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The Biosynthesis of Crustacean Chitin by a Microsomal Enzyme from Larval Brine Shrimp*

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A microsomal preparation from larval stages of the brine shrimp *Artemia salina* was found to catalyze the transfer of *N*-acetyl-D-glucosamine from UDP-*N*-acetylglucosamine to an endogenous acceptor. The product was identified as chitin by its resistance to extraction with alkali and high concentrations of urea and the liberation of chito-oligosaccharides by treatment with purified chitinases. The enzyme requires Mg^{2+} for activity and is inhibited by UDP and diflubenzuron, but not by Polyoxin D. The pH optimum is 7.0. The enzyme is not significantly activated by *N*-acetyl-D-glucosamine nor by trypsin treatment. Incorporation of radioactivity into endogenous acceptor is inhibited by chitodextrins which appear to serve as alternate acceptors. The crustacean enzyme can also utilize exogenous, macromolecular chitin as acceptor. The enzyme, which was partially purified by sucrose step-gradient ultracentrifugation, appears maximally active after 72 h of larval growth.

Chitin is a homopolymer of *N*-acetyl-D-glucosamine linked $\beta 1 \rightarrow 4$, and occurs in microorganisms, fungi, and most invertebrates. In the arthropods, chitin is the major component of the cuticle or exoskeleton (1, 2). The biosynthesis of chitin has been studied in a variety of fungal systems (3–5), but much less data are available on the biosynthesis of arthropod chitin (6–8), owing largely to difficulties in defining an *in vitro* assay system (9, 10). The biosynthesis of chitin in arthropods is a temporal event under hormonal control; to increase in size, the organism resorbs a portion of the shell and initiates the secretion of a new exoskeleton underneath the old cuticle; at this time, chitin synthesis is maximal. After completion of about one-half of the new shell, the animal molts, discarding the old shell, and then completes the synthesis of the new shell. In the present study, larval stages of the brine shrimp *Artemia salina* have been used as a model system to examine the biosynthesis of crustacean chitin.

The purpose of the present report is to describe the isolation, partial purification, and properties of a membrane-associated chitin synthetase from larval brine shrimp. The properties of the crustacean enzyme, including its sensitivity to certain drugs and antibiotics, are compared to published data on the fungal enzyme (3, 5). A preliminary communication of these results has appeared (11).

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EXPERIMENTAL PROCEDURES

Materials

Hepes,[1] *N*-acetylglucosamine, chitinase, chitosan, dithiothreitol, DEAE-Sephacel, and crude chitin were purchased from Sigma. [^{3}H]Acetic anhydride (399 mCi/mmol), UDP-*N*-acetyl-D-[6-^{3}H]glucosamine (6.6 Ci/mmol), and UDP-*N*-acetyl-D-[1-^{14}C]glucosamine (50 mCi/mmol) were the products of New England Nuclear. UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (366 mCi/mmol) was obtained from Amersham-Searle. Bio-gel P-2 and P-4 were the products of Bio-Rad. Brine shrimp eggs (San Francisco Bay Brand) were purchased from Metaframe Corp., Elmwood Park, NJ. Culture bags were obtained from Wards, Inc., Rochester, NY. Diflubenzuron (DIMILIN; Lot PP124) was obtained from the Thompson-Hayward Chemical Co., Kansas City, MO. The nitrogen bomb cavitation apparatus was purchased from Parr Instruments, Moline, IL.

Methods

Growth of Larval Brine Shrimp—Brine shrimp eggs (4 g) were aerated for 72 h at 25°C in culture bags containing 2 liters of artificial sea water: 20 mM Tris·HCl, pH 7.1, containing 0.44 M sodium chloride (rock salt), 23 mM $MgCl_2$, 9 mM KCl, 26 mM $MgSO_4$, 2 mM sodium bicarbonate, and 10 mM $CaCl_2$. After 72 h, the aeration was terminated, and 5 min later, the sea water containing larvae was siphoned off and filtered, leaving unhatched eggs floating in the culture bag.

Homogenization of Brine Shrimp Larvae—Larvae (30 to 50 ml packed volume) were resuspended in 300 ml of chilled Buffer A: 50 mM Hepes, pH 7.1, containing 0.4 M sodium chloride, 30 mM magnesium chloride, 5% (v/v) glycerol, and 0.65 mM dithiothreitol. The larvae were then subjected to nitrogen bomb cavitation at 200 p.s.i. of nitrogen for 15 min at 4°C in a Parr cell disruption bomb (model 4635). Larvae were homogenized by opening the release valve while maintaining internal pressure at 200 p.s.i.; a total of 15 min was allowed for delivery of the sample from the disruption bomb.

Isolation and Purification of Membranes—All operations were carried out at 4°C. The crude homogenate was centrifuged, first at $300 \times g$ (10 min), then at $5500 \times g$ (15 min) to remove shell debris, nuclei, and mitochondria. The supernatant fraction was centrifuged at $30,000 \times g$ (45 min) to obtain a crude microsomal pellet. The microsomes were resuspended in about 90 ml of 25% (w/v) sucrose containing 10 mM Tris·HCl, pH 7.4, and homogenized using three strokes of a loose-fitting Dounce homogenizer. Approximately 15 ml of resuspended microsomes (10 to 20 mg of membrane protein) was then overlayered into each of six nitrocellulose ultracentrifuge tubes containing 5 ml of 48% (w/w) sucrose underneath a 15-ml layer of 43% (w/v) sucrose; both solutions contained 10 mM Tris·HCl, pH 7.4. The refractive index of the latter solution was adjusted to 1.392 as recommended by Crumpton and Snary (12). Following ultracentrifugation (23,000 rpm; 2 h), membranes banding at the 25%/43% interface and at the 43%/48% interface were collected, diluted with 10 volumes of cold 10 mM Tris·HCl, pH 7.1, and centrifuged ($30,000 \times g$; 45 min). The resultant membrane pellets were carefully resuspended in Buffer A (~10 ml) using a plastic pipette.

