of the vocal tract or via the interclavicular air sac that surrounds the syrinx and ramifies through the space between the two bronchi. The coupling may also be structural, for example, involving the cartilaginous pessulus, to which membranes on both sides of the syrinx are attached. A more thorough understanding of syringeal mechanics is necessary, both to characterize the physical basis of this coupling and to assess the extent to which bilateral interactions may be a common property of syringeal operation.

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5. Acoustic evidence typically adduced in support of the two-voice hypothesis is the simultaneous presence of two (or more) nonharmonically related frequency components in a bird sound. An important caveat apparently overlooked by most authors is that many physical oscillation systems generate nonharmonic overtones. Thus, reports of two "voices" in bird sounds must be treated cautiously if unsupported by physiological experimentation.
6. Based on an examination of 1138 syllables, included in 255 calls recorded from 23 individual birds in the field (range, 2 to 22; median, 10 calls per bird; range, 10 to 119; median, 42 "dees" per bird). A fundamental frequency was considered ambiguous if its calculated value deviated from the predicted value by at least 50 Hz (representing, for example, a 25 percent deviation for a predicted fundamental of 400 Hz).
7. In different individuals, and to a lesser extent in different calls of the same individual, the frequencies of these components may vary from 1300 to 1700 Hz and 1700 to 2100 Hz, respectively. However, the two components are always spaced at roughly 350 to 450 Hz, and the spacing of higher frequency components corresponds to the spacing between these two spectral peaks. The frequency values of 1600 and 2000 Hz are realistic and convenient estimates to use for the sake of argument.
8. Either the left or right tracheosyringealis branch was cut approximately 2 mm below the main trunk of the 12th cranial (hypoglossal) nerve. Sectioning this branch has the same effect on syringeal function as cutting the roots above the anastomosis of the 12th and 10th cranial nerves (*3*). Postoperative vocalizations were recorded 2 or 3 days after surgery. Surgical histories and further procedural details may be found in S. Nowicki [thesis, Cornell University (1985)].
9. The frequency components (in hertz) marked in Fig. 2 are: (A) 1 = 1500, 2 = 1875; (E) arrow = 1500; (B) 1 = 1525, 2 = 1950; (F) arrow = 1500; (C) 1 = 1525, 2 = 1925; (G) arrow = 1900; (D) 1 = 1425, 2 = 1900; (H) arrow = 1925. Average values and standard deviations of postoperative fundamental frequencies for these four birds (n = 10 "dees" per bird) are: 1498 ± 51 Hz, 1453 ± 32 Hz (right nerve sectioned, corresponding to 2, E and F); 1878 ± 25 Hz, 1943 ± 44 Hz (left nerve sectioned, 2, G and H). Other spectral components, not harmonically related to the fundamental frequency, sometimes emerge from the noise in postoperative signals, but these components are of lower amplitude and vary extensively among different signals produced by the same bird. The overall syllable syntax of calls (for example, Fig. 1A) remains unchanged in postoperative birds.
10. If $V_1(t) = A_1\cos f_1t + A_2\cos 2f_1t \ldots + A_n\cos nf_1t$ and $V_2(t) = B_1\cos f_2t + B_2\cos 2f_2t \ldots + B_m\cos mf_2t$, where n and m are integers, their multiplication gives rise to the general terms
$$\frac{A_nB_m}{2}\cos(mf_2 + nf_1)t + \frac{A_nB_m}{2}\cos(mf_2 - nf_1)t$$
(The factor 2 π has been omitted from the foregoing terms for clarity.)
11. The sum and difference frequencies generated in the range of interest are: 400, 1200, 1600, 2000, 2400, 2800, 3200, 3600, 4000, 4400, 4800, 5200, 5600, 6000, 6800, 7200, and 7600 Hz, corresponding to the components observed in a normal "dee" syllable with component 1 = 1600 Hz and component 2 = 2000 Hz. Each modulation product also has associated with it a theoretical value for the amplitude of that component, but these predicted values assume no frequency-dependency in the coupling coefficient and are thus of limited value.
12. Field observations and experimentation suggest that the resulting 400 Hz periodicity of the chickadee's "dee" syllable, as reflected in the frequency interval between these components, is an important carrier of information, modifiable through learning, concerning social affiliation [D. L. Mammen and S. Nowicki, *Behav. Ecol. Sociobiol.* **9**, 179 (1981); S. Nowicki, *ibid.* **12**, 317 (1983)].
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Immunization with an Isolate-Common Surface Protein Protects Cattle Against Anaplasmosis

