Chitin and Yeast Budding

PROPERTIES OF CHITIN SYNTHETASE FROM SACCHAROMYCES CARLSBERGENSIS

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SUMMARY

A particulate preparation from a spheroplast lysate of Saccharomyces carlsbergensis was found to catalyze the transfer of acetylglucosamine from UDP-acetylglucosamine to an endogenous acceptor. Uridine diphosphate is liberated in stoichiometric amounts. A divalent cation, Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\), is required for enzymatic activity. Acetylglucosamine stimulates the activity about 5-fold, with a \(K_m\) of 4.7 mM, and can be partially replaced by high concentrations of glucose, mannose, cellobiose, and glycerol. The \(K_m\) for UDP-acetyl-glucosamine is between 0.6 and 0.9 mM and the pH optimum is 6.2. Centrifugation in sucrose gradients indicates that the reaction product remains bound to the same particles which contain the activity. The product was characterized as chitin by its insolubility in alkali, the release of glucosamine on acid hydrolysis, and the liberation of diacetylchitobiose and acetylglucosamine following stepwise enzymatic hydrolysis. The enzyme is inhibited by monovalent and polyvalent anions, the latter being more effective. The antibiotic Polyoxin A is a very potent competitive inhibitor, with a \(K_i\) of \(5 \times 10^{-7}\) M. Polyoxin A affected neither growth of intact cells nor synthesis of chitin by naked spheroplasts. It is concluded that chitin synthetase is not located outside the cytoplasmic membrane.

Previous reports have shown that the small amount of chitin present in the cell wall of yeast is concentrated in an annular area at the bud scar site (1–3). Consequently, the suggestion was made (3) that this polysaccharide may have an important role in the process of yeast budding. It thus became of interest to investigate the mechanism of chitin biosynthesis in yeast at the molecular level in order to approach correlations between biochemical and morphological characteristics. The present report deals with the general properties of the chitin synthetase.1

EXPERIMENTAL PROCEDURE

Materials

UDP-GlcNAc, labeled with \(^{14}C\) in C-1 of the hexosamine moiety, was prepared as directed by O’Brien (5). The same

1 The noncommittal but descriptive term “chitin synthetase” is used in this and the following paper (4), because the endogenous acceptor of the acetylglucosaminyl units has not been identified.

2 The abbreviation used is: UDP-GlcNAc, UDP-N-acetylglucosamine.

nucleotide, labeled with \(^{3}H\) in the acetyl group, was a generous gift of Dr. E. Neufeld. GDP-mannose, labeled with \(^{14}C\) in the sugar moiety, was obtained by a procedure similar to that of Rosen and Zeleznik (6), but with an extract of lyophilized yeast, after passage through Sephadex G-25, as a source of GDP-mannose pyrophosphorylase. Unlabeled UDP-GlcNAc and GDP-mannose were prepared as previously described (7).

Polyoxin A was kindly provided by Dr. K. Isono. Amphotericin A, Amphotericin B, and Nystatin were donated by Squibb and Sons, New York, New York, Griseofulvin by Schering Corporation, Bloomfield, New Jersey, Vancomycin by Eli Lilly and Company, Indianapolis, Indiana, and Ristocetin by Abbott, North Chicago, Illinois. Actidione and Bacitracin were purchased from Mann, and Streptomycin and Penicillin G from Calbiochem.

We are indebted to Dr. L. Glaser for samples of diacetylchitobiose and chitodextrins. The chitodextrins were purified by passage through an MB-3 Amberlite column before use. Yeast RNA was a gift from Dr. Maxine Singer. Inorganic polyphosphate (molecular weight \(\approx 3700\)) was kindly furnished by Dr. W. Carroll. Polyglutamic acid (molecular weight 105,000) and polylysine (molecular weight 195,000) were purchased from Sigma. Dr. P. O’Brien generously supplied samples of glucosamine 6-phosphate, N-acetylglucosamine 6-phosphate, and \(\alpha-N\)-acetylglucosamine 1-phosphate.

Methods

Enzyme Preparation—Saccharomyces carlsbergensis strain 74S (National Collection of Yeast Cultures, England) was grown (g) and collected when the optical density at 600 m\(\mu\) was 0.4 to 0.5; this value corresponds to the late logarithmic phase, about one generation before the onset of the stationary phase.

Saccharomyces cerevisiae A364A and its temperature-sensitive mutant 316\(^{2}\) were grown as described by Hartwell (9).