Enzyme Assays—Fractions from various stages of the isolation and purification scheme summarized above were assayed for several marker enzymes by the following spectrophotometric methods. Leu-

[1] The abbreviations used are: Hepes, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Aces, 2-[(2-amino-2-oxoethyl)-amino]ethanesulfonic acid.

cine aminopeptidase (EC 3.4.11.1) activity was measured at 37°C as described by Basha *et al.* (13). Alkaline phosphodiesterase I (EC 3.1.4.1) was assayed according to the procedure of Roberts and Yuan (14) using *p*-nitrophenyl 5′-thymidylate as substrate. The (Na^+ + K^+)-ATPase was monitored by the technique of Brunette and Till (15). Alkaline phosphatase (EC 3.1.3.9) was estimated using *p*-nitrophenyl phosphate as substrate (14). Glucose-6-phosphatase was assayed as described (16); inorganic phosphate was quantitated by the method of Bartlett (17). Lactate dehydrogenase (EC 1.1.1.27) was monitored as described by Kornberg (18). The specific activity of enzymes releasing inorganic phosphate is given as micromoles of phosphate liberated per min per mg of protein. All other specific activities are expressed as micromoles of substrate (or cofactor) consumed per min per mg of protein.

Protein Determinations—Protein was determined by a modified fluorescamine procedure (19), using bovine serum albumin as standard. Briefly, samples (0.5 to 100 μg of protein) were dissolved in 1 ml of 0.1 M borate buffer, pH 9.0, containing 2% (w/v) sodium dodecyl sulfate by boiling for 3 min. Cooled samples were held on a Vortex-type mixer and 125 μl of 0.05% (w/v) fluorescamine in acetone was added. Fluorescence was measured in an Aminco-Bowman spectrofluorimeter (excitation, 390 nm; emission, 475 nm).

Chitin Synthetase Assay—Membranes at various stages of purification (0.1 to 2 mg of protein) were resuspended in 1 ml of Buffer A in Corex centrifuge tubes (10 × 75 mm). Control samples were capped and boiled for 5 min in a water bath. Thereafter, 0.0625 μCi of UDP-*N*-acetyl-D-[U-^{14}C]glucosamine was added to each tube; the final concentration of nucleotide sugar was 0.186 μM. Samples were incubated at 37°C for 60 to 120 min and the reaction was then terminated by the addition of 1 ml of cold methanol. After mixing, samples were allowed to stand at −20°C for 4 to 15 h. Tubes were then centrifuged (20,000 × *g* for 30 min) and the 50% methanol-soluble material, including buffer salts and residual UDP-G1cNAc, was removed. The pellets were extracted with 5 ml of chloroform:methanol:water (10:10:3; v/v/v) for 5 min at 25°C. After centrifugation (5500 × *g* for 15 min), the liquid was carefully removed, and residual chloroform was removed from the pellets at 37°C under a gentle stream of nitrogen. For certain determinations pellets were resuspended in water and radioactivity measured by liquid scintillation counting. In most cases, the dried pellets were extracted further in 5 ml of 9.5 M urea containing 5 mM potassium carbonate, pH 10.3 (20) at 25°C for 15 min. After centrifugation, the pellets were washed with water (15 ml) and centrifuged again. The final pellets were resuspended in 1 ml of water by blending on a Vortex mixer and, when necessary, were dispersed by low energy sonication. Radioactivity in 0.5 ml of the suspended preparation was determined by liquid scintillation counting. Specific activity is defined as picomoles of *N*-acetyl-D-glucosamine incorporated per h per mg of protein.

Preparation of Chitin Substrates—Granular chitin was prepared from crude powdered chitin by the method of Berger and Reynolds (21). Briefly, crude chitin (15 g) was treated with 2% (w/v) potassium permanganate at 25°C for 8 h; the product was washed with 1% oxalic acid, 2 N HCl, ethanol, and then dried. This material was dissolved in concentrated HCl (200 ml) at 20°C for 15 min, filtered through glass wool, and the clear liquid added to cold, 50% (v/v) ethanol with stirring. The precipitate was collected after 2 h by centrifugation, washed five times with 10 volumes of water, and bleached by the addition of 5 ml of 30% (v/v) H_2O_2 to the chitin slurry. The white product was washed with water and stored at 4°C.

Chitin oligosaccharides were prepared from granular chitin by the procedure of Rupley (22) by partial hydrolysis in concentrated HCl (2 h at 30°C). The hydrolysate was neutralized slowly with solid NaOH and the soluble oligosaccharides (15 ml) were desalted by chromatography on a Bio-Gel P-2 column (4.5 × 25 cm) previously equilibrated with 0.1 M acetic acid. Fractions (5 ml) were assayed for reducing sugar by the procedure of Park and Johnson (23). Oligosaccharide peaks (di- through octasaccharide) were pooled and *N*-acetylated with [^{3}H]acetic anhydride (10 mCi/g of oligosaccharide) according to the method of Molano *et al.* (24). After 1 h of reaction, the labeling was terminated by the addition of an equal volume of 1 M ethanolamine, pH 7.0. The mixture was then applied to a second Bio-Gel P-2 column (2 × 230 cm) previously equilibrated with 0.1 M acetic acid. Labeled oligosaccharides were detected by scintillation counting, pooled, and lyophilized. Re-acetylated [^{3}H]chitosan was prepared by the method of Molano *et al.* (24).

Paper Chromatography—Paper chromatography was carried out with Whatman No. 1 paper. Descending chromatograms were developed for 38 to 40 h with either (*a*) isoamyl alcohol:pyridine:water (10:10:8) or (*b*) *n*-butanol:pyridine:water (6:4:3) (3, 25). The chromatograms were scanned for radioactivity with a Packard model 7201 Radiochromatogram Scanner. Alternatively, chromatograms were cut into 1-cm sections, shredded, and radioactivity determined by liquid scintillation counting in 10 ml of Triton-toluene scintillation fluid (13). Reducing sugars were detected on chromatograms with the silver nitrate procedure (26). The location of various oligosaccharides was verified by the use of external standards.

Chitinase Assays—Incubations with affinity-purified chitinase (27) from *Streptomyces griseus* were carried out in 0.05 M potassium phosphate buffer, pH 6.3, at 37°C for 24 to 48 h as described previously (3). For assays using affinity-purified wheat germ endochitinase (25), samples were incubated with 60 to 180 μg of enzyme in 20 mM Tris·HCl, pH 7.5, containing 0.1 mg/ml of bovine serum albumin at 37°C for 24 to 48 h. Endogenous chitinase was assayed by incubating various membrane fractions with [^{3}H]chitin (24) in Buffer A at 37°C for 2 to 4 h. All incubations were terminated by boiling; after centrifugation, the clarified supernatant were desalted by mixing with an equal volume of Amberlite MB-3 mixed bed ion exchange resin (hydrogen-hydroxyl form).

