugation was repeated for a total of 10 times. The cell walls were then lyophilized and stored at 22 °C in a vacuum desiccator over P_2O_5.

Preparation of Glycol [*acetyl*-^{3}*H*]*Chitin.* Partially O-hydroxyethylated chitin (glycol chitin), radiolabeled in *N*-acetyl groups, was prepared as described by Araki & Ito (1975). Partially O-hydroxyethylated chitosan (glycol chitosan; 80 mg) (Sigma Chemical Co., St. Louis, MO) was combined with 800 mg of $NaHCO_3$ and with 12.5 mCi of [^{3}H]acetic anhydride (100 Ci/mol) (New England Nuclear, Boston, MA) and left for 24 h at 4 °C. Cold acetic anhydride (0.4 mL) was added, and the mixture was left for a further 24 h at 4 °C. After thorough dialysis, the product, glycol [*acetyl*-^{3}H]chitin, was diluted with unlabeled glycol chitin, prepared as described above using unlabeled acetic anhydride.

Preparation of [*acetyl*-^{14}*C*]*Chitin.* Two different preparations were made. (1) Commercial chitosan (derived from shrimp shell) was acetylated with [^{14}C]acetic anhydride (29.2 Ci/mol). The resulting chitin was treated with HNO_2 to eliminate nonacetylated units. (2) Chitin was biosynthesized with a preparation of chitin synthetase in an incubation mixture containing 2 mM UDP-[*Ac*-^{14}C]GlcNAc (1.1 μCi), 20 mM GlcNAc, 0.2 mM ATP, 10 mM $MgCl_2$, 50 mM KH_2PO_4–NaOH buffer, pH 6.5, and 1 mg/mL rennilase in a final volume of 12.5 mL. A drop of toluene was added, and the mixture was incubated for 6 h at 22 °C. The resulting [*acetyl*-^{14}C]chitin was pelleted at 1000*g* for 30 min, washed 5 times with phosphate–magnesium buffer, and resuspended in the same buffer.

Chitosan Assays. Standard chitosan assay mixtures contained either 0.4 mM UDP-[*GlcN*-^{14}C]GlcNAc or 0.4 mM UDP-[*Ac*-^{14}C]GlcNAc, 20 mM GlcNAc, 0.2 mM ATP, 10 mM $MgCl_2$, 50 mM KH_2PO_4–NaOH buffer, pH 6.5, 1 mg/mL rennilase, and enzyme additions, in a final volume of 125 μL. Unless otherwise indicated, 25 μL of chitin synthetase (approximately 30 μg of protein) and 25 μL of chitin deacetylase (300–400 μg of protein) were used. The mixtures were incubated at 22 °C for 30 min, unless otherwise indicated, and processed by either paper chromatography or ethyl acetate extraction. Paper chromatography provided a direct measure of the synthesis of immobile GlcN-containing material. Ethyl acetate extraction was used to measure acetate released in the conversion of UDP-GlcNAc to nonacetylated polymer (chitosan)—an indirect but rapid measure of chitosan synthesis. Calculations for both methods were based on the assumption that chitosan is a pure homopolymer of GlcN, though this may not be the case (Datema et al., 1977b; L. L. Davis and S. Bartnicki-Garcia, unpublished results; see also below).

(*1*) *Paper Chromatography Method.* Two parallel assay mixtures, one containing UDP-[*GlcN*-^{14}C]GlcNAc and the other UDP-[*Ac*-^{14}C]GlcNAc, were prepared for each sample. Incubations were terminated by applying the entire assay mixture to a strip of Whatman 3MM paper. The chromatograms were irrigated as described below. The radioactivity remaining at the origin of chromatograms was used as a measurement of polymer synthesis. Samples incubated with UDP-[*GlcN*-^{14}C]GlcNAc were used to measure the total

polymer synthesized (acetylated + nonacetylated); samples incubated with UDP-[*Ac*-^{14}C]GlcNAc were used to measure only acetylated polymer. The difference in immobile radioactivity between these samples was used as a measure of chitosan synthesis.

(*2*) *Extraction Method.* Chitosan assay mixtures containing UDP-[*Ac*-^{14}C]GlcNAc were incubated in Eppendorf centrifuge tubes, and reactions were terminated by adding 60 μL of glacial acetic acid. Ethyl acetate (0.5 mL) was added to each tube, and the contents were mixed vigorously. The mixtures were centrifuged at 8000*g* for 1 min in a Brinkmann 3200 centrifuge. The ethyl acetate layer was removed to a scintillation vial, and the extraction procedure was repeated once more. Radioactivity in the combined ethyl acetate extracts was determined by liquid scintillation.

Chitin Assays. Chitin synthesis was measured by a filtration method (Ruiz-Herrera & Bartnicki-Garcia, 1976). Standard chitin assay mixtures contained 0.4 mM UDP-[*Ac*-^{14}C]-GlcNAc, 20 mM GlcNAc, 0.2 mM ATP, 10 mM $MgCl_2$, 50 mM KH_2PO_4–NaOH buffer, pH 6.5, 1 mg/mL rennilase, and enzyme additions, in a final volume of 125 μL. Where their addition is indicated, 25 μL of chitin synthetase (approximately 30 μg of protein) and 25 μL of chitin deacetylase (300–400 μg of protein) were used. The mixtures were incubated at 22 °C for 30 min, unless otherwise indicated, and reactions were terminated with 60 μL of glacial acetic acid. The samples were filtered through Reeve Angel 934 AH or Whatman GF/C glass-fiber filters (2.4 cm in diameter) and washed with about 50 mL of 1 M acetic acid–95% ethanol (4:1 v/v). Radioactivity on the filters was determined by liquid scintillation.

Isolation of Biosynthesized Chitosan. Large-scale incubation mixtures contained 2 mM UDP-[*GlcN*-^{14}C]GlcNAc, 20 mM GlcNAc, 0.2 mM ATP, 10 mM $MgCl_2$, 50 mM KH_2PO_4–NaOH buffer, pH 6.5, 1 mg/mL rennilase, chitin synthetase, and chitin deacetylase, in a final volume of 6.25 mL. A drop of toluene was added to prevent microbial growth. This mixture was incubated 4–6 h at 22 °C. The reaction was terminated by adding 6.25 mL of 2 M acetic acid. The acidified mixture was placed in a boiling water bath for 30 min, followed by centrifugation at 1000*g* for 15 min. The supernatant was adjusted to pH 8.5, and the resulting suspension was centrifuged at 1000*g* for 15 min. Acetic acid (1 M) was added to the pellet, and the cycle of acetic acid solubilization and alkaline precipitation was repeated, as described above. The final precipitate was washed thoroughly with water and lyophilized. The lyophilized material was stored at 22 °C in a vacuum desiccator over P_2O_5. Samples were suspended in water for further studies.