In the early part of this study, spheroplasts were prepared as previously reported (10). In more recent experiments 0.55 M mannitol was substituted for 0.6 M KCl as osmotic stabilizer. Spheroplasts prepared and washed with mannitol showed less lysis than those obtained with KCl, and were very well preserved even after storage for several days at 4\(^{\circ}\), with the addition of aureomycin (50 \(\mu\)g per ml) to prevent bacterial growth.

Many preparations of the particulate enzyme were obtained from spheroplasts, as previously described for mannan synthetase.

2 Mutant 316 grows at 24\(^{\circ}\) but not at 37\(^{\circ}\). Both strains of \(S.\) cerevisiae, A364A and 316, were generously provided by Dr. L. H. Hartwell.
(10). More recently, somewhat higher and more reproducible enzymatic activity was obtained by the following procedure. After the last washing with 0.55 M mannitol, spheroplasts were resuspended in the same reagent up to a volume numerically equal in milliliters to two-thirds of the grams of yeast, wet weight used. The spheroplast suspension was added, while stirring with a Vortex mixer, to 5 volumes of Buffer A (50 mM imidazole-chloride, pH 6.5, containing 2 mM MgSO₄) containing 0.385 M mannitol. At brief intervals further additions were made of 3 and 5 volumes, respectively, of Buffer A. The suspension was then centrifuged for 10 min at 20,000 × g and the pellet was washed twice with an amount of Buffer A equivalent to about 4 volumes of the original spheroplast suspension. The final pellet was resuspended, up to the original volume of the suspension, with tritium in the acetyl group (specific activity 200,000 cpm per ml). The enzyme was maintained at about −9°C without freezing. About 50% of the activity was lost in 2 weeks at this temperature.

**Enzyme Assay**—The incubation mixture contained 0.95 mM ¹⁴C or ³H UDP-GlcNAc (see below), 0.05 M imidazole chloride, pH 6.5, 0.8 mM magnesium sulfate, 40 mM acetylglucosamine, and variable amounts of enzyme in a total volume of 50 to 53 μl.

The UDP-GlcNAc used was labeled with ³H in C-1 of the hexosamine moiety (specific activity 107,000 cpm per μmole) or with tritium in the acetyl group (specific activity 200,000 cpm per μmole).

The enzyme contained glycerol, which acts as an activator of chitin synthetase (see below). The glycerol concentration in the incubation mixture was kept constant at 1.36 M, except where indicated otherwise.

After incubation for 1 hour at 30°C the reaction was stopped by the addition of 1 ml of 66% ethanol, and the tubes were centrifuged for 5 min at 1500 × g. The pellets were washed twice with 1 ml of 66% ethanol containing 0.1 M ammonium acetate. Further treatment depended on the sugar nucleotide label. When using tritiated UDP-GlcNAc, the pellets were resuspended in 0.4 ml of absolute ethanol by stirring on a Vortex mixer at maximum speed, and poured into a scintillation vial. The tube was washed with another 0.4-ml portion of absolute ethanol and 12 ml of scintillation mixture was added. The mixture contained, per liter of toluene, 5 g of 2,5-diphenyloxazole, 3.2 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene, and 43 g of CAB-O-SIL thiosulfate gel (Packard Instrument Company). The vials were vigorously stirred with a Vortex mixer and counted in a Packard liquid scintillation spectrometer.

When ³C-labeled UDP-GlcNAc was the substrate, the washed pellets were resuspended in water, plated on copper or stainless steel planchets, dried at 100°C, and counted in a Nuclear-Chicago low background, thin window counter.

In order to account for quenching and self-absorption, the results were multiplied by an experimentally determined correction factor which was 1.56 for ³H and 1.45 for ¹⁴C.

The enzyme was prepared as described under "Experimental Procedure," except that the particles were washed three additional times with 0.05 M imidazole, pH 6.5, to eliminate glycerol and Mg²⁺. The complete incubation mixture was as described under "Experimental Procedure." The activity under those conditions is taken as 100.