Electron Microscopy—Samples of membrane fractions were prepared for transmission electron microscopy by fixation of freshly isolated membranes in 0.1 M cacodylate buffer, pH 7.4, containing 2% (v/v) glutaraldehyde (4°C for 15 h). Samples were treated with 2% osmium tetraoxide (4 h), dehydrated, embedded in Epon-Aryldite, and sectioned. Silver sections were post-stained with uranyl acetate and examined in a Phillips model EM-200 electron microscope at 120 kV.

RESULTS

Formation of radiolabeled product, insoluble in 50% methanol, chloroform:methanol:water (10:10:3), and 9.5 M urea, is shown in Fig. 1 as a function of added microsomal protein. Product formation was linear up to 500 μg of microsomal enzyme and in other experiments, up to a maximum of 1.5 mg of protein. When crude microsomes were incubated with UDP-*N*-acetyl-D-[^{14}C]glucosamine, radioactivity was incorporated into two fractions, one lipophilic and the other chloroform:methanol:water (10:10:3)-insoluble. As shown in Fig. 2,

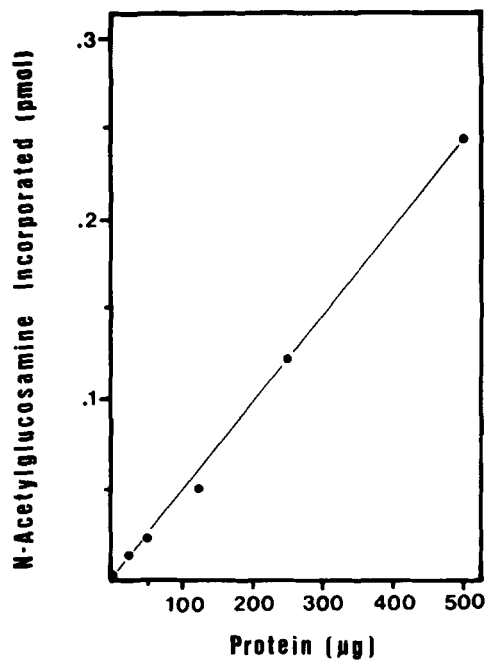

FIG. 1. **Dependence of product formation on added microsomal protein.** Standard assay conditions were used with increasing amounts of crude microsomal enzyme. Radioactivity in the chloroform:methanol:water (10:10:3)-insoluble residue was determined. The radioactivity measured in the control (no added protein) was 34 dpm; this background value was subtracted from all experimental data points to obtain the corrected values shown in Fig. 1.

the amount of radioactivity incorporated into each fraction was dependent on time. Radiolabeled material soluble in chloroform:methanol:water (10:10:3) increased rapidly over the first 10 min of incubation and thereafter continued to slowly increase. On the other hand, incorporation of radioactivity into the chloroform:methanol:water (10:10:3)-insoluble fraction was linear over a 2-h incubation period. Maximal enzyme activity was obtained at pH 7 (Fig. 3).

The general requirements for enzymatic activity are shown in Table I. The enzyme is stimulated by magnesium ions and, to a lesser extent, by manganese. No enzyme activity was detected when the incubation was carried out in the presence of EDTA. Omission of dithiothreitol or sodium chloride from the incubation mixture resulted in slightly lower enzyme activity. Significant activity was observed in the presence of 0.1% (v/v) Nonidet P-40. A slight stimulation of enzyme activity was observed in the presence of 0.1 M *N*-acetyl-D-glucosamine. The enzyme is inhibited strongly by low levels of UDP but is only slightly affected by 10^{-5} M Polyoxin D, a potent inhibitor of fungal chitin synthetase (3). When higher concentrations of Polyoxin D were assayed (up to 2×10^{-4} M), no increase in inhibition was observed. When microsomes were preincubated with and without 10^{-5} M Polyoxin D (2 h) and then assayed for activity, no change in inhibition was detected in the Polyoxin-treated sample. Incubation of *Artemia* microsomes in the presence of 100 μg/ml trypsin caused a slight activation, in comparison to a control incubation containing bovine serum albumin.

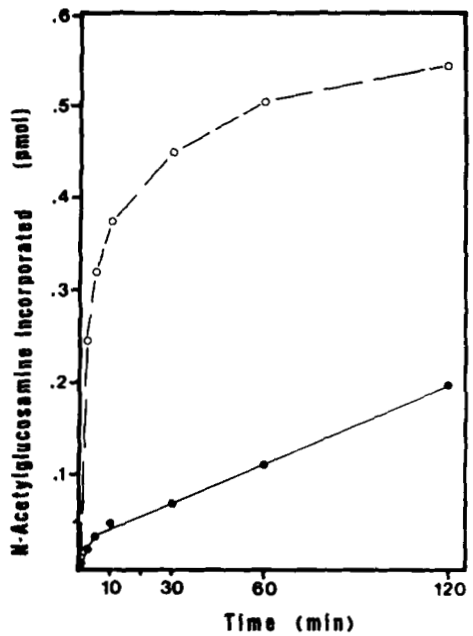

FIG. 2. **Time course of the reaction.** Standard assay conditions were employed using 500 μg of crude microsomal protein. After incubation for the indicated periods of time, radioactivity which was either soluble (○- - -○) or insoluble (●——●) in chloroform:methanol:water (10:10:3) was measured.

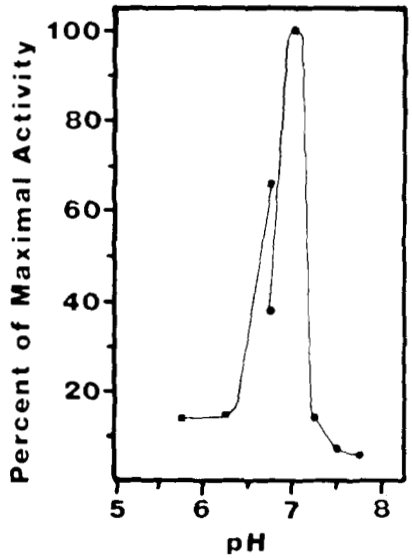

FIG. 3. **Effect of pH on enzyme activity.** Assays were conducted using the standard assay mixture containing either 50 mM Hepes buffer (●——●) or 50 mM Aces buffer (■——■) at the indicated pH values. Each incubation contained 0.146 mg of membrane protein from the 43/48% interface obtained after sucrose step-gradient ultracentrifugation. After 2 h, radioactivity insoluble in chloroform:methanol:water (10:10:3) was determined; results are expressed as percent of maximal activity.

