

CHITIN DIGESTION AND ASSIMILATION BY SEABIRDS

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ABSTRACT.—As a structural component of crustacean exoskeletons, chitin is the most important carbohydrate in the diets of many marine carnivores. To investigate the physiological and biochemical adaptations that may enable seabirds to break down this "prey defense," we estimated chitin digestibilities for Sooty Albatrosses (*Phoebastria fusca*), White-chinned Petrels (*Procellaria aequinoctialis*), Rockhopper Penguins (*Eudyptes chrysocome*), Gentoo Penguins (*Pygoscelis papua*), King Penguins (*Aptenodytes patagonicus*) and Leach's Storm-Petrels (*Oceanodroma leucorhoa*) fed Antarctic krill (*Euphausia superba*). These species retain a substantial proportion ($46.5 \pm \text{SD of } 13.1\%$, $39.1 \pm 4.9\%$, $52.8 \pm 37.6\%$, $45.3 \pm 5.6\%$, $84.8 \pm 11.7\%$ and $35 \pm 12.2\%$, respectively) of ingested chitin. We also obtained preliminary estimates of chitinolytic activity in the gastric mucosae of the above six species by incubating extracts of tissue samples with a chitin substrate and measuring the production of the end product of chitin hydrolysis, N-acetyl-D-glucosamine (NAG). Chitinolytic activity (up to $5,000 \mu\text{g NAG h}^{-1} \text{g}^{-1}$ expressed per gram tissue) was measured from proventricular tissue and within the activity range ($1,350$ to $61,650 \mu\text{g NAG h}^{-1} \text{g}^{-1}$) reported for eight other avian species. In order to assess the energetic and nutritional benefits of chitinolytic activity in seabirds, we studied gastrointestinal absorption of the end products of chitinolysis in Leach's Storm-Petrels. The overall absorption efficiency of NAG and its deacetylated precursor, glucosamine (Gln), in this species was $44.0 \pm 3.0\%$ and $11.0 \pm 1.9\%$, respectively. These absorption efficiencies were significantly less than for glucose, which was absorbed with an efficiency of $90.6 \pm 2.5\%$. No absorption of NAG and Gln occurred in the proventriculus. Overall, we showed that seabirds have a capacity to assimilate a considerable portion of the carbon and nitrogen present as chitin in the exoskeleton of their prey, but we have not demonstrated that assimilation actually occurs. The potential costs and benefits of chitin hydrolysis, as well as the absorption of the breakdown products, need to be assessed. Received 24 May 1991, accepted 18 December 1991.

THE MUCOPOLYSACCHARIDE polymer chitin ([1 β 4-2]-acetamido-2-deoxy- β -D-glucan) in crustacean exoskeletons represents a substantial source of potential energy and carbohydrate for marine predators (Anderson et al. 1978, Rehbein et al. 1986). Estimates of krill biomass, for example, range from 80 to 500 million metric tons (Sahrage and Steinberg 1975), the equivalent of approximately 1.6 to 10 million tons of chitin, and 0.24 to 3 million tons of other carbohydrates (Clarke 1980). Hence, chitin is the most important carbohydrate in the diets of marine predators of krill, and probably of other

crustaceans. Although chitin digestion and assimilation in marine fish are well studied (e.g. Fänge et al. 1976, Lindsay et al. 1984, Lindsay and Gooday 1985), little attention has been paid to utilization of dietary chitin by seabirds whose daily mass-specific energy demands far exceed those of marine fish.

Seabirds such as those that feed on crustaceans in the Southern Ocean would benefit greatly from an ability to penetrate the "structural defenses" of their prey, and to utilize the energy residing in chitinous exoskeletons. By studying species with a range of natural diets (Table 1), we tested the prediction that seabirds known to habitually eat crustaceans also secrete chitinolytic enzymes, which enhance their ability to break down and assimilate this carbohydrate. Although a study of microbial and vertebrate chitin degradation by crabeater seals (*Lobodon carcinophagus*) and Adélie Penguins

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TABLE 1. Predominant prey types of seabirds studied (less-frequently-eaten prey in parentheses).

Species	Predominant prey	Reference
Leach's Storm-Petrel (<i>Oceanodroma leucorhoa</i>)	Fish (crustacea)	Linton (1978)
White-chinned Petrel (<i>Procellaria aequinoctialis</i>)	Fish (squid, crustacea)	Jackson (1988), A. Berruti (unpubl. data)
Sooty Albatross (<i>Phoebastria fusca</i>)	Squid (fish)	Cooper and Klages (unpubl. data)
Rockhopper Penguin (<i>Eudyptes chrysocome</i>)	Crustacea (fish)	Brown and Klages (1987)
Gentoo Penguin (<i>Pygoscelis papua</i>)	Fish (crustacea)	Adams and Wilson (1987)
King Penguin (<i>Aptenodytes patagonicus</i>)	Fish (squid)	Adams and Klages (1987), Croxall and Prince (1980)

(*Pygoscelis adeliae*) is currently under way (Staley 1986), we are not aware of published data other than our own (for a preliminary report, see Place 1990, Place and Jackson 1991) on the levels of chitinolytic activity in seabird guts.

Most forms of chitin are insoluble in many solvents, highly hydrophobic, and relatively inert to biodegradation (Pangburn et al. 1984, Gooday 1990). With only one exception, the β -chitin of diatoms, chitin is always found crosslinked or associated with other structural components. In insects and other invertebrates, chitin is associated with specific proteins through covalent and noncovalent bonding, producing an ordered structure (Blackwell and Weih 1984). Chitin often exhibits varying degrees of mineralization, in particular, calcification or sclerotization involving interactions with phenolic and lipid molecules (Peter et al. 1986, Poulicek et al. 1986). In nature, varying degrees of deacetylation of the polymer result in a structural continuum between chitin (fully acetylated) and chitosan (fully deacetylated; Aruchami et al. 1986, Datema et al. 1977, Davis and Bartnicki-Garcia 1984, Gowri et al. 1986).

