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The Synthesis of Chitin by Particulate Preparations of *Allomyces macrogynus**

Clark A. Porter and Ernest G. Jaworski

ABSTRACT: A particulate enzyme from the mycelium of *Allomyces macrogynus* was shown to catalyze the synthesis of chitin from uridine diphospho-*N*-acetylglucosamine (UDP–GlcNAc). The enzyme was associated with both mitochondrial and microsomal fractions but it exhibited higher specific activity and greater stability in the latter. The enzyme was activated by soluble chitodextrin and GlcNAc. Mercaptoethanol increased the activity of the microsomal fraction and maximum activity was observed after storage at −20° for 24 hr; the activity remained unchanged after 5 months in lyophilized preparations held at −20°. The pH optimum for the reaction was 7.8 and the temperature optimum was 30°. The K_m for the reaction with UDP–GlcNAc was 1.2×10^{-3} M, and activation of the enzyme by GlcNAc did not change this value.

Chitin is an important constituent of the cell wall of fungi (Foster, 1949) but little is known about the biosynthesis of this polymeric material. Glaser and Brown (1957) demonstrated the presence of a particulate enzyme in *Neurospora crassa* that catalyzed the synthesis of chitin from uridine diphospho-*N*-acetylglucosamine[1] labeled with carbon-14. A similar synthetic process was shown to occur in cell-free preparations from *Venturia inaequalis* (Jaworski *et al.*, 1965). This paper reports the results of studies on the properties of chitin synthetase obtained from the mycelium of *Allomyces macrogynus*, a fungus whose cell walls contain 60% chitin.

Materials and Methods

UDP, UDP–GlcNAc, GlcNAc, EDTA, Tris, phosphoenolpyruvic acid (tricyclohexylamine salt), and pyruvate kinase (Type II) were obtained from Sigma Chemical Co. Yeast extract and soluble starch were obtained from Fisher Scientific Co. Chitinase was obtained from General Biochemical and mercaptoethanol from Eastman Kodak Co. [^{14}C]GlcNAc was supplied by New England Nuclear Corp. All other chemicals used were AR grade.

UDP-[^{14}C]GlcNAc labeled in the carboxyl carbon of the acetyl group was prepared according to Glaser and

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[1] Abbreviations used in this work: GlcNAc, *N*-acetylglucosamine; UDP–GlcNAc, uridine diphospho-*N*-acetylglucosamine; UDP uridine 5′-diphosphate.

Brown (1955) and soluble chitodextrins were prepared by the method of Zechmeister and Toth (1931).

Growth of Allomyces. The sexual stage of *A. macrogynus* was maintained on starch–agar slants (Emerson, 1941). Ten-day-old slant cultures were used as inoculum for liquid shake cultures. The slants were approximately two-thirds covered with actively growing mycelium that contained visible orange male gametangia. A single slant was homogenized in 25 ml of the complex liquid medium described by Machlis (1953), except the yeast extract was increased to 10 g/l. (L. Machlis, 1963; personal communication). A Lourdes homogenizer was used at maximum speed for 15 sec. The homogenate was transferred into 75 ml of liquid medium contained in a 500-ml Erlenmeyer flask. The flask was incubated on a gyrotory shaker (New Brunswick Scientific Co., Model G-25) at 30° for approximately 64 hr. During this time mycelial pellets were formed. The pellets were washed with deionized water and filtered on Whatman No. 4 paper. Alternatively, 64-hr-old mycelial pellets, homogenized in a manner similar to that described above, were used as inoculum for new cultures.

Preparation of Particulate Fractions. Mycelial pellets were weighed and blended in 2–3 volumes (w/v) of ice-cold buffer (pH 7.5) containing 0.04 M Tris, 0.01 M $MgCl_2$, 0.003 M EDTA, 0.003 M mercaptoethanol, and 0.25 M sucrose using a Lourdes homogenizer for 1 min at maximum speed. The resulting homogenate was ground in a motor-driven Duall conical glass tissue grinder (Kontes Glass Co.) for 15 sec and centrifuged at 25,000*g* for 20 min (0°) to remove debris and mitochondrial material. The supernatant fluid was centrifuged at 140,000*g* for 1 hr. The microsomal pellet obtained was suspended with the aid of a motor-driven Teflon pestle in the homogenizing medium with sucrose omitted. The final enzyme preparation contained the equivalent of 2 g fresh weight of mycelial pellets/ml.