TABLE I

Requirements for Artemia chitin synthetase

A crude microsomal enzyme fraction was prepared as described under "Methods." Approximately 500 μg of membrane protein/sample was used under standard incubation conditions ("Complete") and with the indicated additions or deletions. The activity under standard conditions is taken as 100.

Incubation mixture	Enzymatic activity
Complete	100
Minus Mg^{2+}, plus 30 mM $MnCl_2$	60
Minus Mg^{2+}, plus 100 mM EDTA	2
Minus 0.4 M NaCl	66
Minus 0.65 mM dithiothreitol	85
Plus 0.1% (v/v) Nonidet P-40	76
Plus 100 mM *N*-acetyl-D-glucosamine	138
Plus 10^{-5} M Polyoxin D	83
Plus 0.5 mM UDP	17
Plus trypsin (100 μg/ml)	170
Plus bovine serum albumin (50 μg/ml)	102

Characterization of Radiolabeled Product as Chitin—As described under "Methods," the radioactive reaction product was insoluble in 50% methanol and in chloroform:methanol:water (10:10:3). When the dried product from several incubation mixtures was extracted with 9.5 M urea containing 5 mM potassium carbonate (20), approximately 70% of the product remained insoluble (Table II). Extraction of practical grade chitin (Sigma) under the same conditions showed that 97% of this material was resistant to urea extraction. Treatment of the radiolabeled fraction insoluble in chloroform:methanol:water (10:10:3) with boiling alkali (1 N KOH, 45 min; Ref. 3) gave variable results, depending upon the membrane preparation (Table II). Approximately 60 to 80% of the material was resistant to alkaline extraction when purified membrane fractions were employed; somewhat lower values were obtained with crude microsomes. Extraction of crude chitin (Sigma) and granular chitin with boiling alkali under the same conditions showed that 87% and 86% of the dry weight was resistant to alkaline treatment. A somewhat higher percentage of the crude, radiolabeled product was solubilized by treatment with boiling sodium dodecyl sulfate solution (Table II). Treatment of crude chitin (Sigma) with sodium dodecyl sulfate under the same conditions showed that 92% of the dry weight was resistant to detergent extraction.

Additional information was obtained by degrading the radiolabeled product (insoluble in chloroform:methanol:water) with affinity-purified chitinase from *Streptomyces*. Following digestion at 30°C for 24 h, the chitinase-treated pellet was collected by centrifugation, washed with water, and radioactivity determined. As shown in Table III, from 73 to 85% of

the radiolabeled product was rendered soluble by chitinase treatment. The supernatants from such incubations were desalted on an Amberlite MB-3 column (hydroxyl-hydrogen form; 0.5 × 3 cm), concentrated, and chromatographed on Whatman No. 1 paper, with *n*-butanol:pyridine:water (6:4:3) as solvent (3). The radiolabeled, soluble material co-chromatographed with authentic *N*-acetylchitobiose, as shown in Fig. 4*A*.

Effect of Endochitinase on Product Formation—The formation of radiolabeled crude product in the presence of affinity-purified wheat germ endochitinase (25) was examined in order to further characterize the material synthesized by the brine shrimp microsomes. Crude microsomes were incubated in the presence and absence of 60 μg of purified endochitinase. This enzyme attacks nascent chains of chitin and exhibits less activity toward pre-existing chains which are significantly hydrogen-bonded; the product is a mixture of di-, tri-, and tetrasaccharides, dependent upon the duration of incubation (25). The results of such experiments using the larval brine shrimp system (Table III) indicate that endochitinase treatment releases about 70% of the radiolabeled product. The solubilized radioactivity migrates as di-, tri-, and tetrasaccharides on paper chromatography (Solvent: butanol:pyridine: water (6:4:3)), as shown in Fig. 4*B*; as noted previously (25),

TABLE II

Chemical extraction of radiolabeled crude product

Agent	Sample source	Control radioactivity	Extracted residue radioactivity	Per cent of material resistant to extraction[a]
		cpm		
A. Urea[b]	1. Crude homogenate	821	621	76
	2. Crude microsomes	351	238	68
	3. 25/43% membrane fraction	243	216	88
	4. 25/43% membrane fraction	549	404	74
B. Alkali[c]	1. Crude homogenate	690	341	49
	2. Crude microsomes	509	261	51
	3. Crude microsomes	582	296	46
	4. 25/43% membrane fraction	270	237	88
	5. 25/43% membrane fraction	270	216	80
	6. 43/48% membrane fraction	546	344	63
	7. 43/48% membrane fraction	471	272	58
C. SDS[d]	1. Crude microsomes	12,045	3,000	25
	2. 25/43% membrane fraction	1,300	520	40
	3. 43/48% membrane fraction	22,585	14,560	64

[a] In all cases, extracted residues were washed three times with deionized water, resuspended in water, and total, insoluble radioactivity was determined by liquid scintillation counting.

[b] Samples of crude product were extracted with 5 ml of 9.5 M urea containing 5 mM potassium carbonate, pH 10.3, at 25°C for 30 min, and centrifuged.

[c] Samples of crude product were treated with 2 ml of 1 N KOH at 100°C for 45 min and centrifuged.

[d] Samples of crude product were extracted with 2 ml of 0.1 M borate buffer, pH 8, containing 2.5% (w/v) sodium dodecyl sulfate and 1% (v/v) 2-mercaptoethanol at 100°C for 5 min and then centrifuged.