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Hemoparasitic diseases are endemic in half the world's livestock production areas and are the greatest obstacle to improved meat, milk, and fiber production in the Third World. The most prevalent of these diseases, anaplasmosis, occurs throughout tropical and subtropical regions and is responsible for 50,000 to 100,000 cattle deaths annually in the United States alone. Despite its prevalence and the severity of the losses, an effective immunoprophylaxis for anaplasmosis has not been developed. A neutralization-sensitive epitope on a surface protein with a molecular weight of 105,000 (Am 105) of the causative rickettsia *Anaplasma marginale* was identified by monoclonal antibody inhibition of infectivity. This epitope was determined to be common to eight isolates with antigenic, morphologic, and protein structural differences. Cattle immunized with Am 105 purified by immunoaffinity chromatography were protected against challenge with virulent *Anaplasma marginale*. The identification of Am 105 as bearing isolate-common epitopes capable of inducing protection in immunized cattle provides the basis for the development of an effective subunit vaccine for bovine anaplasmosis.

RICKETTSIAL INFECTIONS FREquently escape early diagnosis and rapidly progress to cause severe illness and death. Despite the need for immunoprophylaxis to prevent infections, there has been little progress in developing effective rickettsial vaccines. This lack of progress results, in part, from the complexity of the organism's invertebrate vector and vertebrate stages, antigenically variant strains, and their ability to persist in the host. *Anaplasma marginale*, the causative agent of bovine anaplasmosis, demonstrates this complexity and remains without effective immunoprophylaxis. The organism appears as small spherical bodies in the red cells of cattle and is transmitted either directly between cattle by blood-contaminated fomites or via infected ixodid ticks (*1, 2*). After the initial bodies of *A. marginale* have infected the erythrocytes they replicate intracellularly by binary fission and then emerge by exocytosis to infect additional erythrocytes (*3*). A severe anemia develops during acute infection that can result in dramatic weight loss, abortion, and death (*4*). Cattle recovered from acute infection are resistant to challenge with the homologous isolate; however, they remain susceptible to infection from heterologous isolates (*5, 6*). Isolates throughout the United States have antigenic (*7*), morphologic (*8*), protein structural (*9*), and virulence (*10*) differences. Clearly, a vaccine for anaplasmosis would be required to provide protection against all the isolate types in a given livestock region.

Our approach to development of an effective vaccine is to identify one or more isolate-common surface antigens capable of inducing neutralizing antibody and use the isolated immunogen or its cloned, expressed replica as a vaccine. We have identified five surface proteins (molecular weight 105K, 86K, 61K, 36K, and 31K) on the initial

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bodies of *A. marginale* that are recognized by neutralizing antibody (*11*). To identify one or more of these proteins as being capable of inducing neutralizing antibody, we produced monoclonal antibodies reactive with these surface antigens. We identified 26 hybridomas producing antibodies reactive in indirect immunofluorescence with initial bodies from a Florida isolate but not with noninfected erythrocytes or infected erythrocyte membranes (*12*). These hybridoma supernatants were used to immunoprecipitate the ^{125}I–surface labeled initial bodies. The precipitate was subjected to polyacrylamide gel electrophoresis and autoradiography to identify cell lines producing antibody to surface proteins (Fig. 1A). Two cell lines, ANA 15D2 and ANA 22B1 [both immunoglobulin G3's (IgG3's)] were identified that produced antibodies to Am 105. The evidence that Am 105 is a protein on the initial body and is not of erythrocyte origin includes nonreactivity of ANA 15D2, ANA 22B1, or rabbit antibodies to Am 105 with noninfected erythrocytes or infected erythrocyte membranes and failure of these antibodies to immunoprecipitate ^{125}I-labeled erythrocyte ghosts (*11*). In addition, ANA 15D2 and ANA 22B1 immunoprecipitated samples of Florida isolate Am 105 that had been metabolically radiolabeled in vitro during short-term erythrocyte culture (Fig. 1B). We previously demonstrated that, during short-term cultures, ^{35}S incorporation occurs exclusively in initial bodies (*9*).

