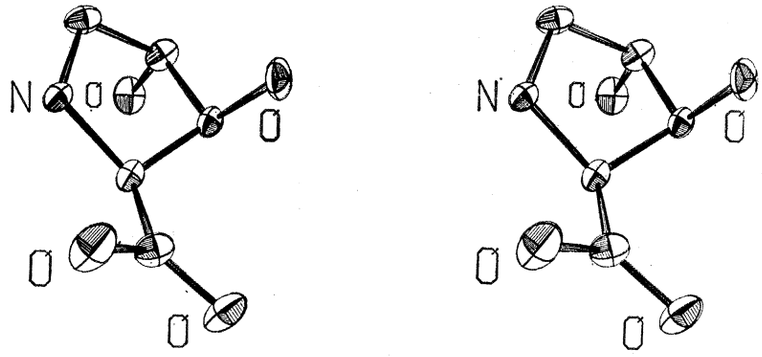
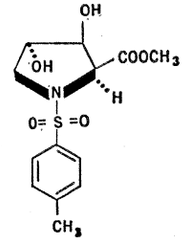


Fig. 4. Computer-made stereodrawing of the configuration of natural 2,3-*cis*-3,4-*trans*-dihydroxy-L-proline as determined by x-ray analysis. The picture should be seen with a three-dimensional viewer for printed stereophotographs (commercially available, Stereo-Magniscope, Inc., Elmhurst, N.Y.).

tion from an acicular prism (0.06 by 0.06 mm cross section) with the multiple-film, equi-inclination Weissenberg technique. Although long exposures were required, because the crystal was very small, the scattering extended to the edge of the Cu sphere. The space group is orthorhombic, $P2_12_12_1$, with cell parameters $a = 8.38$ Å, $b = 8.43$ Å, $c = 8.57$ Å; there are four molecules in the unit cell. The symbolic addition procedure (*3*) for noncentrosymmetric crystals was used to determine the phases of the strong and moderately strong reflections. The initial E-map computed with 214 terms with $|E| > 1.0$ for which phases had been determined revealed the structure of the molecule (Fig. 2). Hydrogen atoms were located in a difference map and the least-squares refinement resulted in an R-factor of 5.8 percent.

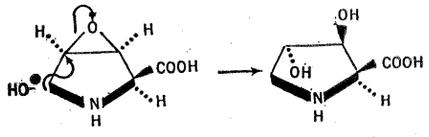
The x-ray diffraction results confirm the structural formula and establish the conformation of the molecule. The bond distances and angles are shown in Fig. 3. Atoms C(4), C(5), N, and C(2) of the five-membered ring lie in a plane, to within ± 0.009 Å, whereas C(3) is 0.60 Å above the plane. This conformation differs from that of L-proline (*4*) and of natural (*trans*) 4-hydroxy-L-proline (*5*), where it is atom C(4) which is out of the plane in the five-membered ring. The carboxyl group is equatorial while each of the two hydroxyl groups is axial to the ring. The N atom is 0.23 Å out of the plane containing the carboxyl group and the α-carbon (Fig. 4). The molecule exists as a zwitterion with an extra proton on the N atom and a negative charge on the carboxyl group. Four different hydrogen bonds, two NH · · · O and two OH · · · O, bind the molecules in the crystal into a tight network, resulting in low thermal factors for the individual atoms.

The closest synthetic analog of the new amino acid is the racemic *trans*-



I

glycol (I) prepared from *N*-tosyl-3,4-dehydro-DL-proline methyl ester (*6*). Such *trans*-glycols (III) are accessible by ring opening of the *cis*-epoxide (II) (*5*). In view of the recent isolation of (arene)oxide intermediates in microsomal metabolism of (aromatic) substrates (*7*) and the existence of a special oxide hydrase (*8*), such a bio-



II III

genetic pathway may now have to be considered, although the biosynthesis of hydroxyproline in collagen (*9*) by proline hydroxylase (*10*) certainly does not involve a dehydroproline precursor (*11*). The new amino acid bears a formal relationship to the antibiotic anisomycin (*12*).