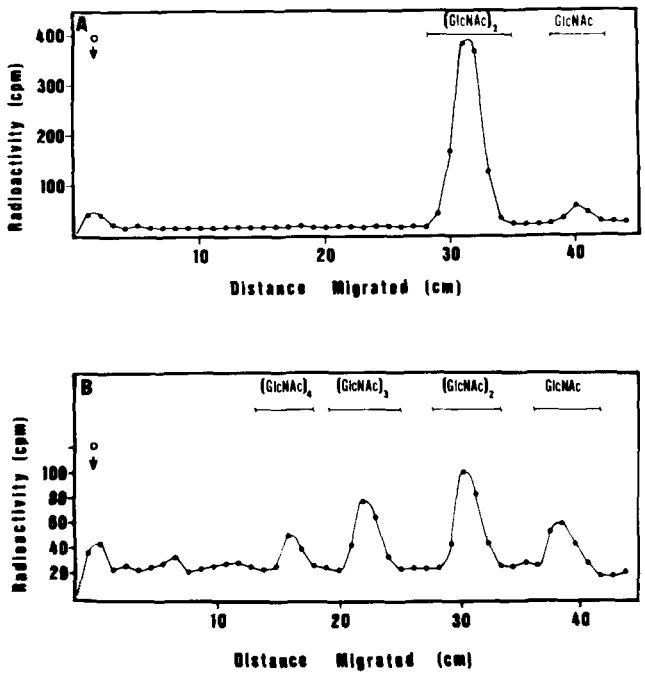

FIG. 4. **Paper chromatography of the chitinase degradation products of the radiolabeled polysaccharide.** *A*, after treatment of crude product with affinity-purified *S. griseus* chitinase (10 μg; 48 h); *B*, after treatment with affinity purified endochitinase from wheat germ (70 μg; 48 h). The positions of standard mono- and oligosaccharides on the two chromatograms are indicated. Origins (0) are *arrowed*.

TABLE III

Treatment of radiolabeled product with affinity-purified chitinases

Enzyme source	Membrane source	Chitinase soluble radioactivity	Chitinase insoluble radioactivity	Per cent of material sensitive to chitinase digestion
		cpm		
A. *S. griseus* chitinase[a]	1. Crude homogenate	626	190	77
	2. Crude microsomes	196	68	74
	3. Crude microsomes	642	182	78
	4. Crude microsomes	296	72	80
	5. 25/43% fraction	224	82	73
	6. 43/48% fraction	144	54	73
B. Wheat germ endochitinase[b]	1. Crude microsomes	475	226	68
	2. Crude microsomes	412	199	67
	3. Crude microsomes	872	346	72
	4. Crude microsomes	578	248	70

[a] Samples of crude product (obtained by incubating 150 to 400 μg of membrane protein) were digested with 10 μg of purified chitinase from *Streptomyces* for 48 h at 30°C ("Methods"). After clarification of the digested preparations by centrifugation, the resultant pellets were washed with water and radioactivity determined by liquid scintillation counting.

[b] Normal incubations containing 300 μg of membrane protein were carried out in the presence and absence of affinity-purified wheat germ endochitinase (60 μg/incubation). The material insoluble in chloroform:methanol:water was prepared after incubation and radioactivity determined. Results are expressed as soluble and insoluble radioactivity in comparison to boiled chitinase control incubations.

digestion of samples with endochitinase for extended periods of time (24 to 48 h) does yield some detectable *N*-acetylglucosamine. When [^{3}H]chitin (24) was digested with the endochitinase preparation and the neutral oligosaccharides were chromatographed, identical results were obtained. Control incubations contained boiled endochitinase.

Enzymatic Transfer to Exogenous Chitin Acceptors—It was of interest to add chemically defined primers to incubation mixtures and attempt to demonstrate extension of the added material by the brine shrimp microsomal enzyme. As shown in Table IV, incubation of various membrane fractions in the presence of 4.5 mg (dry weight) granular chitin increased the accumulation of radioactivity in the crude product. Since the added acceptor is resistant to extraction with aqueous and organic solvents, it is obtained as part of the radiolabeled product. A dramatic increase in radiolabeled product formation was observed when granular chitin was added to membrane fractions purified by sucrose step-gradient ultracentrifugation. As shown in Fig. 5, the incorporation of radioactivity into product increases linearly with added granular chitin acceptor (3.8 to 15 mg of granular chitin/mg of membrane protein). At higher levels of exogenous granular chitin (38 mg of granular chitin/mg of membrane protein), inhibition of transfer is observed. Furthermore, greater than 95% of the radiolabeled product in such experiments was rendered soluble after treatment with affinity-purified *Streptomyces* chitinase (3 μg of enzyme protein/48 h/37°C).

Purified chitodextrins were added to incubation mixtures to determine any effect on the formation of radiolabeled product. As shown in Table IV, addition of microgram quantities of chitin oligosaccharides caused reduced incorporation of radioactivity into endogenous acceptor. Added [^{3}H]chitodextrins are soluble in 50% methanol and not incorporated to any extent into the crude, radiolabeled product (data not shown). In order to determine the fate of the added chitodextrin, ^{3}H-labeled *N*-acetylchitobiose (~50 ng; see "Methods") was incubated with crude microsomes in the presence of UDP-*N*-acetyl-D-[^{14}C]glucosamine. After 2 h, the oligosaccharide fraction was isolated by boiling and passage of the soluble material over an Amberlite MB-3 column (0.5 × 3 cm). The desalted oligosaccharides were analyzed by chromatography on a Bio-Gel P-2 column (1 × 58 cm) previously equilibrated with 0.1 M acetic acid. As shown in Fig. 6, the brine shrimp enzyme converted the added disaccharide to a species eluting in the position of a trisaccharide. Little formation of larger radiolabeled oligosaccharides was detected. This double-labeled oli-

TABLE IV

Enzyme activity toward exogenous chitin acceptors

Membrane fractions at various stages of purification (200 to 500 μg of membrane protein) were incubated for 2 h and the material insoluble in chloroform:methanol:water was prepared ("Methods"). Radioactivity in the crude product was determined by liquid scintillation counting.

Acceptor added	Membrane source	Control radioactivity	Acceptor added	Radioactivity in addition product	Per cent of control
		cpm	*mg/μg*	*cpm*	
1. Granular chitin[a]	Crude microsomes	118	4.5 mg	210	180
2. Granular chitin	25/43% fraction	46	4.5 mg	170	370
3. Granular chitin	43/48% fraction	48	4.5 mg	116	240
4. Chitodextrin II[b]	Crude microsomes	6733	15 μg[c]	284	4
5. Chitodextrin III	Crude microsomes	1188	45 μg[c]	195	12
6. Chitodextrin IV	Crude microsomes	321	23 μg[c]	130	40

[a] Granular chitin was prepared as described under "Methods" and resuspended in fresh assay buffer immediately before use.

[b] Chitodextrins were prepared by partial acid hydrolysis of granular chitin and purified by Bio-Gel P-2 chromatography as described under "Methods"; II, pentasaccharide; III, tetrasaccharide; IV, trisaccharide.

[c] The amount of chitodextrin is given as micrograms of reducing terminal sugar added, as estimated by the method of Park and Johnson (23).