### Table I

**Requirements for yeast chitin synthetase**

The general requirements for enzymatic activity are shown in Table I. The enzyme was stimulated by divalent cations and by acetylglucosamine. No activity was observed when the incubation mixture contained EDTA in the absence of added Mg²⁺. The incorporation of label into material insoluble in 66% ethanol was linear during the 1st hour of incubation and then declined (Fig. 1). Activity was proportional to the amount of added enzyme, at least up to 40 μl of the standard preparation. The maximal incorporation obtained with the best preparations exceeded 30% of the added substrate. The K₉₅ value for UDP-GlcNAc varied between 0.6 and 0.9 mM in different experiments.

The optimal temperature for enzymatic activity was about 37°C, with half-maximal values at 28 and 48°C. The pH optimum was close to 6.2 (see Fig. 2). The highest activity was obtained with 2-(N-morpholino)ethane sulfonic acid. Imidazole-chloride seems to be somewhat inhibitory; in the standard reaction mixture this buffer was present at half the concentration used in determining the pH optimum.

**Specificity of Metal Requirement**—Mg²⁺, Mn²⁺, and Co²⁺ were the only cations among those tested which showed significant stimulation.

**Activators**—As is the case with other chitin synthetases (13–15), the enzyme from yeast was stimulated by acetylglucosamine. The K₉₅ for acetylglucosamine is 4.7 mM. Other compounds had a similar effect albeit at much higher concentration. As shown in Fig. 4, the best activators, aside from acetylglucosamine, were cellulose and glucose. Mannose and glycerol were less effective. Several other compounds were also tested at different concentrations (the maximal concentration used is listed in parentheses). Slight activation was obtained with sucrose (0.29 M). No effect was found with glucosamine (60 mM).

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[^4]: The method used in the preparation of UDP-¹⁴C-GlcNAc results in a product contaminated with the acetylglucosamine analogue (5). The amount of the latter was ascertained by hydrolyzing the nucleotide in 0.1 N acid at 100°C, and separating the free sugars by paper chromatography on borate-treated Whatman No. 1 paper (12), with 1-butanol-pyridine-water (6:4:3) as solvent. The acetylglucosamine peak represented 31% of the total radioactivity; this value was used to correct the specific activity of the sugar nucleotide.

[^6]: With other preparations, the maximal activation obtained with glycerol was only slightly less than with 40 mM acetylglucosamine.
Fig. 1. Time course of the reaction. Conditions as for the standard assay, except that imidazole was replaced by 0.05 M 2-(N-morpholino)ethane sulfonic acid, pH 6.1. Enzyme concentration, 3.8 mg per ml.

Fig. 2. Effect of pH on the activity. Conditions as in the standard assay, except for the buffer used. The potassium salt of phosphate was employed. Enzyme concentration, 3.8 mg per ml. MES, 2-(N-morpholino)ethane sulfonic acid.

Fig. 3. Effect of metal cations on the activity. The enzyme was prepared as described under “Experimental Procedure” and centrifuged again for 10 min at 20,000 × g. The pellet was resuspended in 5 times the initial enzyme volume of 0.1 M EDTA and kept at 0° for 30 min. The suspension was then centrifuged again in the same way. The pellet was washed once, with 5 times the initial enzyme volume of 0.02 M 2-(N-morpholino)ethane sulfonic acid, pH 6.1, and brought back to the initial volume with the same buffer. The assay was carried out under the standard conditions except for the cation used. Enzyme concentration, 3.8 mg per ml.

Fig. 4. Effect of activators on chitin synthetase activity. The enzyme was prepared as described under “Experimental Procedure.” Then, 5 volumes of 0.05 M imidazole-chloride, pH 6.6, were added, the suspension was centrifuged for 10 min at 12,000 × g, and the pellet was washed twice with the same volume of imidazole buffer. The final pellet was resuspended in the imidazole buffer up to the original volume. The assay was carried out under the standard conditions except for the concentration of the activator. Enzyme concentration, 3.8 mg per ml.

Identification of Chitin as Reaction Product—As mentioned under “Experimental Procedure,” the radioactive reaction product was insoluble in 66% ethanol. After boiling for 45 min in 1 N KOH, 60% of the radioactivity was recovered in the alkali-insoluble fraction in two separate experiments; 37 and 18%, respectively, was found in the fraction soluble in alkali but insoluble in 66% ethanol. In each case lyophilized yeast, previously disrupted by sonic disintegration, was added as carrier. Additional information was obtained by degrading the product chemically and enzymatically. For this purpose scaled-up reaction mixtures, prepared as for the standard assay using UDP-[14C]-acetate as substrate, were incubated and treated with 66% ethanol, as described under “Experimental Procedure.” In one case the insoluble pellet was suspended in concentrated hydrochloric acid and heated for 3 hours at 100° in a sealed tube. The hydrolysate was passed through a Dowex 1 (acetate) column and submitted to paper chromatography with 1-butanol-pyridine-water (6:4:3) as solvent. As shown in Fig. 5A, a single
radioactive peak was observed, which migrated at the same Rf as glucosamine.