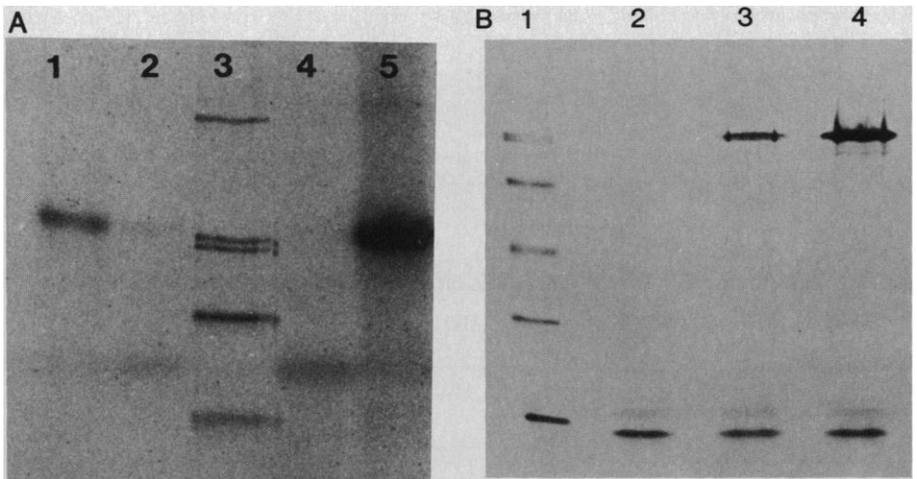

Fig. 1. (A) Monoclonal antibodies ANA 15D2 and ANA 22B1 recognizing ^{125}I-labeled Am 105. Autoradiograph of sodium dodecyl sulfate (SDS)–polyacrylamide gels of radioiodinated *A. marginale* (Florida isolate) initial body surface proteins immunoprecipitated with ANA 15D2 (lane 1), ANA 22B1 (lane 5), and IFA-positive monoclonal antibodies unreactive with Am 105 (lanes 2 and 4). Carbon-14 molecular weight standards (lane 3) are 200K, 92.5K, 69K, 46K, 30K, and 14.3K. Initial bodies were purified, surface-radioiodinated with lactoperoxidase, and disrupted with 1 percent Nonidet P-40 and 0.1 percent SDS (*11*). The radiolabeled antigen was immunoprecipitated with 50 μl of hybridoma supernatant, followed by 10 μl of rabbit antibody to mouse immunoglobulin and protein A–bearing *Staphylococcus aureus*. Identification of the immunoprecipitated antigen was done by autoradiography after SDS–polyacrylamide gel electrophoresis in a 7.5 to 17.5 percent gradient under reducing conditions (*11*). (B) Monoclonal antibodies ANA 15D2 and ANA 22B1 recognizing ^{35}S-labeled Am 105. Fluorograph of SDS–polyacrylamide gels of ^{35}S metabolically labeled initial body proteins immunoprecipitated with ANA 15D2 (lane 4), ANA 22B1 (lane 3), and unrelated monoclonal antibody TRYP 22A1 (lane 2). Carbon-14 molecular weight standards (lane 1) are 92.5K, 69K, 46K, 30K, and 14.3K. *Anaplasma marginale* (Florida isolate)–infected erythrocytes were labeled with [^{35}S]methionine during short-term culture (*9*). The [^{35}S]methionine-labeled initial bodies were disrupted, immunoprecipitated, and subjected to electrophoresis like the ^{125}I-labeled initial bodies described in (A).

To test for neutralization of initial bodies with these two Am 105 monoclonal antibodies, graded numbers of Florida isolate initial bodies (10^7 to 10^{10}) were incubated with a constant amount of antibody (pooled ANA 15D2 and ANA 22B1 ascitic fluids) and injected into splenectomized calves. Am 105 monoclonal antibodies completely neutralized the infectivity of 10^7 initial bodies. There was partial neutralization of the infectivity of 10^8, 10^9, and 10^{10} initial bodies, as judged by the significant prolongation of the prepatent period compared with that in calves given identical numbers of initial bodies incubated with ascitic fluid containing an unrelated monoclonal antibody (Table 1). The complete neutralization at 10^7 and the significant but incomplete protection at 10^8 initial bodies with passive antibody neutralization are similar to findings reported with *Plasmodium* neutralizations (*13*).