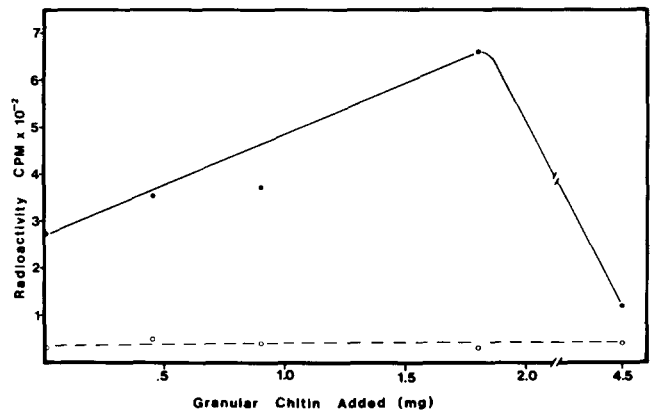

FIG. 5. **Incorporation of radioactivity as a function of exogenous primer.** Granular chitin was prepared ("Methods"), equilibrated with assay buffer and added to standard incubation mixtures containing 0.146 mg of membrane protein (43/48% fraction). After 2 h, the samples (●——●) and boiled controls (○- - -○) were precipitated with an equal volume of cold methanol, collected on a Whatman GF/A glass fiber filters, and washed with (*a*) 50% methanol (10 ml), (*b*) chloroform:methanol:water (10:10:3), and (*c*) hot 2% (w/v) sodium dodecyl sulfate in 0.1 M borate buffer, pH 8.0, before scintillation counting.

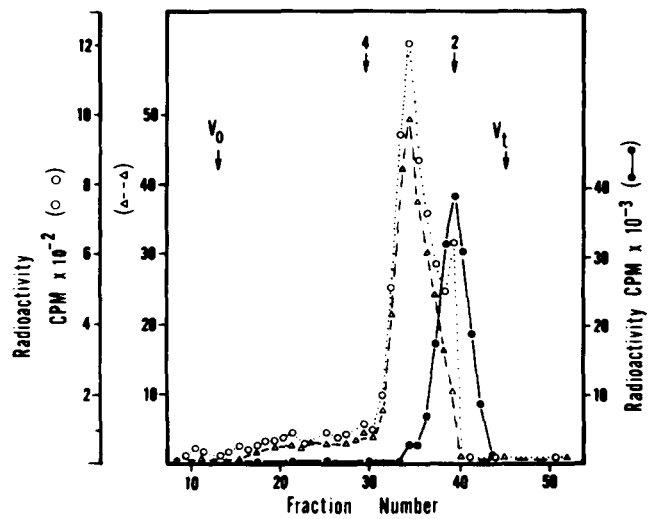

FIG. 6. **Bio-Gel P-2 chromatography of radiolabeled oligosaccharides after incubation with a crude microsomal enzyme preparation.** *N*-Acetyl[^{3}H] chitobiose was prepared as described under "Methods" and chromatographed on a Bio-Gel P-2 column (1 × 50 cm) previously equilibrated with 0.1 M acetic acid (●——●). The labeled oligosaccharide (~50 ng) was incubated with a crude microsomal enzyme preparation (400 μg of membrane protein) plus UDP-[^{14}C]GlcNAc under standard conditions. After 2 h, the sample was boiled, clarified, desalted with Amberlite MB-3, and the uncharged oligosaccharides were chromatographed on the Bio-Gel P-2 column; ^{3}H (○····○) and ^{14}C (△- - -△) appearing in the effluent was measured. The column was previously calibrated with stachyose (4) and *N*-acetylchitobiose (2).

TABLE V

Effect of diflubenzuron on chitin synthetase activity

All samples were preincubated with Dimilin (1 μg/ml final concentration) for 1 h prior to addition of UDP-[^{3}H]GlcNAc. Samples 1 and 2: 2-h incubation; Samples 3 to 6: 72-h incubation. The crude product, insoluble in chloroform:methanol:water (10:10:3) was prepared as described under "Methods."

Sample	Control radioactivity	Dimilin treated radioactivity	Inhibition
	cpm		%
1. Crude microsomes (0.64 mg protein)	880	422	52
2. Crude microsomes (0.22 mg protein)	311	74	76
3. 25/43% membrane fraction (90 μg protein)	733	84	88
4. 25/43% membrane fraction (200 μg protein)	1893	226	88
5. 43/48% membrane fraction (112 μg protein)	1181	94	92
6. 43/48% membrane fraction (950 μg protein)	10019	1793	82

gosaccharide did not bind to DEAE-cellulose and was not cleaved by crude chitinase from *Streptomyces*.

Inhibition of Chitin Synthesis by Diflubenzuron—Since previous studies have indicated the toxicity of the larvicide diflubenzuron ("DIMILIN") and its apparent inhibition of some step in the biochemical pathway leading to cuticular chitin (28, 29), it was of interest to determine the effect of this compound on the *Artemia* enzyme using an *in vitro* assay system. Larval brine shrimp membranes at various stages of purification were preincubated with diflubenzuron (1 μg/ml in assay buffer) which was dissolved immediately before use in absolute ethanol. After 1 h, radiolabeled nucleotide-sugar was added and the normal assay and extraction procedure was carried out. The results of these experiments, summarized in Table V, indicate that prior treatment of *Artemia* membranes with diflubenzuron inhibits the incorporation of radioactivity into endogenous acceptor, insoluble in chloroform:methanol:water. Although the effect on crude microsomes was less dramatic, approximately 90% inhibition of activity was obtained using more purified membrane fractions. Preliminary studies have indicated that diflubenzuron is toxic to larval brine shrimp at a concentration of 10 to 30 parts per billion and causes the larvae to lyse after about 72 h of growth.[2] Similar larval toxicity results have been reported previously (30).