Once considered the action of a nonspecific enzyme for the catabolism of chitin, chitinolysis is now known to involve a complex group of enzymes with discrete phylogenies and subtle differences in specificity, role, and biochemical properties. Chitin degradation requires the action of at least two enzymes, chitinase (E.C. 3.2.1.14; poly- β -1,4-[2-acetamido-2-deoxy]-D-glucoside glycanohydrolyase) and chitobiase (E.C. 3.2.1.30; β -N-acetyl-D-glucosaminidase; Jeuniaux 1961). Chitinase hydrolyzes chitin to yield trimers and dimers (chitotriose and chitobiose) of N-acetyl-D-glucosamine (NAG), and chitobiase hydrolyzes these trimers and dimers to the monomer of NAG. An alternative deg-

radation route, involving the breakdown of chitin into glucosamine by specific deacetylases (Reyes et al. 1986), has not been shown to occur in vertebrates. There are four recognized roles for chitinases in nature, namely morphogenic, nutritional, digestive and defensive (Gooday 1990). In this paper, we use a variety of techniques to assess which of these roles chitinase plays in seabird digestion.

We report the results of chitinolytic assays on stomach contents and tissue samples from five species of seabirds occurring in the Southern Ocean, and one species that breeds on the North Atlantic coast. We examine the nutritional importance of chitin using two approaches, the first of which is a balance study estimating apparent digestibilities of chitin in three penguin species and three procellariiform species found to have gastric chitinase activity. Second, we demonstrate the capacity of a procellariiform to absorb the hydrolytic products of chitin, N-acetyl-D-glucosamine and glucosamine, and compare this capacity to that for absorption of glucose, a structurally similar monosaccharide that is probably the second most important carbohydrate in seabird diets after chitin itself. In addition, we investigated some *in vitro* conditions (i.e. pH and Ca concentrations) that may regulate chitinolytic activity and compared the effects with *in vivo* conditions found in seabird stomachs.

METHODS

We purchased the following chemicals from Sigma Chemical (St. Louis, Missouri): chitin; chitosan; D-(+)-glucosamine hydrochloride; N-acetyl-D-glucosamine; N,N'-diacetylchitobiose; D-glucose; polyethylene glycol 4000; *Serratia marcescens* chitinase; *Helix pomatia* β -glucuronidase; and almond β -glucosidase. Acetic anhydride was purchased from Aldrich Chem-

ical (Milwaukee, Wisconsin). All other chemicals were reagent grade unless specified otherwise. All solvents were either pesticide or HPLC grade. Whatman 3-MM chromatographic filter paper was obtained from VWR Scientific (Philadelphia, Pennsylvania).

Radiolabels and fluors.—We used acetic anhydride [^3H] (50 mCi/mmol), D-[^{14}C (U)] glucose (4.28 mCi/mmol), acetyl-N-[glucosamine-1,6- ^3H (N)]-D-glucosamine (44.2 mCi/mmol), D-[1,6- ^3H (N)] glucosamine hydrochloride (24 mCi/mmol), and [1,2- ^{14}C] polyethylene glycol 4000 (PEG) (0.77 mCi/g) from NEN Research Products (Boston, Massachusetts). All of these were found to have radiopurities greater than 98% by thin-layer chromatography (TLC).

Fluors were Pico-Safe (Packard, Downers, Illinois) and Biosafe II (Research Products International, Mount Prospect, Illinois). Samples were counted on a Packard Tri-Carb 1600A liquid scintillation analyzer. A correction ("quench") curve was derived to determine counting efficiency for different extracts and tissue types. Counting efficiency for ^{14}C in the samples varied from 78 to 82%, and that for ^3H from 38 to 46%. Counting times (3–5 min) were chosen to ensure at least 95% counting accuracy. The coefficient of variation for replicate samples averaged $3.8 \pm 1.3\%$ tritium and $2.1 \pm 0.9\%$ for carbon-14. All radioactivities are expressed in μCi ($1.00 \mu\text{Ci} = 37.0$ kilobecquerels).

Apparent chitin digestibilities.—Chitin digestibilities were determined for: adult and fledgling Sooty Albatrosses; fledgling White-chinned Petrels; and adult Gentoo Penguins and King Penguins at Marion Island ($46^{\circ}52'\text{S}$, $37^{\circ}51'\text{E}$); adult Rockhopper Penguins at Gough Island ($39^{\circ}21'\text{S}$, $09^{\circ}53'\text{W}$); and Leach's Storm-Petrel chicks at Little Duck Island ($44^{\circ}10'\text{N}$, $68^{\circ}15'\text{W}$). Procellariiform chicks were used because: they are easier to capture than are the adult of many species; they must eat what their parent presents to them as food; the energetic and nutritional demands for chick growth are greater than for adult maintenance; and we wished to lessen the impact of our study on breeding adults as far as possible. Removal of a chick from the nest for two to four days does not result in desertion in White-chinned Petrels and Sooty Albatrosses. For the studies on Marion and Gough Island, five adult birds of each species and age class were fasted for 48 h before receiving an homogenized Antarctic krill (*Euphausia superba*, hereafter referred to as krill) meal with a wet mass of 6 to 8% of the birds' body mass. The birds were then fasted for an additional 48 h, during which time all excreta were collected separately for each bird. Samples of food ($n = 6$) and excreta were dried at 45°C for five days, and then weighed and ground finely in an electric coffee grinder.

For studies on Little Duck Island, 12 Leach's Storm-Petrel chicks initially 30 days old were hand-reared for 25 days on homogenized krill. A 20% (weight/volume) homogenate of freeze-dried krill was made with water and supplemented with 35% (weight/

weight) olive oil. Nightly meals of 5 to 8 cc (approximately 88 to 141 kJ/meal) were warmed to 37°C and delivered to the stomach via the esophagus with a disposable 5-ml syringe attached to a 10-cm length of polyethylene tubing. All chicks took the feeding without regurgitation, and their daily wing-chord growth indicated normal development. After ingestion, each chick was placed on a polyethylene mesh (6-mm mesh size) platform suspended in a 9-L Baine Marie polyethylene container (Cole-Palmer) to collect excreta. Containers were kept in the dark and maintained at $14 \pm 3^{\circ}\text{C}$ to simulate the nest environment as much as possible. Meals and daily collected excreta were treated as above.

Digestibilities estimated in this manner are hereafter referred to as "apparent" digestibilities because such balance studies do not take into account retention of unassimilated chitin within the gut (see Discussion). The chitin content of food and excreta from all feeding trials was estimated by equating chitin with fiber, and using the method for crude fiber determination in animal feeds described by Horwitz et al. (1975). The acid-detergent-fiber (ADF) method has been shown to recover chitin completely, at least in shellfish meals (Stelmock et al. 1985, Watkins et al. 1982). We validated our estimates by assessing the chitin content of the crude "fiber" using two hydrolysis routes: enzymatic hydrolysis with *Serratia marcescens* chitinase and either almond β -glucosidase or snail gut β -glucuronidase (Cabib and Sburlati 1988); or acid hydrolysis and reacylation (Lindsay et al. 1984). The N-acetylglucosamine thus formed was quantified colorimetrically according to the procedure of Reissig and Leloir as modified by Boller and Mauch (1988).