The effective use of Am 105 as a protec-

Table 1. Neutralization of infectivity of graded numbers of initial bodies by monoclonal antibodies (ANA 15D2 and ANA 22B1). Mice [BALB/c × B10A (3r)] were injected intraperitoneally with 1.0 ml of Pristane and injected again 1 week later with 2×10^6 to 3×10^6 double-cloned hybridoma cells of lines ANA 15D2, ANA 22B1, or TRYP 1E1. Ascitic fluid was withdrawn, centrifuged to pellet debris, passed over a glass wool column, and heat-inactivated for 30 minutes at 56°C. The IFA titer was 1:16,000 with ANA 15D2 or ANA 22B1; TRYP 1E1 was unreactive. Initial bodies were purified from *A. marginale* (Florida isolate)–infected erythrocytes (*11*) and resuspended in 1.0 ml of RPMI 1640 medium (2 m*M* L-glutamine and 25 m*M* Hepes). The initial bodies were added to 1.0 ml of TRYP 1E1 ascitic fluid or 1.0 ml of a 1:1 mixture of ANA 15D2 and ANA 22B1 ascitic fluid. The initial body–ascitic fluid suspension was briefly vortexed, incubated for 45 minutes at room temperature, and injected into the left semitendinosus muscle of each calf. Blood samples were collected daily for 75 days after inoculation in order to determine packed cell volume (PCV) and parasitemia (1000 erythrocytes counted). The mean number of days between inoculation and 1 percent parasitemia were calculated for all infected cattle in each challenge group. ND, significance not determined.

Monoclonal antibody	Number infected/challenged				Mean number of days between inoculation and 1% parasitemia (range)			
	10^7	10^8	10^9	10^{10}	10^7	10^8	10^9	10^{10}
ANA 15D2/22B1	0/4	4/4	4/4	9/10	>75*	37 (36 to 38)	35 (33 to 39)	33 (29 to 37)
TRYP 1E1	3/3	3/3	3/3	7/7	34 (34 to 36)	30 (32 to 36)	28 (27 to 29)	25 (23 to 28)
P	ND	ND	ND	ND	ND	≤0.01	≤0.01	≤0.01

*Negative at 75 days.

Table 2. Protection against anaplasmosis in Am 105–immunized cattle. Calves were immunized with 100 μg of Am 105 or 100 μg of ovalbumin emulsified in complete Freund's adjuvant and boosted threefold at 2-week intervals with 100 μg of antigen in incomplete Freund's adjuvant. The Am 105 vaccinates developed a titer of 10^4 to 10^5, as determined by radioimmunoassay with ^{125}I-labeled Am 105 (*16*). The calves and four nonimmunized control calves were challenged by intramuscular injection with 10^8 purified *A. marginale* (Florida isolate) initial bodies. Calves were clinically examined each day and PCV and parasitemia were determined at 100 days after inoculation. The mean number of days between inoculation and 1 percent parasitemia was calculated for all infected calves in each group. *P* values were calculated with the pooled *t*-test to compare the responses of Am 105– and ovalbumin-immunized cattle.

Group	Number infected/ challenged	Mean number of days between inoculation and 1% parasitemia (range)	Mean peak parasitemia	Mean low PCV*
Nonimmunized	4/4	29 (26 to 31)	4.2 (2.8 to 6.4)	23 (18 to 26)
Ovalbumin	5/5	33 (31 to 35)	5.4 (3.4 to 11.2)	24.5 (19 to 27)
Am 105	3/5†	†	<0.01, <0.01‡	31‡ (29.5 to 33)
P	ND	$P \leq 0.01$	$P \leq 0.01$	$P \leq 0.01$

*Prechallenge PCV's ranged from 29.5 to 37. †Two of the five Am 105–immunized calves did not become infected and remained hematologically normal. The three Am 105 vaccinates that became infected developed a transient parasitemia of <0.01 percent on day 39 and did not develop clinical disease. ‡These hematologic values are based on the three Am 105 vaccinates with mild infection to demonstrate protection against disease afforded by immunization with Am 105.

tive immunogen to prevent bovine anaplasmosis requires that the determinants recognized by the neutralizing monoclonal antibodies be common to all isolates in a given region. (ANA 15D2 and ANA 22B1 may recognize the same or overlapping determinants, as they completely inhibit binding of each other to ^{125}I-labeled Am 105 in a competition radioimmunoassay and individually neutralize initial body infectivity.) We previously demonstrated similarities and differences in protein and antigenic composition among various isolates of *A. marginale* (*7, 9*); others have reported differences in morphology and ability to cross-protect (*8, 10*). We examined eight isolates from Florida; Okanogan, Washington; south Idaho; north Texas; Clarkston, Washington; Virginia; Kansas; and Oklahoma for the presence of determinants recognized by ANA 15D2 and ANA 22B1 by using indirect immunofluorescence on acetone-fixed blood smears. The determinants were present on 100 percent of the initial bodies (as determined by comparison with initial bodies, stained with Wright's, in an adjacent section of the smear) in all eight isolates. In addition, the determinants were present at all stages of a primary acute infection, from 1 percent parasitemia through peak parasitemia with hemolytic crisis.