In a separate experiment, washed particles were freed from ethanol by evaporation under reduced pressure, and then incubated with purified chitinase for 2 hours at 30° under the conditions described elsewhere (3). The mixture was heated for 2 min at 100° and centrifuged at 2000 X g. The pellet was washed twice with 0.3-ml portions of water. Only 3 to 4% of the previously incorporated radioactivity remained in the chitinase-treated pellet. The supernatant fluid was passed through an MB-3 Amberlite column (acetate-hydrogen form), concentrated, and chromatographed on Whatman No. 1 paper, with 1-butanol-pyridine-water (6:4:3). As shown in Fig. 5B, the radioactive material migrated in a single peak, which coincided with a standard sample of diacetylchitobiose. The radioactive compound was eluted with water, evaporated, and incubated for 1 hour at 30° with Sephadex-treated snail intestinal extract under the conditions previously described for the chitinase assay (3). As already reported (3), snail gut juice contains enzymes which hydrolyze diacetylchitobiose to the monosaccharide. After passage of the enzymatic hydrolysate through an MB-3 Amberlite column and paper chromatography, a single radioactive peak was again obtained, with the same Rf as acetylglucosamine (see Fig. 5C).

To ascertain the fate of the UDP-GlcNAc remaining after incubation, supernatant liquid obtained from the standard assay system, after precipitation of the particles with 66% ethanol, was concentrated and submitted to paper chromatography with neutral ethanol-1 m ammonium acetate (7:3:5) (16). A single radioactive peak was found, corresponding to unreacted UDP-GlcNAc. Thus, hydrolysis of the substrate by the enzyme preparation was not detectable and could not be the cause for the decrease in rate observed in Fig. 1.

**TABLE II**

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>H2O-Acetylglucosamine incorporated (µmole)</th>
<th>UDP liberated (µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>18.5</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>33</td>
<td>34</td>
</tr>
</tbody>
</table>

* In other experiments it was found that phosphatase action was minimal in the presence of Co++.
Polylysine RNA. Polyglutamic acid.

Inorganic polyphosphate.

Inorganic polyphosphate.

The final concentration in the standard assay mixture is given in each case.

<table>
<thead>
<tr>
<th>Inhibitor concentration</th>
<th>Mg²⁺ concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>20</td>
<td>0.8</td>
</tr>
<tr>
<td>RNA</td>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>RNA</td>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>Inorganic polyphosphate</td>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>Inorganic polyphosphate</td>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>Polyglutamic acid</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Polyglutamic acid</td>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>Polylysine</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Polylysine</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Polylysine</td>
<td>2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Compared with a control containing 5 mM magnesium sulfate.  
* At these concentrations of polylysine the particles coagulated.

Stoichiometry of Reaction—The enzymatic preparations contained phosphatases which interfered with the determination of UDP formed in the reaction. A more favorable ratio of chitin synthetase to phosphatase was found in other yeast strains, i.e. S. cerevisiae A364A and a temperature-sensitive mutant derived from it (mutant 316). Strains A364A and 316 yielded, respectively, 2 and 4 to 5 times as much chitin synthetase activity as S. carlsbergensis. The recovery of enzymatic activity was poor, but the distribution of chitin synthetase coincided with that of the labeled chitin. Total protein, when measured, also yielded the same pattern of peaks.

Inhibitors—It would be of obvious interest to find a way of controlling the activity of chitin synthetase in vivo. For this reason the inhibition of the enzymatic system by a number of substances was studied.

Nucleotide sugars depressed chitin synthetase activity somewhat, probably because of their structural analogy to UDP-GlcNAc. UDP-GlcNAc, UDP acetylglucosamine.