In some cases the mitochondrial fraction was isolated by centrifuging the final homogenate at 2000*g* to remove debris. The supernatant fluid was then centrifuged sequentially at 25,000*g* and 140,000*g* to yield mitochondrial and microsomal pellets, respectively. Both were processed further as indicated above for the microsomal pellet. Protein determinations were made by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Assay of Synthetase Activity. The standard reaction mixture contained 0.0004 M UDP–GlcNAc, 0.011 M GlcNAc, 5 mg of chitodextrins, 0.04 M Tris (pH 7.5), 0.03 M mercaptoethanol, 0.01 M $MgCl_2$, 0.003 M EDTA, and enzyme (0.1–0.5 ml) in a final volume of 1 ml. The assays were carried out by determination of the radioactivity incorporated from UDP-[^{14}C]GlcNAc into the insoluble residue of the reaction mixture (Glaser and Brown, 1957), or by measurement of UDP liberated (Leloir and Goldemberg, 1960) from UDP–GlcNAc during the incubation.

Incorporated radioactivity was measured after termination of the reaction by addition of 0.5 ml of 2 N perchloric acid. The insoluble residue was sedimented at 140,000*g* for 30 min and washed twice with 0.5 N perchloric acid and once with distilled water by suspension and centrifugation as before. The radioactivity of the insoluble residue was determined after suspending it in 15 ml of an aqueous counting solution (Kinard, 1957) containing 5% of a thixotropic gelling agent (Cab-O-Sil; Packard Instrument Corp.). All measurements of radioactivity were carried out using liquid scintillation counting with a Tri-Carb liquid scintillation counter. Quenching and background corrections were made by employing internal standardization ([^{14}C])toluene.

Liberated UDP was measured after the reaction was terminated by immersion of tubes in boiling water for 3 min. The reaction tubes were cooled in an ice bath and 0.1 ml of pyruvic kinase (1:100 dilution in 0.1 M $MgSO_4$) and 0.05 ml of 0.005 M phosphoenolpyruvate in 0.2 M KCl were added. The tubes were incubated at 37° for 15 min and then 0.15 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl was added. After 5–10 min at room temperature, 3.6 ml of 95% ethanol containing 0.2 ml of 10 N NaOH was added. The solution was clarified by centrifugation for 5 min at 6000*g* and the absorbancy determined at 520 mμ. Appropriate blanks and UDP standards were treated in the same manner.

Alterations in pH of the reaction mixtures for pH-optimum studies were achieved using Tris–maleate or Tris–HCl buffers (Goniori *et al.*, 1955). The pH was adjusted to 7.5 before the determination of liberated UDP.

Identification of Product of Enzyme Activity. The radioactive residue from 1 ml of enzyme and UDP-[^{14}C]GlcNAc (93,000 dpm) was subjected to peptic and tryptic digestion followed by hydrolysis in HCl to partially solubilize chitin (Glaser and Brown, 1957). One portion of the soluble fraction obtained in this manner was incubated with chitinase (3 mg) and a second portion was incubated without chitinase to serve as a control. The solutions obtained by this procedure were chromatographed in a descending manner on Whatman No. 1 paper strips (3 × 50 cm) with 1-butanol–pyridine–water (2:1.5:0.75) or 70% aqueous phenol. The positions of radioactive materials on the chromatograms were determined by sectioning the strips at 2-cm intervals along the path of solvent flow and counting the individual segments in vials containing 15 ml of aqueous counting solution.

TABLE I: Comparison of Chitin Synthetase Activities Measured by UDP Liberation and ^{14}C Incorporation.

Enzyme Preparation	Substrate Converted (μmoles)	
	UDP Liberated	[^{14}C]GlcNAc Incorporated
1	0.020	0.023
2	0.035	0.032
3	0.022	0.021
4	0.048	0.040

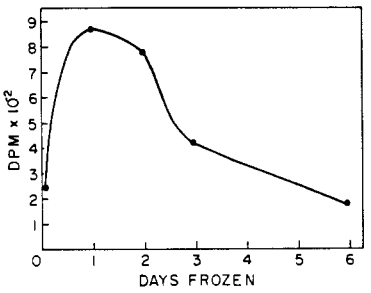

FIGURE 1: Effect of storage of frozen microsomes on chitin synthetase activity. Reaction mixtures contained enzyme (0.2 ml), 3.3×10^{-5} M UDP-[^{14}C]GlcNAc (25,020 dpm), 1.1×10^{-2} M GlcNAc, 5 mg of chitodextrin, 10^{-3} M mercaptoethanol, 4×10^{-2} M Tris (pH 8.0), 10^{-2} M $MgCl_2$, and 10^{-3} M EDTA in a volume of 1 ml. Incubated for 0.5 hr at 30°.