Subcellular Distribution of Chitin Synthetase Activity—It was of interest to determine the subcellular location of the chitin synthetase activity. Previous studies on the chitin synthetase of yeast (31) have indicated that the enzyme is localized in the plasma membrane. In the present study, a variety of homogenization methods were investigated in preliminary experiments. Nitrogen bomb cavitation was found to be superior to Dounce, polytron, and Potter-Elvehjem homogenization. Membranes were then isolated and purified by differential centrifugation and sucrose step-gradient ultracentrifugation (32). Various fractions were assayed for marker enzymes and for chitin synthetase activity, as summarized in Table VI. The membrane fraction banding at the 43/48% (w/v) sucrose interface exhibited a greatly enhanced specific activity (approximately 40-fold) of Na^+, K^+-stimulated ATPase and phosphodiesterase I which are believed to be localized in the plasma membrane of mammalian cells (33). Other putative markers for the plasma membrane, alkaline phosphatase and leucine aminopeptidase, were not as highly enriched. When material banding at the 43/48% interface was diluted and recentrifuged in a continuous sucrose gradient, the preparation gave a single peak with a density of 1.174 g/cm^3. Significantly, chitin synthetase activity in the 43/48% fraction was purified about 10-fold over the crude homogenate. A much lower specific activity was measured in the material banding at the 25/43% interface. To study the nature of the membrane preparations further, the 43/48% fraction was prepared for transmission electron microscopy. As shown in Fig. 7, this fraction consists largely of vesiculated membranes with some contamination by mitochondria and Golgi cysternae.

Since the larval brine shrimp used in these experiments were about to molt, it was necessary to assay the purified membrane preparation for endogenous chitinase which might

[2] M. N. Horst, manuscript in preparation.

TABLE VI

Enzyme activities of subcellular fractions[1]

Samples	Alkaline Phosphatase Specific Activity	Phosphodiesterase I Specific Activity	Na,K-Stimulated ATPase Specific Activity	Leucine Amino Peptidase Specific Activity	Glucose-6-Phosphatase Specific Activity	Lactic Dehydrogenase Specific Activity	Chitin Synthetase nmol/h/mg
Crude Homogenate	.039 (1)	.011 (1)	.322 (1)	5.36 (1)	.000515 (1)	.00032	.047 (1)
600 Supn't	.031 (.79)	.018 (1.64)	.13 (.04)	3.79 (.7)	.000359 (.7)	.00032	.110 (2.3)
600 Ppt (Shells)	.021 (.54)	.037 (3.36	N.A.	N.A.	N.A.		.335 (7.1)
5500 Supn't	.039 (1)	.029 (2.64)	N.A.	N.A.	N.A.		N.A.
Crude microsomes	.233 (7.5)	.28 (25.5)	N.A.	N.A.	.0083 (16.1)	n.d.	.274 (5.8)
25/43%	.0912 (2.34)	.06 (5.45)	4.35 (13.5)	5.23 (1)	.011 (21.4)	n.d.	.03 (.64)
43/48%	.611 (15.7)	.461 (41)	15.31 (47.5)	37.2 (6)	.0089 (17.3)	n.d.	.484 (10.2)

N.A. = not assayed. [1]Specific activity = μmoles substrate (or cofactor) consumed/min/mg protein.
n.d. = not detected. Fold purification is indicated by the value in parentheses.

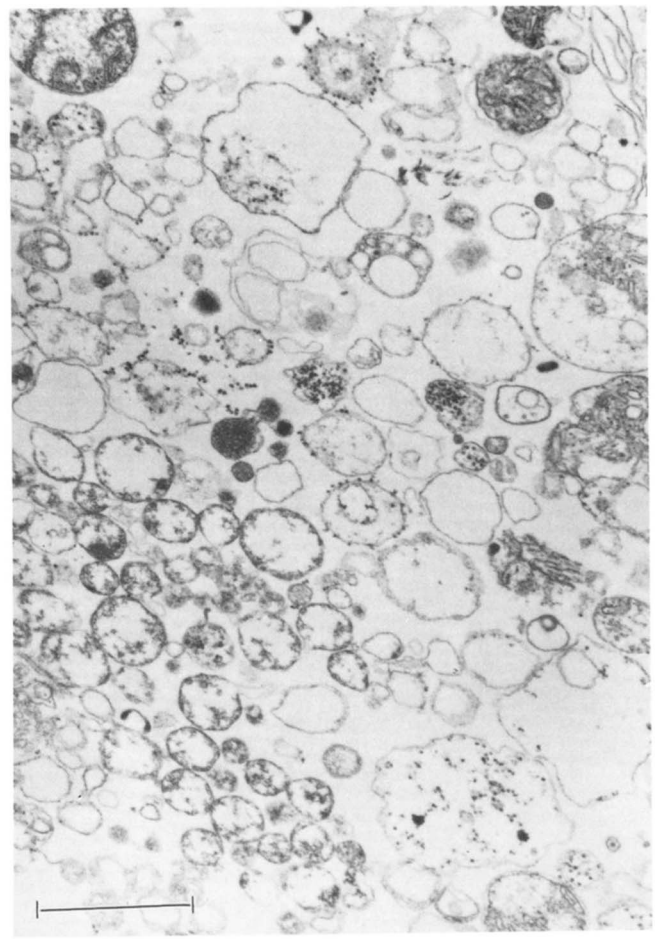

FIG. 7. **Transmission electron microscopy of the 43/48% membrane fraction.** The sample was prepared for electron microscopy as described under "Methods" and examined at 60 kV; *bar* indicates 1 μm.

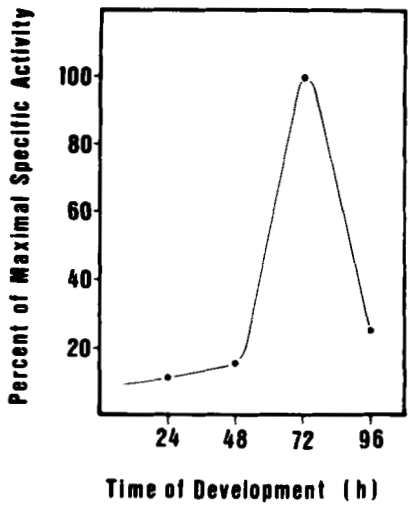

FIG. 8. **Time course of chitin synthetase activity *versus* larval development.** Larvae were grown for indicated periods of time, 43/48% membrane fractions were prepared and stored frozen. After completion of the time course, all samples were thawed, assayed, and specific activity determined. The results are presented as per cent of maximal specific activity; the value for the most active sample, 72 h, was 0.32 pmol/h/mg of protein.

be involved in degradation of the old cuticle. If present, this enzyme might cleave the radiolabeled product synthesized during the *in vitro* incubation period. Accordingly, the 43/48% membrane fraction was assayed for endogenous chitinase activity using [^{3}H]chitin as substrate (see "Methods"). Extremely low levels of endogenous chitinase activity were detected, corresponding to 0.76 milliunit of chitinase/mg of protein when assayed according to the procedure of Molano *et al.* (24). Preliminary experiments indicate that neither chitobiase nor *N*-acetylglucosaminidase activities are detectable in the 43/48% membrane fraction.[2]

Chitin Synthetase Activity during Larval Development—Since chitin biosynthesis in arthropods is a temporal event which is under strict hormonal control (1), it was of interest to study chitin synthetase activity *versus* time of larval development. Accordingly, brine shrimp eggs were incubated as described under "Methods" for 24, 48, 72, and 96 h. At each time point, a 2-liter bag was harvested, larvae were homogenized, and 43/48% membrane fraction was prepared by sucrose step-gradient ultracentrifugation. The specific activity of chitin synthetase at each time point was then determined. The results of this experiment (Fig. 8) show that chitin synthetase activity is maximal at 72 h of development, while much lower levels of activity are detected at earlier time points. The yield of total membrane protein varied with time of larval development, the highest value (11.9 mg) being obtained at 48 h. Nevertheless, the total amount of chitin synthetase present at each time point taken was about 3 times higher at 72 h than at any other point.