Paper Chromatography. Samples were applied to strips of Whatman 3MM paper. Chromatograms were irrigated in descending fashion for 11–12 h with 95% ethanol–1 M acetic acid (7:3 v/v). Chromatograms were cut into 1-cm segments, and radioactivity was determined by liquid scintillation.

Nitrous Acid Degradation of Chitosan. Nitrous acid treatment was as described by Datema et al. (1977a).

Miscellaneous. Protein was measured with Folin's phenol reagent (Lowry et al., 1951) using bovine serum albumin as a standard. UDP-GlcN was kindly supplied by Frank Maley (New York State Department of Health, Albany, NY). Chitinase, from *Streptomyces griseus*, was purchased from Sigma Chemical Co. A sample of highly purified chitosanase, isolated from *Streptomyces* sp. (Price & Storck, 1975), was generously provided by Roger Storck (Rice University, Houston, TX). Polyoxin D was a gift from M. Hori of the Kaken Chemical Co., Ltd., Tokyo, Japan. Rennilase was obtained from the Novo Enzyme Corp., Mamaroneck, NY. Radioactive samples were counted in a Beckman LS 7500 liquid scintillation counter.

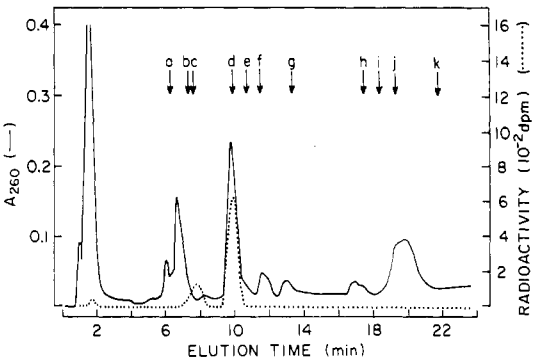

FIGURE 1: Analysis of *M. rouxii* nucleotides and sugar nucleotides by high-performance liquid chromatography. Nucleotides and sugar nucleotides were isolated from *M. rouxii* mycelium that had been labeled with [*GlcN*-^{14}C]GlcNAc. A sample of the nucleotide pool was applied to a Varian AX-10 anion-exchange column. The column was eluted at a rate of 2 mL/min with a linear gradient consisting of 40 mL of 5 mM ammonium phosphate buffer (pH 2.85) in the mixing chamber and 40 mL of 0.75 M ammonium phosphate buffer (pH 4.5) in the reservoir. UV absorbance (—) was monitored continuously at 260 nm. Fractions (0.4 mL) of the column eluate were collected, and radioactivity (···) was determined by liquid scintillation. The elution positions of various standards, determined by the absorbance at 260 nm, are indicated with arrows: (a) UDP-GlcN; (b) UMP; (c) ADP; (d) UDP-GlcNAc, GDP, and CDP-glucose; (e) UDP-glucose, UDP-galactose, and TDP-glucose; (f) ADP-glucose; (g) GDP-glucose and GDP-mannose; (h) UDP-glucuronic acid; (i) UDP; (j) CDP; (k) ATP. UMP and ADP (b and c) were incompletely separated, as were UDP-glucuronic acid and UDP (h and i).

Results

Search for a GlcN Nucleotide Precursor in Chitosan Synthesis. The possibility that chitosan is formed directly from a GlcN nucleotide precursor was investigated. Cultures of *M. rouxii* were amended with [*GlcN*-^{14}C]GlcNAc to study the presence of radioactive amino sugar containing nucleotides. The nucleotide pool was extracted from a cell-free extract of *M. rouxii* mycelium with 50% ethanol and was analyzed by high-performance liquid chromatography with an anion-exchange column (Figure 1). Only one peak of UV-absorbing material had radioactivity associated with it. This peak corresponded to UDP-GlcNAc (Figure 1d). At approximately the elution position corresponding to UDP-GlcN (Figure 1a), there were two UV-absorbing peaks, but no radioactivity was associated with them. Although these peaks were not identified, the absence of radioactivity makes it unlikely that either one would be UDP-GlcN. Apart from UDP-GlcNAc, the only other peak of radioactivity eluted just behind ADP (Figure 1c) but was not identified; it had no UV absorption and may be an *N*-acetylglucosamine phosphate.

Chitin Deacetylase Activity on Preformed Chitin. The activity of chitin deacetylase toward chitin and various GlcNAc-containing samples was tested (Table I). Cell walls labeled in vivo with [*Ac*-^{3}H]GlcNAc were only slightly deacetylated (<1%) upon incubation with chitin deacetylase, indicating that this enzyme has little activity toward native chitin as it exists in the cell wall. Likewise, chitin deacetylase had little activity toward [*acetyl*-^{14}C]chitin prepared by acetylation of chitosan derived from shrimp shell or toward [*acetyl*-^{14}C]chitin biosynthesized in vitro with a chitin synthetase preparation from *M. rouxii*. Only glycol chitin was substantially deacetylated (ca. 18%) upon incubation with chitin deacetylase. Chitin deacetylase was inactive toward GlcNAc, $(GlcNAc)_2$, and UDP-GlcNAc.

Table I: Substrate Specificity of Chitin Deacetylase

substrate[a]	radioactivity released[b] (%)
UDP-GlcNAc (acetyl-^{14}C; 27 000 dpm, 30 μg)	0.02
GlcNAc (acetyl-^{14}C; 8300 dpm, 11 μg)	0
$(GlcNAc)_2$ (acetyl-^{14}C; 12 500 dpm, 21 μg)	0
GlcNAc-labeled *M. rouxii* cell walls[c] (acetyl-^{3}H; 37 000 dpm, 186 μg)	0.39
glycol chitin (acetyl-^{3}H; 24 000 dpm, 11 μg)	17.78
chitin[d] (acetyl-^{14}C; 15 500 dpm, 33 μg)	1.18
chitin[e] (acetyl-^{14}C; 5900 dpm, 38 μg)	0.53

[a] The indicated substrate was incubated with chitin deacetylase, phosphate–magnesium buffer, and 1 mg/mL rennilase for 30 min at 22 °C. Reactions were terminated by the addition of glacial acetic acid. [b] Percentage of initial radioactivity released as acetate per assay; determined by the extraction method. [c] Containing approximately 17.5 μg of chitin. [d] Prepared by acetylating chitosan, derived from shrimp shell, with [^{14}C]acetic anhydride. [e] Biosynthesized in vitro by incubating chitin synthetase with UDP-[*Ac*-^{14}C]GlcNAc for 6 h at 22 °C.