Acid hydrolysis resulted in significant destruction of glucosamine with resulting low levels of NAG being detected. We recovered only $24.2 \pm 5.0\%$ ($n = 11$) of the predicted quantity of amino sugars from purified chitin after acid hydrolysis for 12 h at 105°C (Lindsay et al. 1984). Enzymatic hydrolysis (Cabib and Sburlati 1988) was far more accurate in our hands, yielding nearly $82.2 \pm 22.1\%$ ($n = 6$) of the predicted NAG from reacylated chitin after 4.5 h of treatment. We estimate that 85% of the weight of the fiber was NAG released by enzymatic hydrolysis. The ash content of the acid extracted fiber averaged $0.14 \pm 0.3\%$.

Apparent digestibilities of chitin for individual birds were calculated as percentages using the formula:

$$100 (C_{in} - C_{out})/C_{in} \quad (1)$$

where C_{in} and C_{out} , respectively, are the total dry masses (g) of chitin ingested in the food and chitin excreted.

In vivo chitinolysis in Leach's Storm-Petrel chicks.—We used an isotope-ratio technique to measure *in vivo* chitinolysis of tritiated chitin in seven Leach's Storm-Petrel chicks between 45 and 60 days old. Tritiated chitin was prepared by reacylation of chitosan with

acetic anhydride, as described by Molano et al. (1977). The birds were fed a homogenized meal (4.6 ± 0.9 g, $n = 3$; or 9.6 ± 0.6 g, $n = 4$) containing tritiated chitin ($0.15 \mu\text{Ci/ml}$, $0.5 \mu\text{Ci/mg}$, $0.23 \mu\text{Ci}/\mu\text{mole NAG}$), plus [^{14}C]-PEG ($0.22 \mu\text{Ci/ml}$) in a carrier suspension of 50 mg/ml unlabeled reacylated chitin and 10 mg/ml unlabeled PEG in avian Ringer solution. Both PEG (polyethylene glycol 4000) and the products of chitinolysis are water soluble, but unhydrolyzed chitin is not. We validated this by centrifugation of the meal suspension for 5 min at $200 \times g$; more than 98% of the tritiated chitin and less than 1% of the ^{14}C -labeled PEG sedimented out of the suspension. With the assumption that PEG is nonabsorbable by the proventriculus, we thus quantified *in vivo* hydrolysis of the tritiated chitin by measuring the ratio of dissolved tritium-labeled chitin oligomers to [^{14}C]-PEG in centrifuged proventricular liquids, because 98% of the undigested chitin in the proventricular liquid is not in solution and is removed by centrifugation. Moreover, use of the PEG marker circumvents bias arising from dilution of the original meal by gastric or intestinal secretions.

After feeding, feces were collected as described above. Proventricular samples (ca. 0.2 ml) were obtained by intubation at 30-, 60- and 120-min post-ingestion, centrifuged, and an aliquot removed for scintillation counting. Accumulated excreta (2-, 12- and 24-h post-ingestion) in each container were extracted with 50 cc of distilled water and homogenized with a Polytron homogenizer to uniform composition. Samples were then removed for scintillation counting.

Absorption of products of chitinolysis in Leach's Storm-Petrel chicks.—We estimated glucose, N-acetyl-D-glucosamine (NAG) and D-glucosamine (Gln) absorption efficiencies using both an isotope-ratio technique, and by measuring total isotope recovery. The first method involved feeding five birds 10 μCi of one of the tritiated monosaccharides of interest (NAG, D-glucosamine or D-glucose) simultaneously with 5 μCi of ^{14}C -labeled PEG, in a carrier meal of either avian Ringer solution, or homogenized whole krill. Both types of carrier meal also contained 50 mg of unlabeled PEG, and 0.2 mmol of unlabeled monosaccharide (NAG, Gln or D-glucose), a quantity equivalent to the total amount of chitin contained in a krill meal of 8.6 g, assuming complete hydrolysis of chitin.

Gastrointestinal absorption of [^3H]-N-acetyl glucosamine or [^3H]-glucosamine was calculated from the ratio of [^{14}C] to [^3H] in the daily fecal collection using the formula:

$$\text{Percent absorbed} = (1 - R_{\text{in}}/R_{\text{out}}) \times 100, \quad (2)$$

where R_{in} equals the ratio $^{14}\text{C}/^3\text{H}$ in the test meal, and R_{out} equals the ratio $^{14}\text{C}/^3\text{H}$ in fecal collection. Total recovery of ^{14}C and ^3H in accumulated feces also were measured.

Secondly, total recovery of ^{14}C -labeled glucose and

[^3H]-NAG or [^3H]-D-glucosamine were measured in the excreta of five birds fed a meal of avian Ringer solution carrying the labeled glucose and one of the two labeled monosaccharides. The concentration of each sugar was 25 mM. Assimilation efficiencies of the labeled substances were expressed as percentages of the total label ingested.

In all of the above experiments, the meals were administered *per os* (no chicks regurgitated after feeding), and the feces collected as described above. Proventricular samples removed by intubation at 1- and 2-h post-ingestion. Accumulated excreta in each container were removed at 2-, 12- and 24-h intervals, homogenized, centrifuged, and analyzed as described above.

Chitinolytic activity.—Tissue samples were taken from the stomach (proventriculus) linings of adult Rockhopper, Gentoo and King penguins (in all cases $n = 4$) 12 h after a krill meal, using Olympus FB-15K E biopsy forceps passed through an Olympus GIF type P fiber-optic gastroscope with an Olympus CLE 4U/4E cold light source. This method did not necessitate killing the study animals (Jackson and Cooper 1988). The samples were rinsed a minimum of five times with 67 mM phosphate buffer (pH 7.0), and immersed in a solution of 20 μl of PMSF (phenylmethanesulphonyl fluoride, Merck, RSA) in 10 ml of phosphate buffer. The samples were frozen and stored at -20°C for one to two months before analysis. Two samples were taken from every individual and samples pooled for each species.