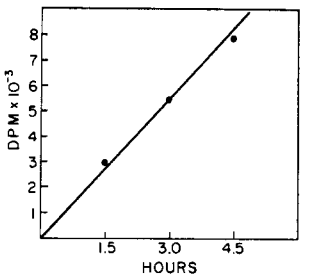

FIGURE 2: Incorporation of radioactivity from UDP-[^{14}C]GlcNAc into chitin as a function of time (*Allomyces* microsomes) at 25°. Reaction mixtures contained enzyme (0.1 ml), 4×10^{-4} M UDP-[^{14}C]GlcNAc (25,020 dpm), 1.1×10^{-2} M GlcNAc, 5 mg of chitodextrin, 10^{-3} M mercaptoethanol, 4×10^{-2} M Tris (pH 7.5), 1×10^{-2} M $MgCl_2$, and 1×10^{-3} M EDTA in a volume of 1 ml.

Results

Comparable results were obtained after a 30-min incubation when chitin synthetase was assayed by measurement of UDP liberated from UDP-GlcNAc and by incorporation of radioactivity from UDP-[^{14}C]GlcNAc in the acid-insoluble residue (Table I).

The reaction was activated by both chitodextrin and GlcNAc and their maximum effects did not vary greatly (Table II). The activation was greater when they were added together and, except at their lowest concentrations, they produced effects that were considerably greater than additive. Concentrations of glucosamine equivalent to those of GlcNAc did not activate the enzyme. Experiments using [^{14}C]GlcNAc showed that free GlcNAc was not incorporated into chitin during the reaction; 0.1% of the added GlcNAc could have been detected. The addition of GlcNAc did not significantly alter the K_m of the reaction.

Both mitochondrial and microsomal preparations exhibited chitin synthetase activity (Table III). The specific activity of fresh microsomal particles was approximately six times greater than that of comparable mitochondrial particles. When these particulate preparations were frozen and stored at −20° for 24 hr, the specific activity of the mitochondrial fraction decreased to approximately 45% of the original activity while the microsomal fraction increased in specific activity by more than 400%. Quick freezing of the microsomal

TABLE II: Activation of *Allomyces* Microsomal Chitin Synthetase by Chitodextrin and *N*-Acetylglucosamine.

Treatment	Chitin (dpm)	
Minus chitodextrin and GlcNAc	146	
Chitodextrin (mg)		
2.5	810	
5.0	1025	
10.0	1118	
GlcNAc (M)		
5.5×10^{-3}	592	
1.1×10^{-2}	1378	
2.2×10^{-2}	1445	
Chitodextrin (mg) + GlcNAc (M)		
$2.5 + 5.5 \times 10^{-3}$	1505	1402[a]
$5.0 + 1.1 \times 10^{-2}$	4500	2403
$10.0 + 2.2 \times 10^{-2}$	4140	2563

[a] Theoretical incorporation if chitodextrin and GlcNAc effects were additive. Reaction mixture contained enzyme (0.4 ml), 4×10^{-4} M UDP-[^{14}C]GlcNAc (25,020 dpm), 4×10^{-2} M Tris (pH 8.0), 10^{-2} M $MgCl_2$, and 10^{-3} M EDTA in a volume of 1 ml; chitodextrin and GlcNAc added as indicated. Incubated 1 hr at 30°.

TABLE III: Chitin Synthetase Activity of Microsomal and Mitochondrial Particulates.

Particulate Fraction	Specific Activity (μmole of UDP/mg of protein/0.5 hr)	
	Fresh	Frozen
Microsomes	0.36	1.53 (15.4)[a]
rehomogenized[b]	1.10	4.67
Mitochondria	0.058	0.032
rehomogenized[c]	1.45	7.85

[a] After removal of inactive aggregates at 2000*g*.
[b] 140,000*g* pellet isolated after rehomogenization.
[c] 140,000*g* pellet isolated after rehomogenization and centrifugation at 25,000*g*.