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7 March 1969

Venom Neutralization by Rattlesnake Serum Albumin

Abstract. *The blood serum of the eastern diamond back rattlesnake* (Crotalus adamanteus) *neutralizes lethal doses of* C. adamanteus *venom in mice. The protective capacity of the serum is associated with the serum albumin, rather than the immunoglobulin fraction of the blood. Neither the serum nor its albumin fraction form precipitin bands on immunophoresis against venom.*

Blood serums of several snakes neutralize the venom of other snakes both in vitro and in vivo (*1*). Useful quantitative data on the effectiveness of specific serums are incomplete or entirely lacking, however. Although commercially available horse serum antivenins function as globulin centered antibodies to venom antigens (*2*), there have been no indications that this is the mechanism in venom neutralization by snake serum. As a result of our investigation into the ability of blood serum and serum fractions of the eastern diamond back rattlesnake (*Crotalus adamanteus*) to protect mice from otherwise lethal doses of *C. adamanteus* venom, we can now report some quantitative data on dosage effectiveness. In addition, we have found that the high degree of protection observed is not due to antibody-antigen interactions and is associated with the albumin rather than globulin fraction of the serum.

Crotalus adamanteus venom was ob-

tained from Ross Allen's Reptile Institute in Silver Springs, Florida. Blood was taken directly from the aorta of several healthy adult specimens and allowed to clot; the serum was separated by centrifugation. Both serum and venom were stored at —100°C in the dark until used. Periodic tests indicated stability of both preparations under these conditions. Our basic procedure for evaluating neutralization involved injecting 6-week-old Swiss albino male mice with doses of various whole and fractionated serum preparations, followed by injection of an LD_{90} (lethal dose for 90 percent of the animals treated) of venom by the technique of Russel and Eventov (*3*). When quantitative analysis rather than qualitative indications were desired, the resulting data on dosage effect were treated by the method of Litchfield and Wilcoxon (*4*), Abbott's formula being used to deal with natural survival after administration of an LD_{90} venom dose. The LD_{50} and LD_{90} with their accompanying 19/20 confidence limits were calculated in a similar manner for the venom stock used in this work and found to be 1.83 (2.43 to 1.38) and 3.10 (2.31 to 4.15) mg of dry venom per kilogram of body weight, respectively.

The undiluted whole blood serum of certain snakes is toxic to mammals on injection (*5*). Our tests indicated that this is the case for rattlesnake serum. Heating serum at 56°C for 30 minutes destroys the toxicity (*6*), and serum so treated was tolerated without incident in mice at dosages as high as could be practically administered (about 500 mg/kg). Heating was not necessary to "activate" the serum, but rather served a detoxification role as stated by earlier workers (*1*). Initial tests indicated that whole, heat-treated rattlesnake serum markedly protected mice from venom injections (Table 1). The ED_{50} (dose effective in protecting 50 percent of the animals treated) of whole serum, determined from a 40-mouse sample, was 11.17 mg of whole serum (lyophilized dry weight) per kilogram with 19/20 confidence limits of 9.07 to 13.75 mg/kg. Although the available data are only roughly comparable, the *C. adamanteus* serum tested here appears to have a markedly higher neutralization ability against *C. adamanteus* venom than any other snake or horse serum preparations reported (*1*).

Use of Sephadex gel filtration for preliminary isolation of the active serum fraction located the venom neutralization capacity in molecules with a molecular weight of 70,000 to 150,000 (Fig. 1). Analysis by electrophoresis (*7*) indicated albumin as the major component of this fraction.

Table 1. Results of venom neutralization tests. For each fraction, ten mice injected with 28 mg/kg of fraction, followed after a 10-minute interval by LD_{90} of venom. Survival ratio is mice alive after 72 hours. Each fraction tested in untreated and heat-treated (ht) form. Bovine serum albumin (fraction V powder, Nutritional Biochemicals Corp.) used as control against nonspecific albumin protection.