Localization of Chitin Synthetase and Reaction Product on Subcellular Particles—In a previous study of yeast mannan synthetase it was found that the mannan formed in the reaction remained attached to water-insoluble particles. Centrifugation of incubation mixtures of chitin synthetase at 80,000 × g also resulted in the recovery of radioactivity in the pellet. Additional information was obtained by submitting the 3H-labeled reaction product to isopycnic centrifugation in a sucrose gradient. The pellet containing tritiated chitin was mixed with particles which had been labeled with ¹⁴C-mannan by previous incubation of GDP-mannose with the same enzymatic preparation (10). It can be observed (Fig. 6) that the radioactivity was distributed over a large portion of the gradient, and that the distribution of chitin and mannan was essentially the same. The specific pattern of peaks was reproducible for each preparation of enzyme, but varied among different preparations. In separate experiments, which are not presented in detail, chitin synthetase activity was also determined in the various fractions. The recovery of enzymatic activity was poor, but the distribution of chitin synthetase coincided with that of the labeled chitin. Zero time control.

The amount of chitin was referred to 1 g of the yeast, wet weight, used to obtain the spheroplasts.

Experimental details given in the text. The amount of chitin was referred to 1 g of the yeast, wet weight, used to obtain the spheroplasts.

**Table I**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Chitin found</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Polyoxin A</td>
<td>mg/g yeast, wet wt</td>
</tr>
<tr>
<td>0.8 × 10⁻⁴ M Polyoxin A</td>
<td>&lt;0.03 &lt;0.03</td>
</tr>
<tr>
<td>Chung and Nickerson's medium</td>
<td>0.64 0.54</td>
</tr>
<tr>
<td>Wickerham's medium</td>
<td>0.60 0.61</td>
</tr>
<tr>
<td>Wickerham's medium, lysed spheroplasts*</td>
<td>0.05 0.05</td>
</tr>
</tbody>
</table>

* The spheroplasts were added to mannitol-free medium and mixed well, after which the medium was supplemented with mannitol to a final concentration of 0.55 M.

**Table II**

| Compounds containing numerous phosphate groups, such as RNA and inorganic polyphosphate, were inhibitory. As shown in Table III, some but not all of the effect may be attributable to chelation of divalent cations. Other inhibitory electrolytes were polyglutamate and polylysine, although the latter caused aggregation of the particulate enzymatic preparation (Table III). Certain anions, e.g. chloride, phosphate, and sulfate, depress enzymatic activity. This can be partly seen in Fig. 2, where a lower curve was obtained with the chloride than with the acetate of imidazole, and phosphate appears to be even more inhibitory.

In other experiments, with standard assay conditions, 0.1 and 0.15 M NaCl resulted in 58 and 87% inhibition, respectively,
whereas the values for 0.1 and 0.15 m NaSO₄ were 91 and 99%, respectively.

A number of antibiotics, some of which were found to depress the biosynthesis of bacterial nucleopeptides, and some of which inhibit yeast growth, were found to be without effect on chitin synthetase. The list included Nystatin, Amphoterin A, Amphoterin B, Streptomycin, and Griseofulvin, all assayed at the 10 and 100 µg per ml levels, Actidione and Penicillin, assayed at 20 and 200 µg per ml, Vancomycin, at 25 and 250 µg per ml, Ristocetin, at 40 and 200 µg per ml, and Dacitacin, at 500 µg per ml.

On the other hand, Polyoxyin A, a metabolite of Streptomyces cacaoi which is endowed with antibiotic activity against phytopathogenic fungi (18), was strongly inhibitory (Fig. 7). The inhibition is of the competitive type and the Kᵢ is 5 × 10⁻⁷ m, about 1000 times smaller than the Kᵢ for UDP-GlcNAc. Complete inhibition could be obtained (97% inhibition at 3 × 10⁻⁶ m Polyoxyin A). The results are similar to those reported recently by Endo and Misato (19) for Polyoxyin D (18) on the chitin synthetase of Neurospora crassa. Whereas Polyoxyin D is also inhibitory in vivo for Neurospora, no effect of Polyoxyin A could be shown on growing yeast cells at a concentration of 0.8 × 10⁻⁴ m.