DISCUSSION

The present study shows that the biosynthesis of crustacean chitin can be demonstrated using a cell-free *in vitro* assay system. The enzyme responsible for this activity, chitin synthetase, has been detected in other crustaceans (6, 7) and the imaginal disks of insects (34), but not in cell-free insect systems (9, 35). The reason for this apparent discrepancy is not clear (8).

Membranes prepared from larval brine shrimp incorporate radiolabeled *N*-acetyl-D-glucosamine from UDP-GlcNAc into a product which is insoluble in water and in chloroform:methanol:water (10:10:3). Approximately 70% of this material, designated the crude product, appears to be chitin based upon its resistance to chemical extraction using alkali and 9.5 M urea. Additional information was obtained by treatment of the crude product with purified chitinases from *Streptomyces* (3) and wheat germ (25). Enzymatic sensitivity to either of these preparations also indicated that about 70% of the crude product is chitin. Solubilized, radiolabeled oligosaccharides produced by the action of these enzymes were uncharged and migrated on descending paper chromatography as disaccharides (*Streptomyces* chitinase) or as di-, tri-, and tetrasaccharides (wheat germ endochitinase). This is the first demonstration that arthropod chitin is sensitive to the wheat germ endochitinase.

The chitin synthetase of *Artemia* larvae is a membrane-bound enzyme which may be purified approximately 10-fold over the crude homogenate by differential centrifugation and sucrose step-gradient ultracentrifugation. The enzyme appears to be localized in a vesicular membrane fraction which is highly enriched in plasma membrane marker enzymes; however, morphological observation (Fig. 7) shows significant heterogeneity in the preparation. It should be noted that valid markers of the plasma membrane in *Artemia* are not really known. The density of this presumed plasma membrane fraction is much higher than that of mammalian plasma membrane purified by comparable procedures (33).

The general requirements for the *Artemia* chitin synthetase are similar to those described for other crustacean enzymes (6, 7) and, in some respects, are like those of fungal chitin synthetase (3, 5). Thus, divalent cations are required for activity, with magnesium being superior to manganese; incubation in the presence of EDTA completely abolishes enzymatic activity. On the other hand, *N*-acetyl-D-glucosamine and trypsin did not cause a substantial activation of the crustacean enzyme when compared to results obtained using fungal chitin synthetase (3). In this respect, the two enzymes appear dissimilar. One of the presumed reaction products is UDP, and as reported earlier, the nucleotide causes 62% inhibition of fungal chitin synthetase activity (3); similar results were obtained with the crustacean enzyme in the present study. Polyoxin D, which causes total inhibition of the fungal enzyme at a concentration of 10^{-5} M, exhibited only a slight diminution of the crustacean enzyme. Higher levels of Polyoxin D caused no change in inhibition. Since preincubation of membranes with Polyoxin D did not affect inhibition, it seems unlikely that the microsomal preparation contains an enzyme which destroys this antibiotic. These results with Polyoxin D are in contrast to the results of Vardanis (35), who noted the inhibition of insect chitin synthetase in a tissue level incubation system. Finally, the formation of chitin by the *Artemia* enzyme appears to be sensitive to the pesticide diflubenzuron ("DIMILIN") while previous studies have shown that the synthesis of fungal chitin is not affected by this compound (36). This is the first case where the action of diflubenzuron at the microsomal level has been indicated. Based upon these observations, it would appear that the crustacean and fungal chitin synthetases are not the same, although both enzymes make the same product.

In the present study, transfer of radiolabeled GlcNAc from UDP-GlcNAc to endogenous acceptor has been demonstrated. The radiolabeled product is not solubilized by high concentrations of urea nor by boiling alkali solutions. However, the product is degraded by treatment with purified *Streptomyces* chitinase. Transfer to exogenous acceptors has also been examined. Addition of water-soluble chitodextrins to incubation mixtures inhibited incorporation of radioactivity into endogenous acceptor; similar results in other arthropod systems have been reported (7). The fate of added chitodextrins was

examined by dual label experiments; added *N*-acetylchitobiose was converted to *N*-acetylchitotriose with no detectable formation of higher oligosaccharides. The mechanistic reason for this observation is not clear; possibly, the trisaccharide is an inhibitor of the *Artemia* chitin synthetase. These results, like those of Wiltse (37) would imply that the crustacean enzyme is not a precessive transferase but rather detaches from the product after each transfer from the nucleotide-sugar to acceptor is accomplished.

Transfer of *N*-acetyl-D-glucosamine from UDP-*N*-acetyl-D-glucosamine to insoluble, granular chitin by the *Artemia* chitin synthetase was also demonstrated in the present study. This is the first report of transfer to a chemically defined, macromolecular acceptor. The radioactivity in the product was sensitive to treatment with either *Streptomyces* chitinase or the endochitinase from wheat germ. It may be argued that added granular chitin stimulates activity by competing for an endogenous chitinase. A low level of chitinase activity was detected in the microsomal preparation; however, the assay conditions (see "Methods") may not be optimal for the endogenous chitinase, *e.g.* pH, salt concentration, and the insoluble nature of both the enzyme and the substrate. On the other hand, the presence of an endogenous chitinase would not explain the decreased activity observed at high levels of granular chitin.

As indicated in Fig. 2, detectable radioactivity can be chased into an organic phase following addition of radiolabeled UDP-*N*-acetyl-D-glucosamine. Further studies are now required to elucidate the possible role of lipid-linked intermediates in the biosynthesis of crustacean chitin.

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