Test fraction	Survival ratio (alive/tested)
Crotalus adamanteus	
Whole serum	8/10
Whole serum, ht	10/10
Sephadex f-II	9/10
Sephadex f-II, ht	10/10
TCA-albumin	9/10
TCA-albumin, ht	10/10
Sephadex f-I	1/10
Sephadex f-I, ht	1/10
Sephadex f-III	1/10
Sephadex f-III, ht	1/10
Bovine	
Albumin	0/10
Albumin, ht	2/10

All serum proteins are precipitated in a solution of 10 percent trichloroacetic acid (TCA), and only albumin and molecules associated with albumin are brought back into organic solvent solution after such precipitation (*8*). Whole rattlesnake serum treated in a similar manner (*9*) yields a product soluble in ethanol composed predominantly of albumin (*10*). This TCA separated albumin fraction offers protection roughly equal to the Sephadex fraction II or unfractionated whole serum (Table 1). Although any of the variously prepared active fractions could be dialyzed against distilled water for 72 hours without loss of effectiveness, the *p*H encountered in ion exchange separation (*11*) partially reduced their protective action. Microzone immunophoresis (*12*) of serum against a concentrated venom sample failed to yield precipitin bands for whole serum or any serum fractions, whereas the expected band between venom and a horse serum antivenin control [Antivenin (Crotalidae) Polyvalent, Wyeth Laboratories] was formed.

Although none of our tests offer positive proof that the observed venom protection is due to the action of serum albumin, the combined evidence leads us to believe that this is so. Such an apparently exclusive role of albumin or albumin-associated molecules or both in venom neutralization by snake serum has not been previously reported. Neither our work nor a general investigation of the chemistry of serum-venom interactions by Crosby (*13*) has shed any light on the mechanism of the observed neutralization. This investigation is concerned only with the effect of rattlesnake serum on venom of the same species. However, other investi-

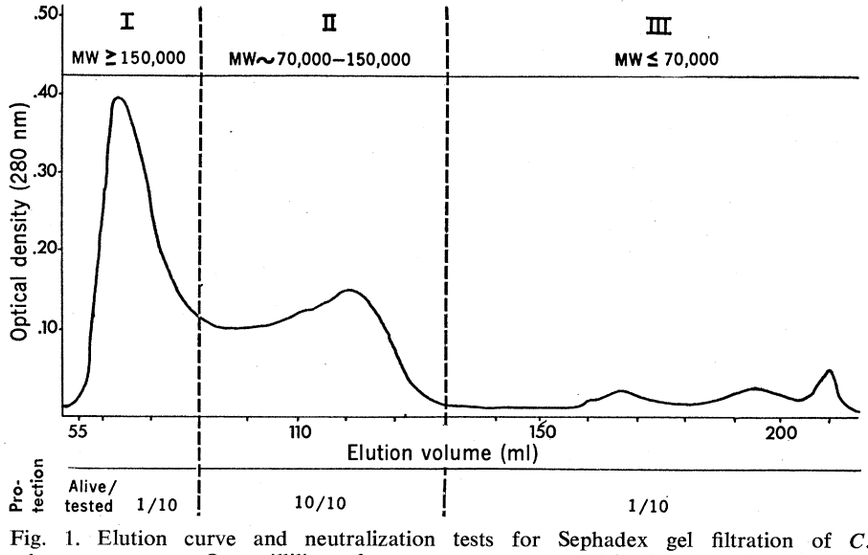

Fig. 1. Elution curve and neutralization tests for Sephadex gel filtration of *C. adamanteus* serum. One milliliter of serum was run on a 45 by 2.3 cm column packed with 40 cm of Sephadex G-100 above and 4 cm of G-75 below. Flow rate was 30 ml per hour in a solvent of 0.006*M* aqueous pyridine. Runs were standardized with blue dextran 2000 (Pharmacia Fine Chemicals, Inc.) and bovine serum albumin. Fractions were lyophilized, reconstituted in an aqueous solution of 0.2 percent trimethylamine, heat treated, and injected at a dose of 28 mg/kg against an LD_{90} of venom as described in the text. Protection data are for 72 hours after injection.

gations of serum neutralization (*1*) and the large amount of data available on the natural resistance of *C. adamanteus* to a wide variety of crotalid venoms (*14*) suggest that at least partial neutralization of other venoms by serum albumin may be expected as well.