Whole sections of stomach and intestinal wall were taken from one Sooty Albatross, two White-chinned Petrel, and six Leach's Storm-Petrel fledglings that had been killed immediately prior to dissection by intravenous injection of "Euthanaze," a stable solution containing 200 mg sodium pentobarbitone per ml (Centaur Laboratories, Johannesburg, South Africa). These samples were frozen separately and stored at -20°C or -70°C , each in 10 ml of a solution with the same proportions of phosphate buffer and PMSF as that used above.

Both squid and krill synthesize chitin (Jeuniaux 1963, Spindler and Buchholz 1988, Grisley and Boyle 1990). To investigate variation in gastric chitinolytic activity resulting from the presence of this activity in the prey itself, the proventriculus contents of captive Sooty Albatrosses were sampled through the esophagus by intubation, 12 h after a krill or a squid meal (in each case, four individuals were sampled). It was necessary to introduce 30 ml of distilled water into each bird's stomach to facilitate withdrawal of the sample. We added 20 μl of PMSF (a serine protease inhibitor) per 10 ml of stomach contents to each of the samples, which were stored as described above.

In vitro chitinolysis.—The tissue samples were thawed, crushed by hand over ice in 4 ml of phosphate buffer using glass tissue grinders, centrifuged at $10,000 \times g$ for 10 min, and the supernatant decanted

for assays. Samples of stomach contents were thawed and centrifuged in the same manner, but were not homogenized. All assays were performed in duplicate. Two methods were used: the end-product colorimetric measurement (Jeuniaux 1966); and the tritiated-chitin method (Molano et al. 1977). The final enzyme-activity values estimated using both methods were expressed as $\mu\text{g NA h}^{-1} \text{mg}^{-1}$ protein.

For the colorimetric measurement, chitin substrate was prepared using the method of Reichenbach and Dworkin (1981). Samples were incubated on a shaker at room temperature, with 1.5 ml of enzyme solution and 600 μl of chitin slurry (10 g/100 ml of phosphate buffer). We added 100 μl of toluene every 24 h to inhibit bacterial activity. At 0, 4, 24, 48 and 72 or 96 h after the start of incubation, 250- μl aliquots of the solution were removed and assayed for the end product N-acetylglucosamine (NAG). The samples were read on a Beckman DU-40 spectrophotometer at wavelength 585 nm, and absorbance was converted to chitinolytic activity ($\mu\text{g NAG}$) using the equation:

$$Y = 9.43X^{0.87}, \quad (3)$$

where Y is $\mu\text{g NAG}$ and X is absorbance at 585 nm. The equation was calculated from a dilution series with standard solutions of NAG (Sigma) in phosphate buffer ($N = 16$, $r^2 = 0.99$). This assay, without the addition of added chitinase, measures the extent to which chitin is completely hydrolyzed to its monomeric constituent. The chitinolytic activities measured in proventricular tissue and luminal contents are based on the production of NAG without added N-acetylhexosamidase to the assay mixture. Protein content of the tissue extracts was determined using the method of Lowry et al. (1951) to permit the standardized expression of NAG concentration per mg of protein analyzed.

For the tritiated-chitin method, substrate was prepared by reacylation of chitosan with acetic anhydride, as described by Molano et al. (1977). Samples were incubated at 41°C on a shaker, in 100 μl of a mixture containing 0.05 M buffer (sodium phosphate, pH 6.3; sodium acetate, pH 4.1; or KCl/HCl, pH 1.0 or 2.0; depending on the desired pH); 3.6 mg/ml of acetyl [^3H] chitin (0.5 $\mu\text{Ci}/\text{mg}$, 0.23 $\mu\text{Ci}/\mu\text{mole NAG}$); and tissue extract. After incubation, 0.3 ml of 10% (w/v) trichloroacetic acid was added, and the suspensions centrifuged for 5 min at 500 $\times g$. We removed 200 μl of the supernatant with a micropipette, and transferred this aliquot to a scintillation vial for radioactivity determination. This assay procedure measures the production of all chitin oligomers with chain lengths less than 7 and, thus, does not require the production of NAG monomers for activity determinations. The activities of both endochitinase (which cleaves chitin within the polysaccharide chain) and exochitinase (which cleaves the dimer chitobiose from the ends of the chitin polymer) are thus determined. The release of soluble chitin oligomers was linear both with respect to enzyme addition,

$$\mu\text{g NAG} = 7.4 \pm 0.63/\text{mg protein (crude extract)} \\ + 0.336 \quad (R^2 = 0.98, n = 10),$$

and to time,

$$\mu\text{g NAG} = 0.009 \pm 0.0004/\text{min} + 0.083 \\ (R^2 = 0.995, n = 14)$$

for at least 180 min. The production of the soluble tritium label did not result from deacetylation of [^3H] chitin, because only $0.5 \pm 0.1\%$ ($n = 12$) of the tritium was ethyl-acetate extractable (Araki and Ito 1988). Protein content of the tissue samples was determined using the method of Bradford (1976) when we assayed using the [^3H] chitin method.

pH and calcium dependence of chitinolytic activity.—The mucosal linings from samples of proventricular tissue from two White-chinned Petrels and six Leach's Storm-Petrels were stripped away from the outer muscle by scraping with a glass microscope slide. The lining was weighed, minced, and homogenized with an Ultra-Turrax T25 homogenizer in a volume of 2% acetic acid adjusted to double the mass of tissue. The extract was centrifuged at 20,000 $\times g$ for 60 min and the supernatant collected and stored at -70°C . The extracts prepared with 2% acetic acid showed higher specific activities than did those extracted with phosphate buffer, because 200- to 300-fold less protein is obtained with the former technique. The acid extracts lose activity with storage, even when kept at -70°C . Lysozyme activity was measured using a 0.25 mg/ml suspension of freeze-dried *Micrococcus luteus* as a substrate. Upon addition of the mucosal extract, clearing of the suspension reflects lysing of the *Micrococcus* cell walls, which can be quantified by recording the change in absorbance of the suspension at 540 nm with a Beckman DU8 spectrophotometer at 37°C (Dobson et al. 1984). A unit of activity is defined as a 1% change in absorbance per minute. For studies of activity as a function of pH at constant ionic strength of 0.133, a series of buffers were made by combining five parts of the appropriate buffer (HCl/NaCl, pH 1–2.5, Acetate, pH 3–4.5, Phosphate, pH 6–7.5, Miller and Golder 1950) at ionic strength of 0.1, with one part of 0.03 M NaCl.