Table II: Chitosan and Chitin Syntheses by Chitin Synthetase and Chitin Deacetylase[a]

enzymes	chitosan synthesis (nmol/min)[b]	chitin synthesis (nmol/min)[c]
chitin deacetylase	0	0.001
chitin synthetase	0.003	0.293
chitin synthetase + chitin deacetylase	0.197	0.026[d]

[a] Chitosan and chitin syntheses were determined in standard assay mixtures containing 46.6 nmol of UDP-[*Ac*-^{14}C]GlcNAc. [b] Chitosan synthesis was determined by the extraction method and is expressed as nanomoles of acetate released per minute. [c] Chitin synthesis is expressed as nanomoles of GlcNAc incorporated into chitin per minute. [d] This value refers to chitin accumulation rather than to chitin synthesis since, in the presence of chitin deacetylase, much of the chitin that is synthesized is converted to chitosan.

Chitosan Synthesis by Chitin Synthetase + Chitin Deacetylase. In the course of testing the activity of chitin deacetylase on chitin, the deacetylase was added to incubation mixtures in which chitin was being synthesized from UDP-GlcNAc by chitin synthetase. This addition caused a drastic reduction in the amount of chitin recovered and a concomitant increase in chitosan formation (Table II). When the two enzymes—chitin synthetase and chitin deacetylase—were incubated simultaneously with UDP-GlcNAc, 10–15% of the substrate was converted to chitosan in a 30-min incubation (Table II). When either enzyme was incubated alone with UDP-GlcNAc, there was essentially no chitosan synthesis. Inactivating the chitin synthetase or the chitin deacetylase by boiling in a water bath for 10 min reduced the chitosan synthesizing capacity of the mixture by 99.2 or 96.9%, respectively.

Paper chromatography of assay mixtures incubated with both chitin synthetase and chitin deacetylase revealed two major bands of radioactivity: a mobile band of unused substrate (UDP-GlcNAc) and an immobile band of biosynthesized polymer (Figure 2). By comparing the amount of polymer formed from substrate labeled in the acetyl moiety (Figure 2B) with substrate labeled in the sugar ring (Figure 2A), we estimated that nearly 80% of the total polymer synthesized consisted of nonacetylated glucosamine units.

The total polymer (chitin + chitosan) recovered when both chitin synthetase and chitin deacetylase were incubated with UDP-GlcNAc was 25% less than when chitin synthetase was incubated alone with the substrate (Table II). The loss was probably due to chitin destruction by traces of chitinase introduced with the crude deacetylase. [Chitinase is present in the cytosol of *M. rouxii* (Lopez-Romero et al., 1982).]

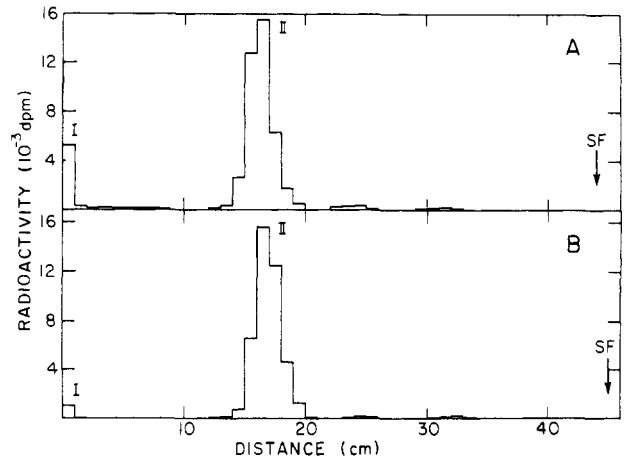

FIGURE 2: Paper chromatography of chitosan assay mixtures. Standard assay mixtures containing chitin synthetase and chitin deacetylase were incubated with either (A) UDP-[*GlcN*-^{14}C]GlcNAc or (B) UDP-[*Ac*-^{14}C]GlcNAc. The chromatograms were irrigated 11 h in 95% ethanol–1 M acetic acid (7:3 v/v). SF = solvent front.

Identification of Biosynthesized Chitosan. The product formed from UDP-[*GlcN*-^{14}C]GlcNAc by chitin synthetase + chitin deacetylase was isolated from a large-scale incubation mixture and suspended in water. Paper chromatography of a sample of the radioactive suspension revealed only immobile radioactivity. The radioactive suspension was used for the following treatments:

(i) A sample was hydrolyzed with 6 M HCl at 105 °C for 8 h. The HCl was evaporated in vacuo over NaOH pellets at 50 °C. The hydrolysate was redissolved in water. About 89% of the initial radioactivity was recovered in solution. Paper chromatography revealed only one major band of radioactivity, which was identified as GlcN by cochromatography.

(ii) A sample was treated with nitrous acid. About 94% of the initial radioactivity became mobile on paper chromatograms. At least 75% of the initial radioactivity was present in a single, fast moving band (R_f 0.76) presumed to be 2,5-anhydromannose. Nitrous acid simultaneously deaminates and depolymerizes polysaccharides at monomer units containing unsubstituted 2-amino groups (Shively & Conrad, 1970); it would degrade a polysaccharide containing only GlcN units to 2,5-anhydromannose.

(iii) The product had solubility properties typical of chitosan: it was soluble in dilute (1 M) acetic acid, insoluble at alkaline pH, and insoluble in acetone.

(iv) Samples were incubated with chitinase or purified chitosanase at 0.2 mg/mL in 25 mM KH_2PO_4–NaOH buffer, pH 6.5, with 5 mM $MgCl_2$, for 3 h at 30 °C. The digestion mixtures were applied to paper chromatograms. In the chitinase-treated sample, all of the applied radioactive material remained immobile (Figure 3A). In the chitosanase-treated sample, 75% of the radioactive sample separated into three distinct mobile bands (Figure 3B). Band IV had the same chromatographic mobility as GlcN. Bands II and III were not identified but may represent the trimer and dimer of GlcN (chitotriose and chitobiose).