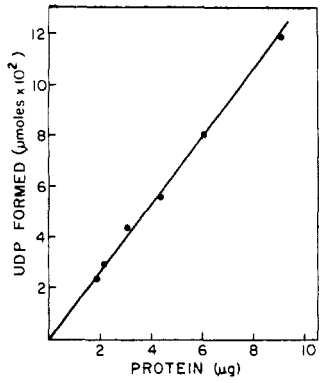

FIGURE 3: Relationship between UDP formation and enzyme concentration. Reaction mixtures contained 4×10^{-4} M UDP-GlcNAc, 1.1×10^{-2} M GlcNAc, 10^{-3} M mercaptoethanol, 4×10^{-2} M Tris (pH 7.5), 1×10^{-2} M $MgCl_2$, 1×10^{-3} M EDTA, and protein as indicated in a volume of 1 ml. Incubated for 0.5 hr at 30°.

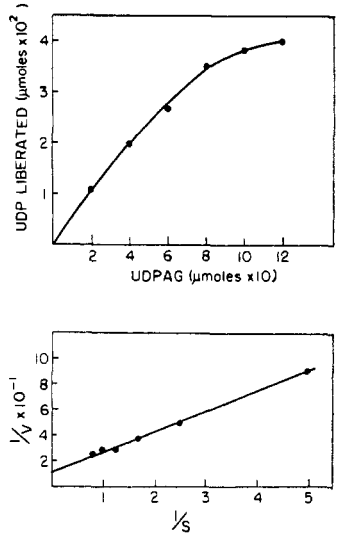

FIGURE 4: Effect of substrate concentration on the chitin synthetase reaction. Conditions of the experiment were those in Figure 3 except UDP-GlcNAc concentration was varied as indicated.

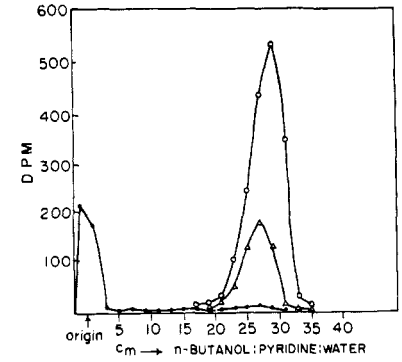

FIGURE 5: Chromatography on paper of the soluble fraction derived from the insoluble reaction product of chitin synthetase and UDP-[^{14}C]GlcNAc by HCl treatment: ●—● soluble fraction; △—△ soluble fraction treated with chitinase; ○—○ chitinase treated material cochromatographed with [^{14}C]GlcNAc.

preparations with Dry Ice-acetone and immediate thawing increased their activity by only 20%. Freezing the microsomal preparations for 24 hr at −20° produced protein aggregates that were visible upon thawing and could be removed by centrifugation at 2000*g* for 5 min. These aggregates did not exhibit chitin synthetase activity and their removal increased the specific activity of the microsomal fraction by 10-fold. The activity remained unchanged for 5 months when such preparations were lyophilized and stored at −20°.

The specific activity of isolated microsomes was increased by rehomogenization in a ground glass homogenizer (Table III). Isolated mitochondria rehomogenized in the same manner and centrifuged sequentially at 25,000*g* and 140,000*g*, yielded a 140,000*g*-particulate fraction of higher specific activity than the microsomes and had the same characteristic observed for the microsomes of increased activity after 24 hr at −20°.

The stability of frozen microsomal preparations during storage at −20° is illustrated in Figure 1. An increase in activity during the initial 24 hr was apparent and was followed by a decrease during the next 5 days to a level approximately equivalent to that of the freshly prepared particles.

Enzyme preparations containing all of the particulates sedimented between 2000 and 140,000*g* exhibited a lower degree of stability than the microsomal particles when stored at −20°. Such preparations exhibited approximately 10% of their initial activity after storage for 6 days. The presence of mercaptoethanol (0.003 M) or the substrate, UDP-GlcNAc, during storage did not protect against this decrease in activity. Preparations containing only the mitochondrial particles exhibited a similar instability.

The reaction rate was linear during 4.5 hr at pH 7.5 and 25° with an initial UDP-GlcNAc concentration of 0.0004 M (Figure 2). Similarly, a linear relationship was observed between formation of UDP and enzyme concentration at saturating levels of substrate (Figure 3). The effect of UDP-GlcNAc concentrations upon the reaction velocity is shown in Figure 4. The value obtained for K_m is 1.2×10^{-3} M.