RESULTS

Apparent chitin digestibilities.—The krill used in our diet contained $2.9 \pm \text{SD}$ of 0.4% chitin (dry mass) based on the fiber content, and the wet-mass-to-dry-mass ratio for the krill was 4.25 ± 0.04 . The calcium content (percent dry mass) was $1.8 \pm 0.2\%$ and the energy content was 17.87 ± 1.91 (kJ g^{-1} dry mass). These figures are consistent with other analyses of krill composition (Clarke 1980, Fricke et al. 1984). All six seabird species exhibited appreciable, although highly variable, apparent chitin digestibilities,

with values for King Penguins and Sooty Albatross chicks higher than those for other species (Fig. 1).

In vivo chitinolysis in Leach's Storm-Petrel chicks.—In the two sets of experiments where chicks were fed different-sized meals of radio-labeled chitin, average rates of proventricular chitinolysis determined by *in situ* solubilization of tritiated chitin in the first hour postingestion were 28.5 ± 11.2 ($n = 3$) and 220 ± 87.7 ($n = 4$) $\mu\text{mole NAG equivalents h}^{-1} \text{ ml}^{-1}$ of proventricular liquid for the two meal sizes (4.6 and 9.6 ml containing 50 mg/ml chitin), respectively. If we assume the average molecular weight of a purified chitin chain is 1,000,000, then approximately 4,500 moles of NAG make up each chain. This translates to a chitinolytic rate (expressed as a percent of ingested chitin) of approximately 13%/h and 98%/h for the two meal sizes, respectively. Very little NAG (<10%) was produced; the major product being a polymer six or more units in length as assessed by TLC of the radioactive products (Molano et al. 1979). These chitinolytic activities are not diet-derived, because purified chitin was used as food.

In vivo chitin digestibility in Leach's Storm-Petrel chicks.—The aqueous marker (PEG) emptied rapidly from the proventriculus (Place et al. 1989), with recovery of $38 \pm 13.5\%$ of the original dose in excreta 2 h after feeding. High proportions of the original doses were recovered 24 h after feeding in excreta ($88.4 \pm 3.0\%$ and $76.2 \pm 5.4\%$ for the 4.6 g and 9.6 g meals, respectively). Tritiated chitin was retained in the proventriculus, with only 1% recovered in excreta after 2 h. After 24 h, $42.6 \pm 5.7\%$ and $49.4 \pm 16.3\%$ of the ingested tritiated chitin was recovered in excreta, for the two meal sizes, respectively. The recovered tritiated chitin in excreta was sufficiently nondegraded to be completely precipitated by 10% trichloroacetic acid. Apparent digestibilities of radio-labeled chitin estimated from these excretion rates (50–58%) are similar to those obtained using the crude-fiber measurement with krill meals (Fig. 1). The reacylated chitin is a better substrate for chitinolysis than is the native-protein-associated chitin in the krill meals, accounting for the slightly higher apparent digestibilities yielded by the former technique.

Absorption of D-glucose and products of chitinolysis in Leach's Storm-Petrels.—The proventriculus of this species exhibits no capacity to absorb N-acetyl-D-glucosamine (for all results given below, $n = 5$ unless otherwise stated). The ratio

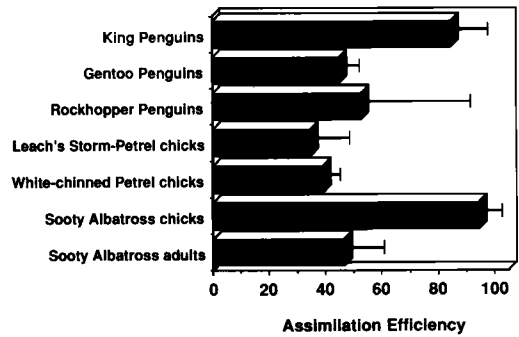


Fig. 1. Mean apparent digestibilities (with SD indicated by whiskers; in all cases $n = 5$, except for King Penguins where $n = 6$) of chitin in King Penguins, Gentoo Penguins, Rockhopper Penguins, Leach's Storm-Petrels, White-chinned Petrels, and Sooty Albatrosses fed meals of Antarctic krill.

of ^3H in NAG to ^{14}C in PEG in the meal (2.55 ± 0.05) is statistically indistinguishable from that in the proventriculus at 1-h (2.60 ± 0.05) and 2-h (2.57 ± 0.08) postingestion. Similar results were obtained for glucosamine and glucose.

In contrast, absorption of all three sugars was proven to occur across the entire gut. When estimated by the marker ratio method, $46 \pm 8.1\%$ ($n = 3$) of ingested NAG fed in avian Ringer solution is absorbed, whereas the corresponding estimates using the total radio-isotope recovery method is $39.4 \pm 6.9\%$. The aqueous marker (PEG) is rapidly evacuated from the gastro-intestinal tract, evidenced by the recovery of $46.3 \pm 8.1\%$ of this marker in the excreta 2 h after ingestion. After 24 h, $78.1 \pm 4.7\%$ of the ingested dose of this marker was excreted.

When fed as a mixture with equal molar concentrations of $[^{14}\text{C}]$ -D-glucose, a similar absorption efficiency was recorded for $[^3\text{H}]$ -NAG. D-glucose is absorbed at $90.6 \pm 2.5\%$ of the ingested dose, and NAG at $44.3 \pm 3.0\%$ of the ingested dose. Similarly, when $[^{14}\text{C}]$ -D-glucose and $[^3\text{H}]$ -D-glucosamine are fed as an equal molar solution, absorption efficiencies of $89.7 \pm 1.2\%$ and $12.5 \pm 11.0\%$ were observed.

The absorption efficiency for NAG increased to $59.9 \pm 17.9\%$ when this substance was fed with a meal of homogenized krill, whereas the corresponding value for D-glucosamine was unchanged at $11 \pm 1.9\%$. The $[^{14}\text{C}]$ -PEG recoveries after 48 h were $116 \pm 20.6\%$ and $94.5 \pm 21.5\%$, respectively, when fed with krill meals.