Acetyl Content of Biosynthesized Chitosan. Chitosan was extracted from two parallel large-scale incubation mixtures containing chitin synthetase + chitin deacetylase. One of the mixtures contained UDP-[*GlcN*-^{14}C]GlcNAc, and the other contained UDP-[*Ac*-^{14}C]GlcNAc. By comparing the radio-

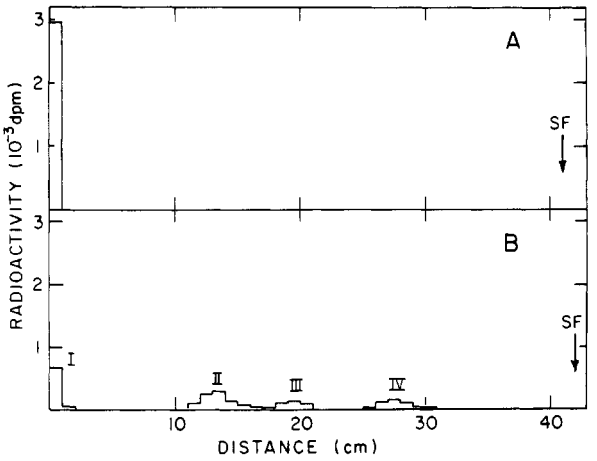

FIGURE 3: Paper chromatography of biosynthesized chitosan after digestion with chitinase or chitosanase. [^{14}C]Chitosan, synthesized from UDP-[*GlcN*-^{14}C]GlcNAc by chitin synthetase + chitin deacetylase, was treated with chitinase (A) or chitosanase (B) for 3 h at 30 °C. The digestion mixtures were subsequently chromatographed 11 h in 95% ethanol–1 M acetic acid (7:3 v/v). SF = solvent front.

Table III: Effect of Chitinase on Chitosan and Chitin Syntheses[a]

enzyme addition	chitinase (mg/mL)	chitosan (nmol)[b]	chitin (nmol)[c]
chitin synthetase + chitin deacetylase	0.0	4.18	1.26
	0.1	2.03	0.21
	0.2	0.53	0.06
chitin synthetase	0.0	0	7.65
	0.1	nd[d]	1.48
	0.2	nd[d]	0.39

[a] Chitinase was added to standard chitosan and chitin assay mixtures containing 46.6 nmol of UDP-[*Ac*-^{14}C]GlcNAc. [b] Chitosan synthesis was determined by the extraction method and is expressed as nanomoles of acetate released in 30 min. [c] Chitin synthesis is expressed as nanomoles of GlcNAc incorporated into chitin in 30 min. [d] nd = not determined.

activity of the two chitosan samples, it was determined that 26–29% of the GlcN units in this acid-soluble product were acetylated.

Role of Chitin in Chitosan Synthesis. Although the foregoing evidence indicated that chitin synthetase and chitin deacetylase are involved in chitosan synthesis, the question remained as to the exact relationship of the deacetylating and polymerizing activities. Were the GlcNAc moieties of UDP-GlcNAc deacetylated before they were polymerized? Were they deacetylated and polymerized simultaneously? Were the GlcNAc units first polymerized into a chitin chain and then deacetylated? An experimental answer to these questions was obtained by adding chitinase to chitosan synthesizing mixtures containing chitin synthetase + chitin deacetylase. Addition of chitinase reduced chitosan synthesis dramatically (Table III). This is strong evidence that chitin chains are first formed and then deacetylated. Addition of chitinase reduced the amount of chitin recovered by 81–95% in assay mixtures containing either chitin synthetase alone or chitin synthetase + chitin deacetylase (Table III).

Deacetylation of Nascent vs. Preformed Chitin. To compare the activity of chitin deacetylase on nascent vs. preformed chitin, chitin deacetylase was incubated with chitin synthetase in two different sequences: (1) Both enzymes were incubated simultaneously with UDP-[*Ac*-^{14}C]GlcNAc. Deacetylation of nascent chitin proceeded rapidly, with an initial rate of 0.24 nmol of acetate released/min (Figure 4A, solid circles). There was little accumulation of chitin as determined in parallel assays (Figure 4B, solid circles). (2) Chitin was first allowed

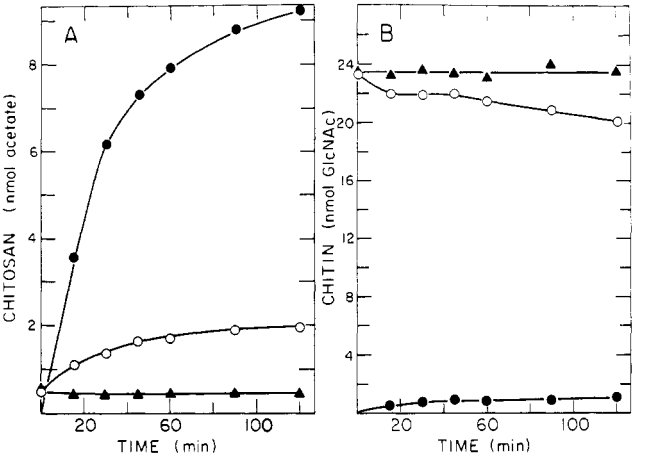

FIGURE 4: Chitin deacetylase activity on nascent vs. preformed chitin. Chitosan (A) and chitin (B) syntheses were determined in three parallel assay mixtures all containing UDP-[*Ac*-^{14}C]GlcNAc (50 nmol) as substrate but differing in the time and type of enzyme added: (i) chitin deacetylase and chitin synthetase were both added at the outset (●); (ii) an assay mixture containing only chitin synthetase was preincubated for 4.5 h, and polyoxin D (final concentration 60 μM) was added to stop any further chitin synthesis; chitin deacetylase was then added, and the incubation was continued for 2 h as shown (○); (iii) same as (ii) except that phosphate–magnesium buffer was added instead of the deacetylase (▲). Chitosan synthesis was determined by the extraction method and is expressed as nanomoles of acetate released per assay. Chitin synthesis is expressed as nanomoles of GlcNAc incorporated into chitin per assay.