The enzyme exhibited maximum activity at pH 7.8 with a secondary peak of activity at pH 8.6. The magnitude of the secondary peak was variable from preparation to preparation and represented as much as 65% of the activity of the primary peak. In other cases it was only a slight inflection on the activity curve. Maximum activity was observed at 30°. At 20 and 37° the activity was approximately 65% of the maximum.

The activity of microsomal particles prepared in the absence of mercaptoethanol was increased by approximately 60% when mercaptoethanol was added during the assay. Similar particles prepared in the usual manner and subjected to either Tris (0.04 M) or phosphate (0.05 M) buffer, pH 7.5, without additional additives during the assay showed that the activity in phosphate buffer was only 30% of the activity observed in Tris buffer.

Limited attempts to solubilize chitin synthetase from microsomal or mitochondrial particles by combination treatments of sonic oscillation, freezing and thawing, and incubation at refrigerator and room temperatures in the presence of a noninhibitory concentration of taurocholic acid (0.05%) were not effective.

The chromatographic patterns obtained with chitinase-treated and nontreated portions of the soluble fraction from the partial acid hydrolysis of the radioactive insoluble residue are illustrated in Figure 5. In the control, which was not treated with chitinase, 85% of the radioactivity remained at the origin, 7% coincided with the position of GlcNAc, and the remaining 8% was distributed between these positions. The chromatogram of the chitinase digest showed all of the radioactivity at the position of GlcNAc and none at the origin. Cochromatography of the chitinase digest with [^{14}C]GlcNAc resulted in a single radioactive peak. These same patterns were evident following chromatography individually in both 1-butanol–pyridine–water and 70% phenol.

Discussion

The results of these studies show that a particulate enzyme from the mycelium of *A. macrogynus* catalyzes the biosynthesis of chitin from UDP–GlcNAc in a manner analogous to that reported for *N. crassa* (Glaser and Brown, 1957) and *V. inaequalis* (Jaworski *et al*, 1965). The characteristic activity was found in both the mitochondrial and microsomal fractions of *Allomyces*. This suggested that the enzyme activity in the microsomal fraction could be due to contamination by mitochondrial fragments produced during homogenization. Indeed, rehomogenization of isolated mitochondria did produce a 140,000*g*-particulate fraction that showed higher specific activity than the microsomes and the same characteristic of increased activity after 24 hr at −20°. However, the total activity was only 7% of that found in the microsomal fraction. This indicates that if the activity in the microsomal fraction were due to mitochondrial fragments, the fragmentation occurred much more readily during the initial homogenization of the intact tissue than during the successive homogenization of isolated mitochondria. The low yield of activity in the latter case may have been due to the liberation of microsomes adsorbed to the surface of mitochondria rather than to mitochondrial fragmentation.

The relative instability of the enzyme in *Allomyces* preparations containing all particulates between 2000 and 140,000*g* when stored at −20° is in contrast to the results obtained with similar preparations from *Neurospora*. The latter preparations retained their activity for several weeks under these conditions (Glaser and Brown, 1957). Since the enzyme was considerably more stable in *Allomyces* microsomal preparations than in mitochondrial or mixed preparations, it would appear that freezing and thawing mitochondrial particles may liberate an inhibitor or a hydrolytic enzyme that acts upon the substrate or the chitin synthetase enzyme.

The enzyme from *Allomyces* was activated by GlcNAc and soluble chitodextrin in a manner similar to that observed in *Neurospora* preparations (Glaser and Brown, 1957). The activation by these materials is different since their combined effects were greater than additive even at saturating concentrations of both materials. The activation produced by soluble chitodextrin may result from an increased number of nuclei to which GlcNAc from UDP–GlcNAc can react. The activation produced in microsomal preparations by freezing must be caused by still other factors since approximately the same relative response to added GlcNAc and chitodextrin was observed with fresh and frozen preparations. Freezing and thawing may disrupt the integrity of the particles sufficiently to allow better penetration of substrate to enzyme sites. However, the decrease in activity observed in phosphate buffer may indicate that too severe disruption results in loss of activity. Phosphate buffer is known to cause the breakage of pea hypocotyl microsomes into smaller units by removal of magnesium through complex formation with phosphorus (Tso *et al.*, 1958). A similar breakage may have occurred when *Allomyces* microsomal particles were exposed to phosphate buffer.