In vitro chitinolytic activity.—Four of the five Southern Ocean species and the single North-

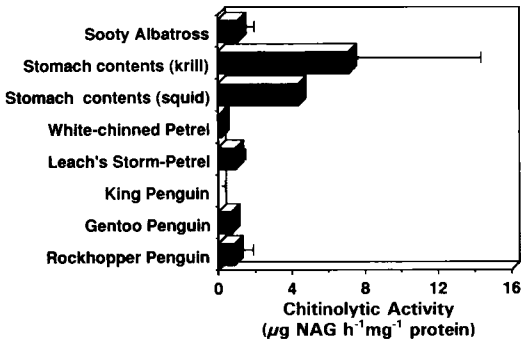


Fig. 2. Chitinolytic activities ($\mu\text{g NAG h}^{-1} \text{mg}^{-1}$ protein; 1 SD indicated) in the gastric mucosa and stomach contents of Sooty Albatrosses, and in the gastric mucosae of White-chinned Petrels, Leach's Storm-Petrels, King Penguins, Gentoo Penguins, and Rockhopper Penguins. In all cases $n = 4$, except Leach's Storm-Petrels ($n = 6$) and White-chinned Petrels ($n = 2$).

ern Hemisphere species showed similar levels of chitinolytic activity in the gastric mucosa (Fig. 2, overall mean of $0.77 \pm 0.38 \mu\text{g NGA h}^{-1} \text{mg}^{-1}$ protein), despite wide differences in their natural diets (Table 1). The chitinolytic activity in the stomach contents of krill- and squid-fed Sooty Albatrosses was significantly higher than were mucosal activity levels, and was highly variable among the krill-fed seabirds.

Recall that the colorimetric assay measures the production of NAG monomers, and does not detect the higher NAG polymers resulting from enzymatic hydrolysis unless chitobiase is added. The values presented in Figure 2 underestimate total chitinolytic activity; when similar mucosal extracts from Leach's Storm-Petrels were assayed using acetyl [^3H] chitin, the chitinolytic activity increased nearly four-fold ($3.8 \pm 1.6 \mu\text{g NAG equivalents h}^{-1} \text{mg}^{-1}$ protein).

pH and calcium dependence of chitinolytic activity.—Chitinolysis is fully functional even with the pH at less than 2 (Fig. 3). There appears to be a bimodal pH dependency, although in general chitinolytic activity declines as neutrality is approached. The chitinolytic activity is enhanced nearly threefold when calcium is added to the reaction mixture (Fig. 4).

Both the acid extracts and the phosphate-buffered extracts from White-chinned Petrels and Leach's Storm-Petrels exhibited lysozyme activity. The specific activity varied from 0.322 ± 0.007 units/mg protein for the acid extracts to

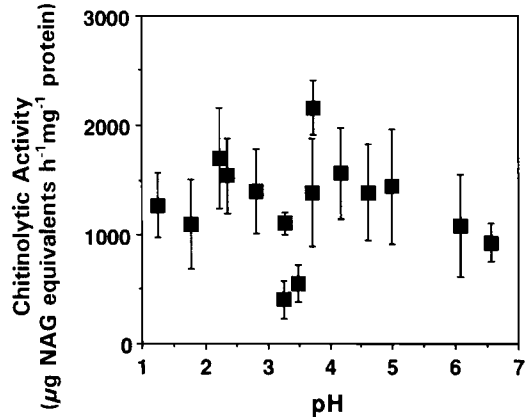


Fig. 3. pH dependence of chitinolytic activity ($\mu\text{g NAG equivalents h}^{-1} \text{mg}^{-1}$ protein; SD indicated) extracted from the proventricular mucosa of White-chinned Petrels. Activity measured at indicated pH's at constant ionic strength using acetyl [^3H] chitin (Molano et al. 1977). Specific activities represented here (and in Fig. 4) are three orders of magnitude greater than those measured with the colorimetric method (Fig. 2), because the tritium assay is more sensitive for two reasons: it detects all chitin oligomers with chain lengths less than 7, not just monomers; and the acetic acid extract assayed has 200- to 300-fold less protein (see Results section for greater detail).

0.011 ± 0.004 units/mg protein for the phosphate-buffered extracts.

DISCUSSION

Exogenous versus endogenous chitinolytic activity in seabirds.—When examining the digestive machinery responsible for assimilation of a particular dietary component, it is important not to underestimate the possible contribution of enzymes present in the food to an item's eventual digestion (Vonk and Western 1984). We believe that the exogenous chitinolytic activity present in the food items contributes substantially to complete chitin digestion, especially in the hydrolysis of chitin oligosaccharides to product free NAG. Both chitinases and chitobiasis have been described in krill (Spindler and Buchholz 1988) and cephalopods (Grisley and Boyle 1990, Jeuniaux 1963). The high levels of chitinolytic activity (as evidenced by production of NAG) we found in stomach contents (of Sooty Albatrosses fed krill or squid; Fig. 2) as opposed to the mucosal tissue of this and all other species studied, suggest dietary origin in these samples.

However, we also have demonstrated endogenous chitinolytic activity in the proventricular tissue of Leach's Storm-Petrels and White-chinned Petrels (see below).

Endogenous chitinase synthesis by vertebrates was first reported by Jeuniaux (1961), and has since been documented in marine fish (Okutani 1966, Rehbein et al. 1986, Seiderer et al. 1987), as well as terrestrial mammals and birds (Cornelius et al. 1975, 1976, Jeuniaux and Cornelius 1978). The synthesis of chitinolytic enzymes (chitinase and chitobiase) is thought to be a primitive, retained characteristic rather than one that has evolved secondarily, and is prevalent among invertebrates (Jeuniaux 1971). Chitinase production is thought not to be inducible in vertebrates that habitually eat food devoid of chitin, and synthesis of this enzyme persists in animals that normally feed on chitinous prey, once chitin has been excluded from their diets (Jeuniaux 1971).

The mucosal chitinolytic activities presented here (see also Place 1990, Place and Jackson 1991) are the first evidence of substantial carbohydrase activity in any seabird. Kerry (1969) reported very low levels of disaccharidase activity in five Southern Ocean seabirds: Rockhopper Penguins, Gentoo Penguins, King Penguins, Brown Skuas (*Stercorarius skua*) and Kelp Gulls (*Larus dominicanus*).