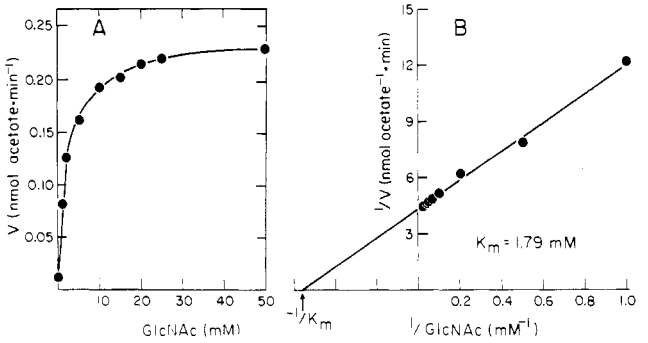

FIGURE 5: Effect of GlcNAc concentration on chitosan synthesis. Velocity (V) was determined in standard assay mixtures containing chitin synthetase, chitin deacetylase, and the indicated concentrations of GlcNAc. Chitosan synthesis was determined by the extraction method. Velocity is expressed as nanomoles of acetate released per minute. (A) Direct plot. (B) Lineweaver–Burk plot.

to accumulate for 4.5 h in assay mixtures containing chitin synthetase and UDP-[*Ac*-^{14}C]GlcNAc; further synthesis was blocked with polyoxin D. During this time, about 23 nmol of GlcNAc was incorporated into chitin (Figure 4B, open circle at zero time). Chitin deacetylase was then added to the mixtures. Despite the abundance of preformed chitin, deacetylation was very slow, with an initial rate of 0.04 nmol of acetate released/min (Figure 4A, open circles). During the 2-h incubation, only 6% of the preformed chitin was converted to chitosan. There was, however, a 14% loss in preformed chitin (Figure 4B, open circles). The discrepancy was probably due to chitin destruction by traces of chitinase present in the crude deacetylase (as described above in relation to Table II).

Kinetics of Chitosan Synthesis. In standard assays, chitosan synthesis was linear for about 20 min, after which the reaction rate fell off gradually (Figure 4A, solid circles). Chitosan synthesis was an approximately linear function of enzyme concentration in the range of 0.1–0.5 mg of protein.

GlcN had no effect on chitosan synthesis at concentrations from 1 to 50 mM. GlcNAc, on the other hand, stimulated

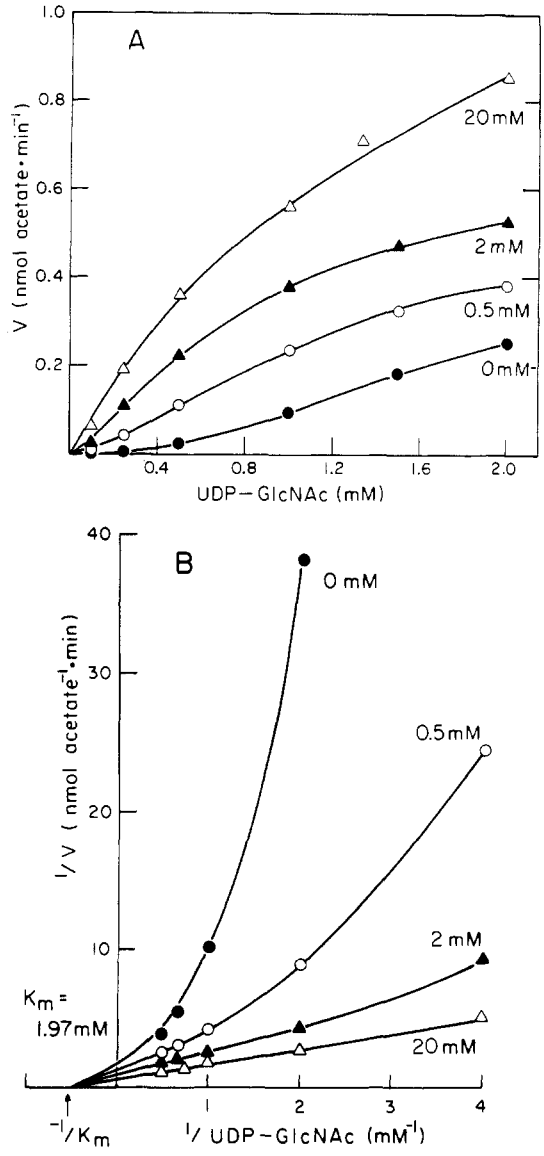

FIGURE 6: Effect of UDP-GlcNAc and GlcNAc concentrations on chitosan synthesis. Chitosan synthesis was determined in standard assay mixtures containing chitin synthetase, chitin deacetylase, the indicated concentrations of UDP-GlcNAc, and 0 (●), 0.5 (○), 2 (▲), or 20 (△) mM GlcNAc. The mixtures were incubated at 22 °C for 10 min. Chitosan synthesis was determined by the extraction method. Velocity (V) is expressed as nanomoles of acetate released per minute. (A) Direct plot. (B) Lineweaver–Burk plot.

chitosan synthesis over this same concentration range (Figure 5A). A K_m of 1.79 mM was estimated from the double-reciprocal plot relating the velocity of chitosan synthesis to GlcNAc concentration (Figure 5B).

The velocity of chitosan synthesis was a sigmoidal function of substrate (UDP-GlcNAc) concentration (Figure 6A). Addition of increasing concentrations of GlcNAc diminished the sigmoidicity. When the data were plotted by the double-reciprocal method, there was a nonlinear relationship between UDP-GlcNAc concentration and the velocity of chitosan synthesis, especially at low GlcNAc concentrations (Figure 6B). At 20 mM GlcNAc, however, this relationship approached linearity. A K_m of 1.97 mM was estimated by extrapolating the linear portions of the curves (i.e., values at high substrate concentration). Since the kinetics of chitosan synthesis are analogous to those reported for chitin synthesis [e.g., see Glaser & Brown (1957), Porter & Jaworski (1966), Keller & Cabib (1971), McMurrough & Bartnicki-Garcia (1971), and Ruiz-Herrera et al., (1977)], it is likely that

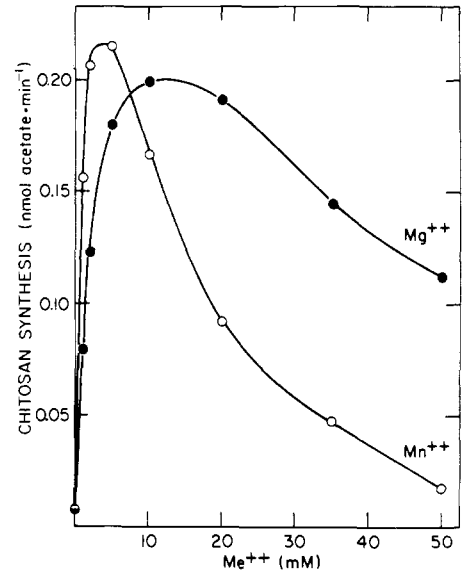

FIGURE 7: Effect of Mg^{2+} and Mn^{2+} on chitosan synthesis. Samples of chitin synthetase and chitin deacetylase were dialyzed separately against 4 L of 50 mM KH_2PO_4–NaOH buffer, pH 6.5, for 4 h at 4 °C to eliminate Mg^{2+}. Samples of the dialyzed enzymes were incubated together in standard assay mixtures containing 20 mM KH_2PO_4–NaOH buffer, pH 6.5, and the indicated concentrations of $MgCl_2$ (●) or $MnCl_2$ (○). Chitosan synthesis was determined by the extraction method and is expressed as nanomoles of acetate released per minute.