To test whether Am 105 could induce protection in immunized cattle, we isolated Am 105 by immunoaffinity chromatography on an ANA 15D2–Sepharose 4B column (Fig. 2). Calves immunized with this purified protein developed high titers of antibody to Am 105 (Table 2) and were significantly protected from challenge with 10^8 Florida isolate initial bodies compared with ovalbumin-immunized control or nonimmunized control calves. Two Am 105–immunized calves did not show any parasitized erythrocytes in blood smears stained with Wright's, while the other three calves showed 0.01 percent parasitemia. Furthermore, the three Am 105–immunized calves that were transiently infected had significantly prolonged prepatent periods and did not show clinical disease, as judged from hematological measures or clinical appearance, whereas all control calves developed clinical disease (Table 2).

Our challenge of 10^8 initial bodies greatly exceeds both the typical and minimum infective dose of initial bodies in the field (*1, 14*); the effect on Am 105–immunized calves of repeated challenges in the field with multiple isolates remains to be determined. Current whole-organism vaccines bearing Am 105 neutralization-sensitive epitopes are poorly cross-protective (*6*); however, immunization with Am 105 devoid of extraneous antigens should direct the immune response to the critical epitopes. Determination of the ability of Am 105 immunization to induce cross-protection is a priority in development of a subunit vaccine for anaplasmosis. The presence of critical Am 105 epitopes on multiple isolates indicates that cross-isolate protection is feasible.

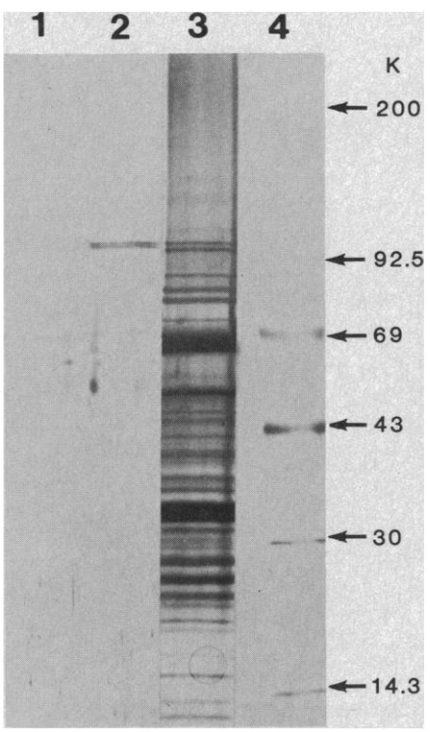

Fig. 2. Purification of Am 105 by immunoaffinity chromatography. Silver-stained SDS–polyacrylamide gel (*15*) of *A. marginale* (Florida isolate) detergent extract is shown before (lane 3) and after (lane 2) chromatography on ANA 15D2–Sepharose 4B. Molecular weight standards (lane 4) are indicated; lane 1 was not loaded to control for silver-stained artifacts. Initial bodies (10^{12}) were purified from parasitized erythrocytes, disrupted in a buffer containing 1.0 percent Nonidet P-40, 0.1 percent SDS, and proteolytic inhibitors (*11*), and applied to a 1.0-ml column of ANA 15D2–Sepharose 4B (10 mg of purified IgG3 per milliliter of Sepharose) at 25 ml/hour. Unbound proteins were removed by washing in 20 m*M* tris-HCl buffer (*p*H 7.6) containing 5 m*M* EDTA and 0.1*M* NaCl, followed by specific elution of Am 105 with tris buffer (*p*H 8.0) containing 0.5 percent deoxycholate and 2*M* KSCN. The eluted protein was dialyzed for 72 hours against PBS (*p*H 8.0) to remove the KSCN and deoxycholate. Extracts of noninfected erythrocytes did not bind to the ANA 15D2–Sepharose 4B.

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Hemocyanin Respiratory Pigment in Bivalve Mollusks

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Hemocyanins, high molecular weight oxygen-binding proteins, were identified in two species of protobranch bivalve mollusks, *Acila castrensis* and *Yoldia limatula*. Although hemocyanins have been reported in chitons, gastropods, and cephalopods, they have not been observed in the Class Bivalvia. In *A. castrensis* the dissociation products of hemocyanin, characterized by gel electrophoresis, had a subunit molecular weight of approximately 250K. Negatively stained preparations of extracted hemocyanin formed protein aggregates in the shape of cylinders measuring 35 by 38 nanometers. X-ray microanalysis of hemocyanin aggregates in thin sections of *Y. limatula* demonstrated the presence of copper in the molecules. The discovery of hemocyanin in the protobranchs reinforces the primitive nature of the taxon and is further evidence that the major molluscan classes have a common ancestry.

