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++, Mn++, or Cu++, 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 nor synthesis of chitin by naked spheroplasts. It is concluded that chitin synthetase is not located outside the cytoplasmic membrane.

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 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 Zeleznick (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, Grisefundin by Schering Corporation, Bloomfield, New Jersey, Vancomycin by Eli Lilly and Company, Indianapolis, Indiana, and Ristocetin by Abbott, North Chicago, Illinois.

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 $\sim$ 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 748 (National Collection of Yeast Cultures, England) was grown (8) and collected when the optical density at 600 nm 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 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°C, with the addition of aureomycin (50 μg per ml) to prevent bacterial growth.

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

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 Mutant 316 grows at 24°C but not at 37°C. Both strains of S. cerevisiae, A364A and 316, were generously provided by Dr. L. H. Hartwell.
to about 4 volumes of the original spheroplast suspension. The final pellet was resuspended, up to the original volume of the spheroplast suspension, in Buffer A containing 33% glycerol.

The protein content of such preparations ranged between 10 and 13 mg 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 $^{14}$C or $^{3}H$ UDP-GlcNAc (see below), 0.06 mM 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 $^{14}$C 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 and counted in a Packard liquid scintillation spectrometer.

When $^{14}$C-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 $^{14}$C and 1.45 for $^{3}H$.

**Specificity of Metal Requirement**—Mg$^{2+}$, Mn$^{2+}$, and Co$^{2+}$ were the only cations among those tested which showed significant stimulation. Several other compounds were also tested at different concentrations (the maximal concentration used is listed in parentheses). The $K_m$ for acetylglucosamine was 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 cellobiose 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).

**Results**—Protein was determined according to the method of Lowry et al. (12). Mannan synthetase was assayed as previously reported (10). Radioactivity on paper chromatograms was measured with a Vanguard scanner.

| Incubation mixture | Enzymatic activity |
|--------------------|--------------------|
| Complete           | 100                |
| Minus acetylglucosamine | 21                |
| Minus acetylglucosamine, plus 1.4 M glycerol | 57                |
| Minus Mg$^{2+}$    | 27                |
| Minus Mg$^{2+}$, plus 1 mM Mn$^{2+}$ | 94                |
| Minus Mg$^{2+}$, plus 8 mM EDTA  | 1                 |
| Minus Mg$^{2+}$ and acetylglucosamine | 1                 |

**Miscellaneous**—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 Mg$^{2+}$. 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 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 Mg$^{2+}$. The complete incubation mixture was as described under "Experimental Procedure." The activity under those conditions is taken as 100.

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| Minus Mg$^{2+}$, plus 8 mM EDTA  | 1                 |
| Minus Mg$^{2+}$ and acetylglucosamine | 1                 |
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 X 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 X 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]-GlcNAc 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

acetylgalactosamine (60 mM), acetylgalactosamine 6-P (24 mM), trehalose (0.2 M), and mannitol (0.2 M). Glucose-6-P (40 mM), glucosamine-6-P (80 mM), and α-N-acetylglucosamine 1-P (32 mM) were inhibitory. Adding 30 mM acetylgalactosamine in the presence of 1.36 M glycerol or 0.56 M glucose increased the activity by only about 10 to 15%. Chitodextrins, which stimulated chitin synthetase from other sources (13-15), were practically ineffective in this case. Addition of 0.13 mg of chitodextrins to the standard incubation mixture resulted in a stimulation of only 22% in the absence of acetylglucosamine, and 5% in its presence.

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]-GlcNAc 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 $R_F$ 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 $\times g$. 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 and paper 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 peak was again obtained, with the same $R_F$ 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 60% ethanol, was concentrated and submitted to paper chromatography with neutral ethanol-1 M ammonium acetate (7:3:8) (10). 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**

Slochiometry of reaction

Chitin synthetase was obtained from *S. cerevisiae* 316. Incubation was carried out under standard conditions except for the replacement of Mg$^+$ by 1 mM Co$^{2+}$. For the measurement of acetylglucosamine incorporation, the standard assay procedure was used. For UDP determination the reaction was stopped by heating for 3 min at 100° and the mixture was centrifuged for 15 min at 60,000 $\times g$. UDP was measured in the supernatant fluid with the method used by Lowry et al. (17) for ADP.

| Incubation time (min) | $\Delta$-Acetylglucosamine incorporated (mмоles) | UDP liberated (mмоles) |
|-----------------------|-----------------------------------------------|-----------------------|
| 15                    | 18.5                                         | 15                    |
| 30                    | 33                                           | 34                    |

* In other experiments it was found that phosphatase action was minimal in the presence of Co$^{2+}$.

**FIG. 5.** Paper chromatography of degradation products of the synthesized polysaccharide. A, hydrolysis with concentrated HCl; B, after chitinase attack; C, radioactive peak from B, after incubation with snail enzyme. For explanations, see text.