The role of free GlcNAc in the reaction is not apparent since it was not incorporated into insoluble chitin. However, activation by GlcNAc suggests the possibility that chitin synthetase consists of subunits which may be aggregated by GlcNAc. Such aggregation would provide rapid chitin biosynthesis in response to GlcNAc biosynthesis without UDP–GlcNAc levels being rate limiting. Since UDP–GlcNAc has been shown by Kornfeld *et al.* (1964) to be a feedback inhibitor in the pathway to GlcNAc biosynthesis, the leap-frogging activation by GlcNAc would prevent premature feedback inhibition due to a buildup of UDP–GlcNAc before the insoluble end product was accumulated to the required level. The regulation of chitin synthetase enzyme and the role of GlcNAc will require further studies, some of which are currently in progress.

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Conversion of Proline to Collagen Hydroxyproline*

James M. Manning and Alton Meister

ABSTRACT: Puromycin inhibits the incorporation of proline into the collagen proline and collagen hydroxyproline of carrageenan granuloma minces; however, the formation of soluble hydroxyproline-containing peptides continues, suggesting that hydroxylation of proline takes place prior to the release of the collagen polypeptide from the ribosome. Chlortetracycline inhibits the incorporation of proline into collagen and the hydroxylation of proline to about the same extent, suggesting that hydroxylation takes place on the ribosome. Evidence was obtained indicating that hydroxyproline can occur at the carboxyl-terminal position of the growing peptide chain attached to soluble ribonucleic acid (s-RNA) on the ribosome. Free hydroxyprolyl s-RNA could not be demonstrated, while, under the same experimental conditions, prolyl s-RNA was invariably obtained. When labeled proline was incubated with a granuloma mince from an ascorbic acid deficient guinea pig, the collagen isolated from the microsome fraction contained somewhat less labeled proline as compared to the control, while the incorporation into hydroxyproline was markedly reduced; this suggests formation of a proline-rich polypeptide. A cell-free system was obtained which incorporates proline into a peptide form which does not contain hydroxyproline. The available data support the conclusion that ribosome-bound peptidyl-prolyl s-RNA is a substrate for hydroxylation.

Collagen differs from many other proteins both in its amino acid composition and in the manner of its biosynthesis. Thus, collagen contains certain amino acids (4-hydroxy-L-proline, 3-hydroxy-L-proline, and 5-hydroxy-L-lysine) that are not usually found in other proteins; these are not derived from the corresponding free amino acids, but there is evidence that proline and lysine are hydroxylated during the biosynthesis of collagen (Stetten and Schoenheimer, 1944; Stetten, 1949; Sinex and Van Slyke, 1955). Stetten (1949) suggested that collagen hydroxyproline might arise by hydroxylation of peptide-bound proline. Subsequent studies led to consideration of the possibility that proline is hydroxylated at an intermediate step prior to its incorporation into the peptide chain (Robertson *et al.*, 1959; Green and Lowther, 1959; Stone and Meister, 1962). More recently, experimental support for the belief that proline is hydroxylated in peptide linkage has come from experiments with a cell-free chick embryo system capable of incorporating proline into peptide-bound proline and hydroxyproline (Peterkofsky and Udenfriend, 1963, 1965; Juva and Prockop, 1964; Prockop and Juva, 1965). On the other hand, work in several laboratories has provided evidence for the existence of hydroxyprolyl soluble ribonucleic acid (s-RNA) (Manner and Gould, 1962, 1963; Coronado *et al.*, 1963; Jackson *et al.*, 1964; Urivetzky *et al.*, 1965).

Earlier work in this laboratory showed that the incorporation of tritiated proline into collagen hydroxyproline by minces of carrageenan granuloma is directly related to the release of tritiated water from tritiated proline, and therefore that the formation of tritiated water can be used as a valid measure of hydroxyproline formation in this system (Stone and Meister, 1962). Subsequent studies indicated that puromycin inhibits the formation of collagen hydroxyproline markedly, but that hydroxylation (as determined by the formation of tritiated water and tritiated hydroxyproline) continues (Meister *et al.*, 1964). The experiments reported here support this conclusion; thus, evidence is presented that,

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