GlcNAc stimulates chitosan synthesis by acting as a positive allosteric effector of chitin synthetase.

Effect of pH and Buffer Concentration on Chitosan Synthesis. The pH of standard chitosan assay mixtures was varied with sodium succinate–HCl, KH_2PO_4–NaOH, or imidazole–HCl buffers, each at a final concentration of 50 mM. The combined data showed a sharp curve extending from about pH 5 to 8 with optimum activity at pH 6.5. The same pH optimum was found for chitin synthetase from *M. rouxii* (McMurrough & Bartnicki-Garcia, 1971; Ruiz-Herrera et al., 1977), whereas the pH optimum of chitin deacetylase is 5.5 (Araki & Ito, 1975). Highest activities were attained in the phosphate buffer. Thus, at pH 6.2, the velocity of chitosan synthesis measured in imidazole or succinate buffer was about 81% or 60%, respectively, of that in phosphate buffer. Chitosan synthesis was quite sensitive to the concentration of KH_2PO_4–NaOH buffer employed. Maximum activity was obtained with the lowest concentration of phosphate buffer tested (20 mM). A similar relationship was found between phosphate buffer concentration and crude mycelial chitin synthetase activity (McMurrough & Bartnicki-Garcia, 1971).

Effect of Cations on Chitosan Synthesis. Standard chitosan assay mixtures prepared with samples of chitin synthetase and chitin deacetylase previously dialyzed against 50 mM KH_2PO_4–NaOH buffer exhibited practically no activity (Figure 7) because of a requirement for divalent metal ions. Chloride salts of various cations were added to a final concentration of 5 mM to standard assay mixtures containing 20 mM KH_2PO_4–NaOH buffer, pH 6.5, and 4 mM $MgCl_2$. Of the cations tested, only Mg^{2+} and Mn^{2+} were stimulatory to chitosan synthesis; K^+, Na^+, or NH_4^+ had little effect; others ($Cu^{2+} > Fe^{3+} > Zn^{2+} > Ca^{2+}$) were inhibitory. The final concentration of $MgCl_2$ or $MnCl_2$ in standard chitosan assay mixtures was varied between 1 and 50 mM, and chitosan synthesis was determined. The optimal concentration of $MgCl_2$ was 10 mM, though there was a fairly broad range of concentrations at which Mg^{2+} strongly stimulated chitosan synthesis (Figure 7). $MnCl_2$ stimulated chitosan synthesis

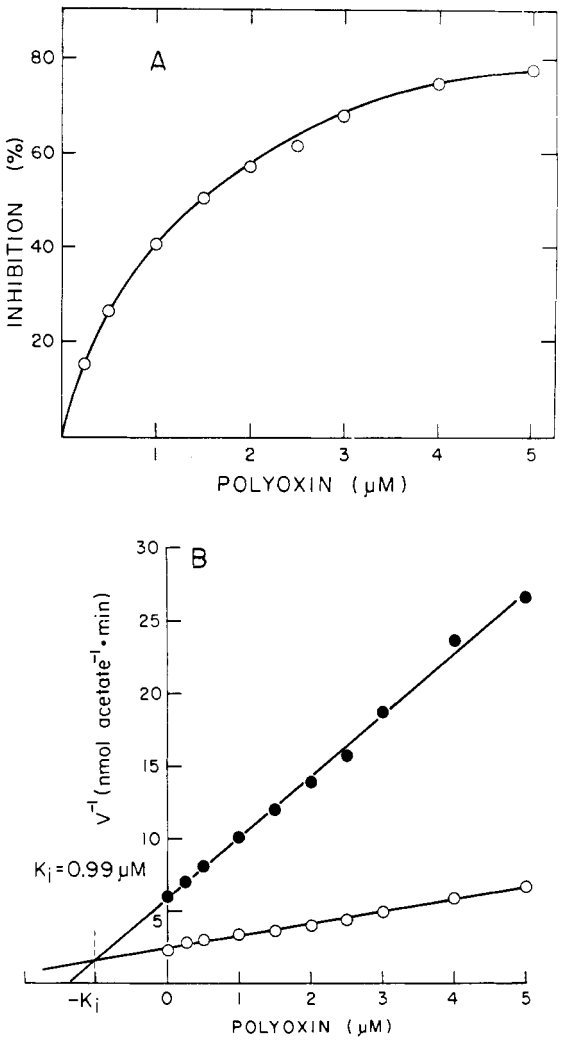

FIGURE 8: Inhibition of chitosan synthesis by polyoxin D. (A) Chitosan synthesis was determined in standard assay mixtures containing chitin synthetase, chitin deacetylase, and the indicated concentrations of polyoxin D. Chitosan synthesis was determined by the extraction method. Percentage inhibition was calculated relative to a control without polyoxin D. The rate of chitosan synthesis in control assays was 0.168 nmol of acetate released/min. (B) Determination of K_i. Reaction velocity (V = nanomoles of acetate released per minute) was determined in assay mixtures containing either 0.4 mM (●) or 2.0 mM (○) UDP-[*Ac*-^{14}C]GlcNAc and the indicated concentrations of polyoxin D. Data were plotted according to Dixon (1953).