**FIG. 6.** Density gradient centrifugation of particles containing labeled mannan and chitin. Reaction mixtures for mannan synthetase (10) and chitin synthetase, each equivalent to 10 standard assays, were prepared and incubated separately for 1 hour at 30°. The respective substrates were GDP-mannose labeled with $^{14}$C in the mannose moiety and UDP-GlcNAc labeled with $^3$H in the acetyl group. After incubation the reaction mixtures were diluted with 20 volumes of Buffer A and centrifuged at 22,000 $\times g$ for 10 min. The pellets were combined and washed with the same buffer. The combined pellet was resuspended in 16% sucrose and layered on top of the gradient. The latter was formed by layering successively the following sucrose solutions: 0.5-ml portions of 65, 60, and 57% (w/w); 1-ml portions of 55 to 39%, decreasing the concentration in steps of 2%; 0.5-ml portions of 37, 34, 30, 25, and 20%. A gradient obtained in this way allowed maximal resolution of the peaks. Centrifugation was performed at 5° for 16 hours, using International rotor SB-269 at 30,000 rpm (105,000 $\times g$). The position of the bands which were visible after centrifugation is shown in the upper part of the graph. The tube contents were collected from the bottom in 0.5-ml fractions. The particles were reisolated by 66% ethanol precipitation and the radioactivity of each entire fraction was determined by scintillation counting. Sucrose concentration was determined by refractometry in an identical gradient.
TABLE III
Inhibition of chitin synthetase by polyelectrolytes

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

| Inhibitor concentration | Mg\(^{2+}\) concentration | Inhibition |
|-------------------------|---------------------------|-----------|
| mg/ml                   | mM                        | %         |
| RNA                     | 20                        | 0.8       | 82        |
| RNA                     | 5                         | 0.8       | 56*       |
| RNA                     | 5                         | 5         | 48*       |
| Inorganic polyphosphate | 2                         | 0.8       | 41        |
| Inorganic polyphosphate | 5                         | 0.8       | 99        |
| Inorganic polyphosphate | 5                         | 5         | 44*       |
| Polyglutamic acid       | 5                         | 0.8       | 36        |
| Polyglutamic acid       | 5                         | 0.8       | 58        |
| Polysine                 | 0.1                       | 0.8       | 22        |
| Polysine                 | 1                         | 0.8       | 74*       |
| Polysine                 | 2                         | 0.8       | 88*       |

* Compared with a control containing 5 mM magnesium sulfate.

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 74S per g of yeast. It can be seen from Table II that an enzyme from mutant 316 liberated an amount of UDP equivalent to the radioactivity incorporated into the insoluble fraction.

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 x 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. 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. The closely related UDP-glucose, at 2.7 mM, inhibited 61%, while 2.7 mM GDP-mannose and 3.8 mM GDP-glucose resulted in 36 and 20% inhibition, respectively. UDP, a product of the reaction, was strongly inhibitory, whereas UMP was less effective. At 0.5 mM, UDP inhibited 62% and UMP 11%; at 1.5 mM the values were 94 and 42%, respectively.

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 polysine, 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 mM NaCl resulted in 58 and 87% inhibition, respectively.
whereas the values for 0.1 and 0.15 mM NaSO₄ were 91 and 99%, respectively.

A number of antibiotics, some of which were found to depress the biosynthesis of bacterial mucopeptides, and some of which inhibit yeast growth, were found to be without effect on chitin synthetase. The list included Nystatin, Amphotericin A, Amphotericin 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, Rifamycin, at 40 and 200 μg per ml, and Dacitacin, at 500 μg per ml.

On the other hand, Polyoxin 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 \( K_i \) is 5 \( \times 10^{-7} \) M, about 1000 times smaller than the \( K_m \) for UDP-GlcNAc. Complete inhibition could be obtained (97% inhibition at 3 \( \times 10^{-5} \) M Polyoxin A). The results are similar to those reported recently by Endo and Misato (19) for Polyoxin D (18) on the chitin synthetase of Neurospora crassa. Whereas Polyoxin D is also inhibitory in vivo for Neurospora, no effect of Polyoxin A could be shown on growing yeast cells at a concentration of 0.8 \( \times 10^{-4} \) M.

On the assumption that removal of the cell wall might facilitate the access of the antibiotic to the enzyme site the effect of Polyoxin A on yeast spheroplasts was examined. Eddy and Williams (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 Polyoxin A was added at a final concentration of 0.8 \( \times 10^{-4} \) 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 \( \times g \) 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 \( \times g \), 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. cerevisiae 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 Polyoxin A to the spheroplast incubation mixture.

Polyoxin A, at a concentration of 0.8 \( \times 10^{-4} \) 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 acetylglucosaminase.

One of the reaction products is UDP, as reported for the chitin synthetase of Allomyces macrogynus (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 diacetylchitobiose 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 alkali-soluble material may correspond to the synthesis of a relatively shorter 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 Polyoxin A is a very strong inhibitor. As noted by Isono, Asahi, and Suzuki (18), Polyoxin 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 Polyoxin D on the growth of Neurospora (19), is probably caused by lack of penetration through the cell membrane. Conversely, the ineffectiveness of Polyoxin A on chitin formation by yeast spheroplasts indicates that chitin synthetase is not located on the outside of the cytoplasmic membrane.

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CORRECTION

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

imidazole-chloride, pH 6.5, containing 2 mM MgSO$_4$) containing