HEMOCYANINS ARE HIGH MOLECULAR weight, copper-containing pigments in the hemolymph of mollusks (*1–4*) and arthropods (*5*). In mollusks, hemocyanins have been found in chitons, gastropods, and cephalopods but to our knowledge have not been reported in bivalves. However, while investigating the ultrastructure of protobranch pericardial glands (*6*), we noted that the hemolymph contained structures that resembled characteristic molluscan hemocyanin molecules (*7*). Further studies were undertaken to characterize the respiratory protein in two protobranch species, *Acila castrensis* (Hinds) and *Yoldia limatula* (Say). We examined the molecular weight, size, and shape of the aggregates and tested for the presence of copper in the protein.

Preparations enriched in the respiratory pigment were obtained from *A. castrensis* and *Y. limatula* by a modification of the procedure of Waxman (*8*). Pellets from the final centrifugation were resuspended in collecting fluid and examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*9*). Polypeptides of *A. castrensis* hemocyanin comigrated with those of the hemocyanin of the limpet, *Megathura crenulata*, at an estimated molecular weight of 250K (Fig. 1). The *Y. limatula* preparation had two prominent polypeptides of approximately 250K and 200K, the latter possibly being a breakdown product of hemocyanin or of an unrelated polypeptide (Fig. 1).

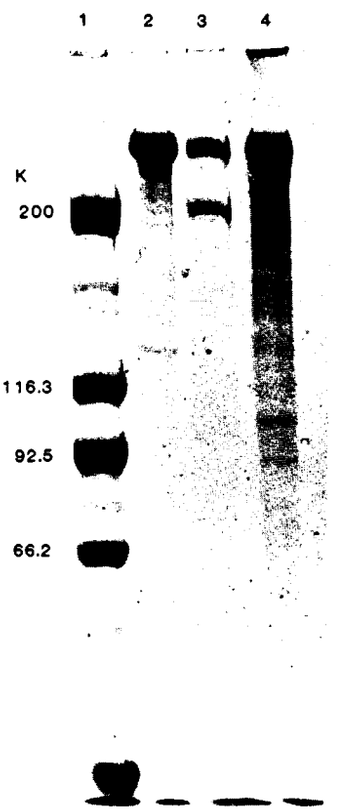

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of hemocyanin from *A. castrensis* (lane 2) and *Y. limatula* (lane 3). For comparison, limpet hemocyanin (Sigma) is shown in lane 4. Lane 1 contains molecular weight markers: myosin (200K), β-galactosidase (116K), phosphorylase B (92.5K), bovine serum albumin (66K), and ovalbumin (45K) (Pharmacia).

Samples of the hemocyanin-enriched preparations of *A. castrensis* and *Y. limatula* used to determine molecular weights also were negatively stained and viewed with a transmission electron microscope. The hemocyanin of *A. castrensis* (Fig. 2A) showed the characteristic cylindrical configuration of the macromolecular aggregates, and each aggregate was made up of six tiers. Hemocyanin of the heart hemolymph of *A. castrensis* (Fig. 2B) also showed the characteristic shape of the protein. The cylinders appeared circular in end view and rectangular in side view; they averaged 35 nm in height and 38 nm in width ($n = 5$). The negatively stained hemocyanin from the preparation extracted from *Y. limatula* was not as well defined as that from *A. castrensis*; however, the cylindrical configuration of the molecular aggregate was again evident (Fig. 3C).

Thin sections of the auricle of *Y. limatula* (Fig. 3A) were used for x-ray microanalysis (*10*) to establish the presence of copper in the aggregates. The microanalysis results (Fig. 3B) indicate the presence of significant energy peaks of both the K- and L-shell electrons of copper. Analysis from nearby nonhemal tissues revealed no significant peaks for copper. Furthermore, during the procedures to isolate hemocyanin from *A. castrensis* (*8*), it was observed that the hemocyanin pellet was bright blue. As noted by Mangum (*3*), oxygenated hemocyanin molecules appear blue because of the presence of copper atoms.

The uniqueness of hemocyanins in arthropods and mollusks has led to numerous investigations into the structure and function of the proteins and speculation as to their evolutionary importance in mollusks. Investigators have searched for hemocyanins

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