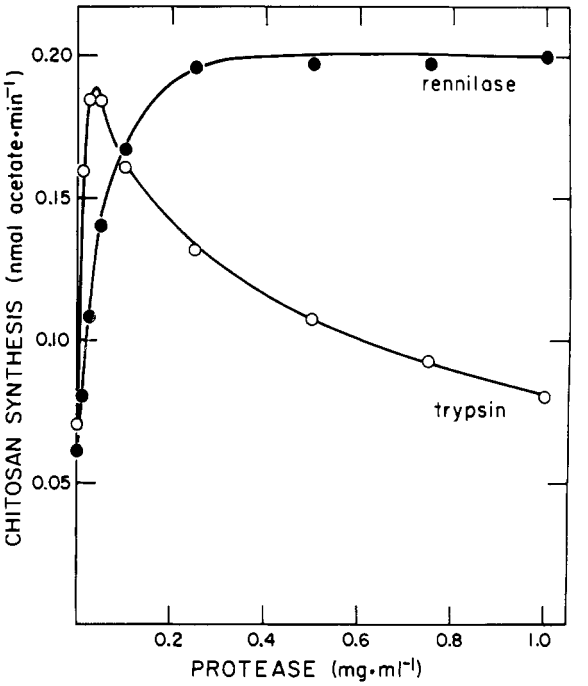

FIGURE 9: Activation of chitosan synthesis by proteases. Chitosan synthesis was measured in standard assay mixtures containing chitin synthetase, chitin deacetylase, and the indicated concentrations of rennilase (●) or trypsin (○). Chitosan synthesis was determined by the extraction method and is expressed as nanomoles of acetate released per minute.

slightly more than $MgCl_2$, with the optimal concentration at 5 mM. The concentration range at which Mn^{2+} strongly stimulated chitosan synthesis was much narrower, however. Above 10 mM $MnCl_2$, the velocity of chitosan synthesis decreased sharply. Mg^{2+} or Mn^{2+} is also required for chitin synthetase activity (McMurrough & Bartnicki-Garcia, 1971; Ruiz-Herrera et al., 1977). In contrast, chitin deacetylase shows no metal ion requirement (Araki & Ito, 1975).

Effect of Polyoxin D on Chitosan Synthesis. Polyoxin D is a powerful inhibitor of chitosan synthesis. At concentrations as low as 1.5 μM, polyoxin D caused 50% inhibition of chitosan synthesis (Figure 8A). The kinetics of polyoxin D inhibition of chitosan synthesis were investigated at various polyoxin concentrations, and at two different concentrations of UDP-GlcNAc. By plotting the data according to Dixon (1953), a K_i of 0.99 μM was estimated (Figure 8B). The effect of polyoxin D on chitin deacetylase activity was tested with glycol [*acetyl*-^{3}H]chitin as substrate. Polyoxin D, at concentrations from 0.25 to 60 μM, had no effect on chitin deacetylase activity.

Effect of Proteases on Chitosan Synthesis. Rennilase or trypsin greatly enhanced chitosan synthesis (Figure 9). Rennilase, a crude acid protease from *Mucor miehei*, had maximal effect over a wide concentration range, from 0.25 to 1.0 mg/mL. Trypsin was most effective in a much narrower concentration range, from 0.01 to 0.1 mg/mL; higher concentrations resulted in rapidly decreasing rates of chitosan synthesis. The differential response to trypsin and to rennilase closely approaches that observed for chitin synthetase preparations from *M. rouxii* (Ruiz-Herrera & Bartnicki-Garcia, 1976; Ruiz-Herrera et al., 1977). Presumably, the stimulatory effect of proteases on chitosan synthesis is due to proteolytic activation of zymogenic chitin synthetase (Cabib & Farkas, 1971; Bartnicki-Garcia et al., 1978).

Discussion

Sugar Nucleotide Precursor for Chitosan Synthesis. The evidence in vivo indicates that UDP-GlcNAc is the natural precursor for chitosan synthesis. Growing cells of *M. rouxii* utilize GlcNAc for the synthesis of both chitin and chitosan (Bartnicki-Garcia & Lippman, 1969). UDP-GlcNAc was the only radiolabeled nucleotide we detected in the cytosol of *M. rouxii* grown with [*GlcN*-^{14}C]GlcNAc; neither UDP-GlcN nor any other GlcN nucleotide was detected. It is therefore unlikely that chitosan synthesis proceeds by an independent pathway of direct incorporation of GlcN from a GlcN nucleotide. It is questionable whether UDP-GlcN exists at all in the cytosol of fungi. We found only one report that claimed the presence of UDP-GlcN in a fungus. Urbanek (1967) analyzed the nucleotides in the mycelium of *Aspergillus oryzae* by column chromatography on Dowex-1 and identified one of the peaks as a mixture of UDP-GlcNAc and UDP-GlcN. This identification was probably incorrect, however, since UDP-GlcN separates well from UDP-GlcNAc on such anion-exchange chromatography (Maley et al., 1956; see also Figure 1).

In Vitro Synthesis of Chitosan. By incubating UDP-GlcNAc with a mixture of chitin synthetase and chitin deacetylase, we demonstrated that this nucleotide is a substrate for chitosan synthesis in vitro. Substantial quantities of a polymer were made and characterized conclusively as chitosan by its solubility in acid, digestion by chitosanase but not by chitinase, and degradation by nitrous acid. C. Calvo and J. Ruiz-Herrera (personal communication) have also collected evidence indicating that UDP-GlcNAc is a precursor for the synthesis of both chitin and a nitrous acid sensitive polymer (i.e., chitosan) by crude membrane fractions of *M. rouxii*.

In most properties (pH optimum, Mg^{2+} or Mn^{2+} requirement, activation by GlcNAc and by proteases, inhibition by phosphate ions and by polyoxin), the behavior of the binary enzyme system (chitin synthetase + chitin deacetylase) responsible for chitosan synthesis closely paralleled the behavior of chitin synthetase. Particularly significant was the finding that chitosan biosynthesis was inhibited by polyoxin D, a highly specific and powerful competitive inhibitor of chitin synthetase, at concentrations comparable to those reported for inhibition of chitin synthetase from various fungi (Endo et al., 1970; Keller & Cabib, 1971; Bartnicki-Garcia & Lippman, 1972). As a whole, these observations reinforce our conclusion that chitin synthetase is the glycosyl transferase involved in chitosan synthesis and point to glycosyl transfer as the rate-limiting step for the in vitro synthesis of chitosan.

Biosynthesized chitosan was not a homopolymer of GlcN but contained a high proportion (26–29%) of acetylated residues. This is probably not an artifact of the biosynthetic system since chitosan isolated from the cell walls of *M. rouxii* also contains a substantial proportion (7–27%) of acetylated units (L. L. Davis and S. Bartnicki-Garcia, unpublished results). What determines the proportion of acetylated units in native chitosan is a question that has yet to be investigated. Presumably, it might be established by the ratio of chitin synthetase to chitin deacetylase at the site of synthesis.