Jeuniaux (1961, 1963) and Jeuniaux and Cornelius (1978) reported chitinase activities in eight bird species ranging from 1,350 to 61,650 μg NAG liberated $\text{h}^{-1} \text{g}^{-1}$ fresh mass of tissue. The highest values that they reported were for the European Starling (*Sturnus vulgaris*), and the lowest were for the chicken (*Gallus gallus*). The main source of the avian chitinase was the proventricular mucosa, with levels decreasing progressively from the gizzard and intestinal lumen contents to the intestinal mucosa (Jeuniaux 1963). These chitinase activities measured by Jeuniaux and his colleagues use added chitobiase in the assay to release NAG for colorimetric determination. Our colorimetric assays of chitinolytic activity reported in Figure 2 do not incorporate added chitobiase. To compare their activities with values reported by us for seabirds, we must compare the seabird activities measured with acetyl [^3H] chitin as substrate. In addition, since our values are reported on a mg-protein basis, a conversion to grams of tissue mass must be made. For the White-chinned Petrels activities reported in Figures 3 and 4,

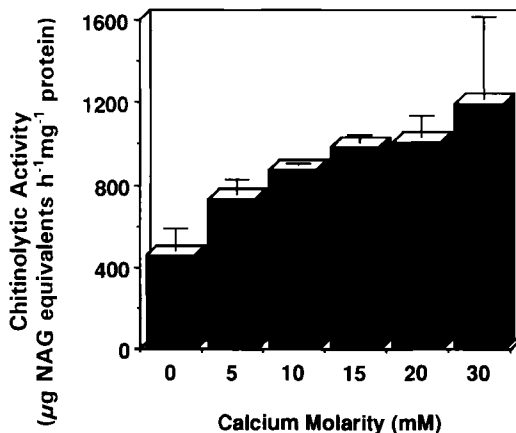


Fig. 4. Calcium dependence of chitinolytic activity (μg NAG equivalents $\text{h}^{-1} \text{mg}^{-1}$ protein; SD indicated) extracted from proventricular mucosa of White-chinned Petrels. Activity measured at pH 2.0 using [^3H] chitin.

the conversion factor is approximately 2.5. Hence, the chitinase activity of White-chinned Petrels is in the range of 2,000 to 5,000 μg NAG $\text{h}^{-1} \text{g}^{-1}$ expressed per gram tissue. Bird gastric chitinases have an optimum pH of 4.7 to 5.4, retaining considerable activity at lower pH values, but showing a sharp decrease in activity at pHs above 6. Seabird stomach pH is between 0.50 and 2.75 (van Dobben 1952, Place et al. 1986), below the optimum levels for the chitinolytic enzymes described by Jeuniaux and Cornelius (1978). However, the pH optimum of the endogenous chitinolytic activity that we report in White-chinned Petrels and Leach's Storm-Petrels is well within the pH range for seabird stomachs.

Role of chitinolytic bacteria in seabird guts.—Chitinolytic bacteria have been implicated in the digestion of chitin by fish (Okutani 1966, Goodrich and Morita 1977), although several studies using antibiotics have shown that fish gut flora may be insignificant in chitin digestion (Danulat 1986). Soucek and Mushin (1970) isolated gram-negative bacteria from the guts of eight species of penguin and two species of skua. Although *Escherichia coli* predominated, 7 of 95 Adélie Penguins and 1 of 10 Rockhopper Penguins sampled had *Enterobacter* in their intestines. *Enterobacter* is known to produce large amounts of chitinase (Monreal and Reese 1969).

In our study, tissue biopsies yielded positive chitinase assay results for Rockhopper and Gentoo penguins, whereas stomach contents sam-

pled within minutes of the biopsies and from the same individual birds yielded negative results. This suggests that the chitinase was secreted by the birds' gastric mucosae, rather than produced by bacteria in the stomach lumen. In addition, we have used tritiated chitin to demonstrate chitinolytic activity in extracts from the mucosa of Leach's Storm-Petrels and White-chinned Petrels. Finally, all tissue samples were thoroughly rinsed before freezing, and assays were carried out in the presence of toluene, which inhibits bacterial activity. Overall, we attribute a substantial component of initial chitin breakdown (i.e. cleavage of chain into smaller fragments) to the endogenous gastric activities possessed by the seabirds.

Chitinolytic activity in relation to natural diet.—Chitinase activity has been demonstrated in seven species of raptors (Leprince et al. 1979) and in insectivorous birds, but is absent in two granivores, the domestic pigeon (*Columba livia*) and the African Grey Parrot (*Psittacus erithacus*; Jeuniaux 1961, 1963, Jeuniaux and Cornelius 1978). Of the five species of Southern Ocean seabirds that we sampled, all but the King Penguin showed chitinolytic activity in the gastric mucosa. Among seabirds, secretion of this enzyme appears widespread irrespective of the proportion of crustaceans in the natural diet, but further study of species that do not eat crustaceans would be instructive.

Why digest chitin?—There are few published data on the digestibility of chitin in birds. Japanese Nightingales (i.e. Red-billed Leiothrix, *Leiothrix lutea*) digest 56.8% of the chitin in mealworm *Tenebrio molitor* larvae, and 15-day-old chickens digest 23.5 to 31.7% of purified shrimp chitin added to their normal diets (Jeuniaux and Cornelius 1978). Digestibilities as high as 92% have been reported for adult and juvenile chickens fed chitin and chitosan dietary supplements (Hirano et al. 1984). In our study, most Southern Ocean species exhibit digestibilities that agree closely with the values for Leach's Storm-Petrels that were estimated using purified chitin and a double-isotope technique to circumvent biases arising from retention of exoskeleton. However, digestibility estimates for King Penguins and Sooty Albatross chicks are higher than those for all seabirds, and may be artifacts resulting from the extended gastric retention of undigested krill exoskeleton which has been observed in these species (Jackson and Cooper 1988, Jackson 1992). Sooty Albatross chicks also

exhibit prolonged retention of neutral lipids when compared with adult conspecifics fed the same diet (Jackson and Place 1990). Retention of exoskeletal material for longer than the duration of the fecal-collection period might have resulted in underestimation of chitin content of the feces and, consequently, overestimation of digestibilities. However, apparent chitin digestibilities as high as 90% have been recorded for chickens (Hirano et al. 1984), and equally high values for these two seabird species are possible.