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7 March 1969; revised 24 April 1969

Cartilaginous Dermal Scales in Cephalopods

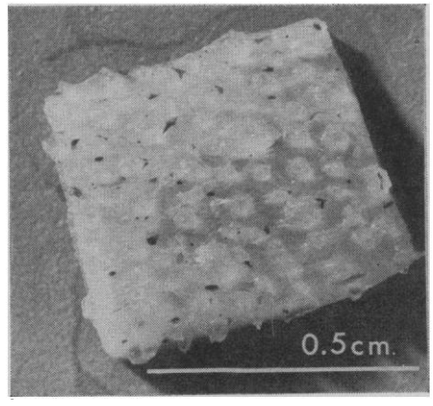
Abstract. *Epidermal scales of the squid* Cranchia scabra *are composed of tissue which stains metachromatically with toluidine blue, and which is similar in appearance to hyaline cartilage.*

The origin of cartilage tissues in the vertebrates is a problem whose solution would contribute significantly to our knowledge and understanding of the earliest stages of vertebrate evolution. Because of the lack of information concerning the existence of cartilage tissues in early vertebrates, there is current disagreement on whether cartilage preceded bone in vertebrate evolution or vice versa and on whether the earliest fishlike vertebrate ancestors had bony or cartilage skeletons, or both. Romer (*1*) first proposed, and recently (*2*) reemphasized, that cartilage probably arose as an embryonic adaptation of early vertebrates, which permitted them to successfully cope with the problem of a rigid endoskeleton in a rapidly growing and transforming embryo. In Romer's view, bone preceded cartilage in the evolution of vertebrates. Denison (*3*) on the other hand, has supported the opposite view. Recently, Moss (*4*) attempted to reconcile these opposing views, as follows—"Romer (1964) forcefully states his position that cartilage did not precede the first appearance of bone (but rather is neotenous); others (Denison, 1963) adopt a contrary point of view. In evaluating this argument, it seems to me that an eclectic position may be held. It seems to be reasonably certain that no one seriously contends that *any* ancestral form possessed a cartilaginous *dermal skeleton*, and similiarly, no one doubts that the earliest Agnatha, as well as their presumed ancestors, possessed a cartilaginous *endoskeleton*. If these two skeletal sites are differentiated, it appears that both points of view may be reconciled." Moss's suggestion has been accepted by many workers as reasonable.

Until recently most biologists believed that cartilage, like bone, was a uniquely vertebrate tissue and was not to be found in the invertebrates (*5*). However, true endoskeletal cartilage does indeed occur in the invertebrates (*6*). Dermal cartilage tissues also exist in the invertebrates. In a study of cephalopods, Clarke (*7*) reported a squid *Lepidoteuthis grimaldii* with dermal scales similar in many ways to the scales of fish. We now report the existence of cartilaginous dermal scales in a squid *Cranchia scabra* from Florida coastal waters (*8*).

The transparent dermal scales of *C. scabra* are easily seen as individual projections (Fig. 1). The scales are made of rounded and ovoid cells (some apparently recently divided) suspended in a relatively homogeneous matrix (Fig. 2). Scale components stain metachromatically with the cationic dye toluidine blue, indicating the probable presence of acid mucopolysaccharides. The histological and histochemical appearances of this tissue are typical for a variety of hyaline cartilages, both invertebrate and vertebrate.

Although this finding does not bear



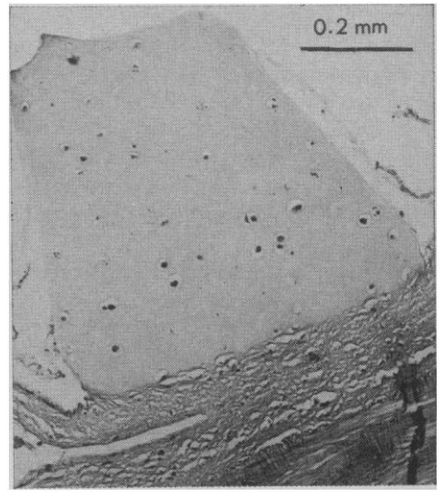


Fig. 1 (left). An excised square of skin of *Cranchia scabra* showing gross appearance of cartilage scales. Fig. 2 (right). Hematoxylin-eosin stained section through a cartilage scale and the connective and muscle tissues beneath. Histologic appearance of the scale is that of typical hyaline cartilage.