Relationship between Chitin Synthetase and Chitin Deacetylase: A Tandem Mechanism. The inability of chitin deacetylase to act alone on preformed chitin and the success in producing chitosan when it was coincubated with chitin synthetase led us to consider three possibilities to explain the joint action of the glycosyl transferase and the deacetylase. (1) The first possibility is deacetylation prior to polymerization. The deacetylase would act on UDP-GlcNAc to produce UDP-GlcN, which would immediately be polymerized into chitosan. (2) Simultaneous deacetylation and polymerization is another possibility. The two enzymes would form a complex that would simultaneously deacetylate and polymerize the GlcNAc units from UDP-GlcNAc. (3) A third possibility is deacetylation following polymerization. GlcNAc units would first be linked into a transient chitin chain that would subsequently be attacked by the deacetylase. None of our evidence supported the first two options. But the finding that chitinase markedly reduced chitosan biosynthesis was convincing proof that the third alternative was correct, i.e., that a chitin polymer is first formed and subsequently deacetylated. The possibility that the reduction in chitosan recovery was due to chitosanase activity in the chitinase sample was ruled out: the chitinase did not digest biosynthesized chitosan. Since microfibrillar chitin is refractory to the deacetylase, deacetylation must occur before the *nascent* chains have a chance to crystallize into microfibrils. We conclude that the glycosyl transferase and the deacetylase must therefore operate consecutively in tandem fashion.

Deacetylation of Preformed vs. Nascent Chitin. Our evidence indicates that preformed chitin is a very poor substrate for the soluble chitin deacetylase from *M. rouxii*. This is consistent with the results of Araki & Ito (1975), who found that chitin deacetylase has little activity toward insoluble forms of chitin, such as colloidal or powdered chitin. The low activity of chitin deacetylase toward preformed chitin can be explained by the near inability of this enzyme to attack crystalline chitin. Preformed chitin, which is highly crystalline, consists of individual chitin chains which are tightly associated with one another through hydrogen bonding. Presumably, few acetyl groups in preformed chitin are accessible to the deacetylase. Individual chains of nascent chitin, on the other hand, are acted upon by chitin deacetylase before they have time to undergo extensive hydrogen bonding. The rate of deacetylation of nascent chitin vis-à-vis preformed chitin is difficult to compare since the actual concentrations of nascent chitin could not be determined but were probably extremely low.

In conclusion, we have found that chitin synthetase and chitin deacetylase, operating in tandem, catalyze the conversion of UDP-GlcNAc to chitosan. UDP-GlcNAc serves as the common substrate and chitin synthetase as the common glycosyl transferase to both chitin and chitosan syntheses. The pathway of chitosan synthesis appears to proceed by deacetylation of chitin as proposed by Araki & Ito (1975), but with one crucial modification: significant chitosan synthesis occurs only when chitin deacetylase acts on *nascent* chitin; once the chitin chains crystallize into microfibrils, they cease to be an effective substrate for the deacetylase.

Registry No. GlcNAc, 7512-17-6; UDP-GlcNAc, 528-04-1; chitosan, 9012-76-4; chitin synthetase, 9030-18-6; chitin deacetylase, 56379-60-3; polyoxin D, 22976-86-9.

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Effects of Ultraviolet Light on the in Vitro Assembly of Microtubules†

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ABSTRACT: Exposure of microtubular protein to ultraviolet light inhibits its assembly into morphologically normal microtubules. This effect appeared to result primarily from damage to the tubulin dimers. The damage consisted of a conformational change, a loss of two free sulfhydryl groups, a production of higher molecular weight cross-linked species, and the formation of aggregated amorphous material upon polymerization.

Previous studies in this laboratory have indicated that damage induced in microtubular protein by ionizing radiation is due primarily to the loss of two free sulfhydryl groups in the tubulin dimer (Zaremba & Irwin, 1981). These studies have been confirmed by Coss et al. (1981). The results suggested that the mitotic delay observed following exposure of cultured cells to ionizing radiation could be due to damage of microtubular protein. Studies in several laboratories have demonstrated that the microtubules of the mitotic spindle are formed from a pool of tubulin subunits rather than by de novo protein synthesis (Brinkley et al., 1975; Fulton & Simpson, 1979). Therefore, radiation-induced damage of tubulin could result in a delay in the formation of the mitotic spindle. In addition, since microtubules have been reported to function in cell shape, orientation of cell surface receptors, transport of neurotransmitters [for reviews see Olmsted & Borisy (1973), Roberts (1974), and Sloboda (1980)], the immune response (Snyderman & Goetzl, 1981; Malawista, 1975), hormone release (McDaniel et al., 1980; Wolff & Bhattacharyya, 1975), and regulation of adenylate cyclase (Rasenick et al., 1981) and microtubule depolymerization has been reported to initiate DNA synthesis (Crossin & Carney, 1981), such damage could have far-reaching effects in vivo.

Ultraviolet (UV) light administered at various stages of the cell cycle has also been reported to cause mitotic delay, spindle destruction, and inhibition of chromosome movement (Carlson, 1961; Smith, 1964; Inoue, 1964; Rustad et al., 1964; Brown & Zirkle, 1967; Sillers & Forer, 1981). The greatest delay in the onset of mitosis has been demonstrated to occur if cells are irradiated during S phase and is apparently due to repair of damaged DNA (Djordjevic & Tolmach, 1967; Domon & Rauth, 1968; Scaife, 1970; Bootsma & Humphrey, 1968; Han et al., 1971). However, some mitotic delay has also been shown to occur following exposure of cells to UV light during the G_2 phase of the cell cycle (Djordjevic & Tolmach, 1967; Domon & Rauth, 1968; Scaife, 1970; Bootsma & Humphrey, 1968; Carlson, 1976a,b) and therefore could be due to a mechanism other than DNA repair.

In this report we demonstrate that UV light inhibits microtubule assembly apparently by causing a loss of two sulfhydryl groups in the tubulin dimer, and therefore, a common mechanism of action could explain the effects of ionizing radiation on mitosis and UV light on spindle destruction and on mitotic delay during G_2.

Materials and Methods

Materials

Guanosine 5′-triphosphate (GTP), 2-(*N*-morpholino)-ethanesulfonic acid (MES), dimethyl sulfoxide (Me_2SO_4), and dithiodinitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. Acrylamide, methylenebis(acrylamide), ammonium peroxydisulfide, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratories. Glycerol, 2-mercaptoethanol (BME), and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) were obtained from Eastman-Kodak Chemical Co. Other chemicals used were reagent grade from Baker or Fisher Chemicals. Calf brains were obtained from a local slaughterhouse within 2 h of slaughter.

Methods

Isolation of Microtubular Protein. Microtubules were isolated from calf brains by successive cycles of polymerization and depolymerization in the presence of glycerol according to the method of Shelanski et al. (1973). The microtubular protein was stored in 0.1 M MES buffer (0.1 M MES, 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 6.5) containing 2 M glycerol

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