The seabirds that we studied are able to hydrolyze a substantial proportion and to retain nearly one-half of the chitin they ingest. Chitin itself may not be a significant source of energy to seabirds, because crustaceans such as krill contain a small proportion of chitin (between 2.1 and 2.9%, dry mass; Clarke 1980, this study). Even assuming that chitin has the same energy value (17.9 kJ g^{-1} dry mass) as whole krill (Karasov 1990), an overestimate, seabirds able to digest 40 to 90% of ingested chitin and fully assimilate all digestive products would derive a maximum of between 1.2 and 2.7% of their total energy gain from the chitin fraction of each meal. Our data on absorption of NAG and glucosamine suggest that intestinal absorption of the products of chitinolysis is far from complete in at least one planktivorous seabird species, further reducing the likelihood that chitin is of significant energetic value to seabirds. Chitinolysis probably increases the rapidity of mechanical breakdown of the crustacean exoskeleton, thereby facilitating digestion of soft prey tissues in shorter time than otherwise would be possible. Also, cleavage of the chitin polymer in the exoskeleton would allow easier access by proteolytic enzymes to associated proteins in the cuticle. Increased gastric evacuation rates may be the major benefit of chitinase secretion accrued by seabirds, given the energetic advantages of rapid passage of digesta in all avian species (Sibly 1981).

Chitin and its breakdown products also may influence the gut flora of seabirds. As a dietary supplement, chitin improves the utilization efficiency of whey by chickens (Spreen et al. 1984), apparently by enhancing intestinal growth of lactase-producing *Bifidobacteria* spp.

Despite these benefits of chitin digestion, absorption of the products of chitinolysis may carry a cost; juvenile chickens fed chitin and chitosan supplements gained mass at a rate

equivalent respectively to only 12 and 77% of the rate shown by birds in a control group fed an otherwise identical diet free of these compounds, despite apparent chitin digestibilities as high as 92% (Hirano et al. 1984). Similarly, the growth rates of rainbow trout (*Salmo gairdneri*) smelts fed diets containing 4, 10 and 25% chitin were significantly lower over a 12-week period than the growth rates of fish fed a diet containing 25% starch; however, high chitinase and chitobiase activities were found in the guts of these fish (Lindsay et al. 1984). These reduced growth rates may have resulted from nutrient or energy deprivation arising from "dilution" of the digestible components in the diet by chitin, but the high levels of chitinolytic enzymes in trout guts and high apparent chitin digestibilities in chickens suggest that the energy in chitin is available for assimilation in both species. Alternatively, absorption of the end products of chitin digestion may have an inhibitory effect on growth. In mammals, amino sugars like NAG and Gln are respired much less rapidly than is D-glucose (Kohn et al. 1962). Consideration of chitobiase activities in avian guts may be illuminating here; recall that this enzyme accomplishes the final breakdown of NAG dimers and trimers into absorbable monomers.

Chitobiase has a predominantly luminal distribution in the gizzards and intestines of five bird species studied by Jeuniaux (1963), with activity levels ranging from 0 to 200 $\mu\text{g NAG h}^{-1} \text{g}^{-1}$ tissue. Jeuniaux (1963) considered the highest values that he reported (1,160 $\mu\text{g NAG h}^{-1} \text{g}^{-1}$ tissue in the cecal lumen of the chicken) to be of bacterial origin. On the basis of the low chitobiase concentrations in all species that they studied, Jeuniaux and Cornelius (1978) concluded that the extent of metabolic utilization of the products of chitin hydrolysis by vertebrates is uncertain. Their conclusion is highly significant in view of possible growth-related costs of chitin end-product absorption.

Furthermore, our results suggest low absorption efficiencies of the products of chitinolysis, D-glucosamine in particular, reinforcing the conclusion that the major role of chitinase in seabirds is its acceleration of exoskeleton breakdown rather than the freeing of assimilable nutrients in the chitin polymer. Low absorption efficiencies are consistent with earlier measurements on the rate of intestinal absorption of carbohydrates from isolated intestinal segments of the rat (*Rattus norvegicus*; Crane 1968). The

mean relative absorption for NAG as a percentage of glucose absorption was 34%, and for glucosamine (Gln) was 22%. Similar findings were obtained when rats were fed molar solutions of NAG and Gln (Capps et al. 1966). More recently, Karasov (1989) documented low NAG uptake rates in mice (*Mus musculus*). Is this inefficiency adaptive for a species ingesting chitin? Further comparative studies using equicaloric diets differing in chitin content are needed to assess the influence of the end products of chitinolysis on the growth of juvenile birds.

Do birds digest chitin more efficiently than do mammals?—Among mammals, gastric chitinases are found in omnivorous and insectivorous species belonging to the orders Insectivora, Chiroptera, Carnivora, Rodentia and primates, and pancreatic chitinases have been found in the pig (*Sus domesticus*; Jeuniaux and Cornelius 1978). There is a close relationship between chitinase secretion and the normal diet of the species considered, and the ability to secrete chitinase is not inducible by manipulation of the diet (Jeuniaux and Cornelius 1978). Chitinase activity in the gastric mucosa is generally lower in mammals than in birds (Jeuniaux 1963, Jeuniaux and Cornelius 1978, LePrince et al. 1979). Few data are available on chitin digestibilities among mammals: values for mice are $19.4 \pm 27.6\%$ (Jeuniaux and Cornelius 1978), and for musk shrews (*Suncus murinus*) and pygmy hedgehog tenrecs (*Echinops telfairi*), respectively, are 1.8% and 19.8% (Allen 1989), substantially lower than both the overall mean value of 56.8 ± 23.2 that we report for seabirds, and values among terrestrial birds (Jeuniaux and Cornelius 1978).

Further considerations and caveats.—We clearly have documented that seabirds have the capacity to digest a considerable portion of the chitin in the exoskeletons of their prey. Our study yields no evidence that seabirds actually assimilate the carbon and nitrogen thus released; we merely demonstrate that a significant portion of dietary chitin is retained within the gut, and that much of the biochemical machinery exists for assimilation of the breakdown products of this polymer by seabirds. It is possible that a significant portion of NAG is utilized by gut microflora for cell-wall synthesis and/or as a metabolic fuel source (e.g. Capps et al. 1966). Until a species is fed uniformly labeled carbon-14 chitin, and respired carbon-14 is recovered in addition to the label deposited in tissue, there

is no evidence for chitin assimilation by an animal.

Further research into the chitinolytic activity extracted from the proventriculus of Leach's Storm-Petrels and White-chinned Petrels also is warranted. Some portion of this chitinolytic activity may be attributable to a lysozyme. Recruitment of lysozyme as a bacteriolytic enzyme in the stomachs of both ruminants and colobine monkeys is well documented (Stewart et al. 1987), as is the chitinolytic activity of some lysozymes (Jollés and Jollés 1984). We propose that seabirds may have recruited lysozyme as a gastric digestive enzyme in addition to its normal bacteriolytic function. Current efforts to purify and characterize this activity will provide a test for this hypothesis.

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