On the assumption that removal of the cell wall might facilitate the access of the antibiotic to the enzyme site the effect of Polyoxyin A on yeast spheroplasts was examined. Eddy and William-son (20) showed that spheroplasts were able to produce aberrant cell walls with a high content of hexosamine, when incubated in a growth medium. A quantitative estimate of the synthesized chitin was obtained as follows. Spheroplast samples, representing 70 mg of whole yeast, wet weight, were incubated in 5 ml of Wickerham medium (21), or Chung and Nickerson's medium (22) to which 0.3% yeast extract was added. Both media contained 0.55 m mannitol as osmotic stabilizer, plus 250 units per ml of Penicillin and 250 µg per ml of Streptomycin. In each case duplicate samples were used, to one of which Polyoxyin A was added at a final concentration of 0.8 × 10⁻⁴ m. After overnight incubation at 30° with gentle shaking, the spheroplasts were found to be greatly swollen and to have collected in large clumps. The suspension was centrifuged at 1000 × g and the spheroplasts were lysed in 0.5 ml of water. The particulate fraction was isolated by the following steps: centrifugation for 10 min at 10,000 × g, resuspension in water followed by heating for 5 min at 100°, recentrifugation, and, again, suspension in water. Chitin was determined enzymatically (3) in the insoluble material. The spheroplasts did accumulate chitin, as shown in Table IV. For comparison, the chitin content of intact S. carlsbergensis is about 0.6 mg per g of yeast, wet weight (3). Less than 10% of the increase shown in Table IV was found when spheroplasts were lysed prior to incubation. The table also shows that there was no difference in chitin formation upon addition of Polyoxyin A to the spheroplast incubation mixture.

Polyoxyin A, at a concentration of 0.8 × 10⁻⁴ m, did not significantly affect the activity of yeast mannan synthetase (10) or glycogen synthetase (23).

**DISCUSSION**

The chitin synthetase from yeast resembles those obtained from other sources (13-15) in its general requirements. Thus, divalent cations are necessary for the reaction, and acetylglucosamine is also an activator. On the other hand, chitodextrins, which were stimulatory with other preparations, appear to be ineffective in the yeast system. It is noteworthy that acetylglactosaminase was without effect (cf. 13). In contrast, compounds which are less closely related structurally to acetylglucosamine, e.g. glycerol and glucose, were active albeit at high concentrations, and their effect was not additive to that of acetylglucosamine.

One of the reaction products is UDP, as reported for the chitin synthetase of Allozymes macrosporum (14). For the determination of liberated UDP, an enzyme from mutant 316 was employed here because of its high specific activity. The increased activity indicates that the enzyme from the mutant differs somehow from that of the parent strain. Yet, it seems safe to assume that the two enzymes catalyze the same reaction.

The other reaction product was identified as chitin mainly on the basis of the compounds released after acid or enzymatic hydrolysis. The fact that radioactive disaccharide was isolated from the chitinase digest, shows that the transferred acetylglucosamine was linked to another residue of the same monosaccharide. The insolubility of most of the polysaccharide after alkaline digestion is also in agreement with the properties of chitin. On the other hand, the smaller amount of alkalinsoluble material may correspond to the synthesis of a relatively short chain.

When mannan synthetase was studied in the same organism it was found that the product was sedimentable by centrifugation, in agreement with the conclusion that the mannan was attached to a particulate fraction (10). The same observation was made with synthesized chitin. However, since chitin itself is insoluble, the interpretation is less clear. Nevertheless, the finding that synthesized chitin is distributed in the sucrose gradient in exact correspondence with the mannan-carrying fractions, as found in the double isotope experiment, suggest that both polysaccharide products remain attached to particles.

Yeast chitin synthetase is inhibited by anions, especially by those in polymeric form. Of more potential usefulness is the observation that the antibiotic Polyoxyin A is a very strong inhibitor. As noted by Isono, Asahi, and Suzuki (18), Polyoxyin A may be considered as a structural analogue of UDP-GlcNAc, and that is in agreement with the competitive character of the inhibition. The absence of action in vivo, in contrast to the effect of Polyoxyin D on the growth of Neurospora (19), is probably caused by lack of penetration through the cell membrane. Conversely, the ineffectiveness of Polyoxyin A on chitin formation by yeast spheroplasts indicates that chitin synthetase is not located on the outside of the cytoplasmic membrane.

**Acknowledgments**—We are indebted to Dr. L. B. Robinson-Denes for many useful discussions and for help in some experiments. We are also grateful to Doctors G. Ashwell and W. B. Jakoby for a critical reading of the manuscript.

**REFERENCES**

5. O'Brien, P. J., in E. F. Neufeld and V. Ginsburg (Editors),

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p. 161, left hand column, line 8 should read:

imidazole-chloride, pH 6.5, containing 2 mM MgSO4) containing
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