Thermal Vent Clam (*Calyptogena magnifica*) Hemoglobin

Abstract. *A heterodont bivalve mollusk* Calyptogena magnifica, *from the East Pacific Rise and the Galápagos Rift hydrothermal vent areas, contains abundant hemoglobin in circulating erythrocytes. No other known heterodont clam contains a circulating intracellular hemoglobin. The hemoglobin is tetrameric and has a relatively high oxygen affinity, which varies only slightly between 2° and 10°C. The presence of hemoglobin in the clam may facilitate the transport of oxygen to be used in chemoautotrophic hydrogen sulfide metabolism.*

One of the more conspicuous animals encountered in recent explorations of the deep-sea hydrothermal vents of the East Pacific Rise and the Galápagos Rift was a large (26 cm long) white clam (*1*). This heterodont bivalve resembles members of the family Vesicomyidae, a group of clams related to the common veneroid clams found in shallow water. The thermal vent clams, which live in depressions between basalt pillows on the ocean floor at depths of 2.5 km, have been identified as *Calyptogena magnifica* (Boss and Turner) (*2*). The environment of these animals is variable, with conditions ranging between that of the vent water (up to 22°C, 350 μM H_2S, *p*H 6.5, and anoxic) and the surrounding water (2°C, no H_2S, *p*H 7.5, and 110 μM O_2) (*1*).

An obvious feature of *C. magnifica* is the red color of its body tissues, which is due to an intracellular hemoglobin present in the hemolymph. The presence of abundant hemoglobin within erythrocytes of this clam provided the opportunity to study the structure and function of an intracellular hemoglobin from a heterodont bivalve—one that, in addition, lives in an unusual and extremely variable environment. Clams used in the following study were collected from the East Pacific Rise during November 1979 and the Galápagos Rift Valley during November and December, 1979.

Blood was removed from the clams either by cutting into the tissues of the animal and collecting the hemolymph or by using a syringe to withdraw blood samples from the clam's circulatory system. The blood cells were stored frozen with their hemoglobin in the oxygenated state. A hemoglobin sample from thawed lysed cells can be separated by gel chromatography on Sephadex G-100 or Bio-Gel A-1.5M into a major hemoglobin peak (fraction I) with an apparent molecular weight (M_r) of 68,000 and a minor hemoglobin peak (fraction II) with a molecular weight of 28,000, which appears as a trailing shoulder to the major peak (Fig. 1a). Chromatography of a dilute sample of fraction I results in a shift of its elution peak to a lower apparent molecular weight (M_r, 42,000). Chromatography of fraction II shows no change in its elution position. Sodium dodecyl sulfate slab gel electrophoresis of fraction I and fraction II shows two protein bands in each fraction, with apparent molecular weights of 14,200 and 13,400 (lanes B and C in Fig. 2a). Electrophoresis of fractions I and II in the same well shows that the two bands migrated together. The two fractions were also indistinguishable on electrophoresis in urea. Thus, *C. magnifica* hemoglobins appear to be tetrameric molecules, and it is likely that the tetramer dissociates into dimers as a result of either methemoglobin formation or having been frozen.

Purified tetrameric carbonylhemoglobin (fraction I) can be resolved into three

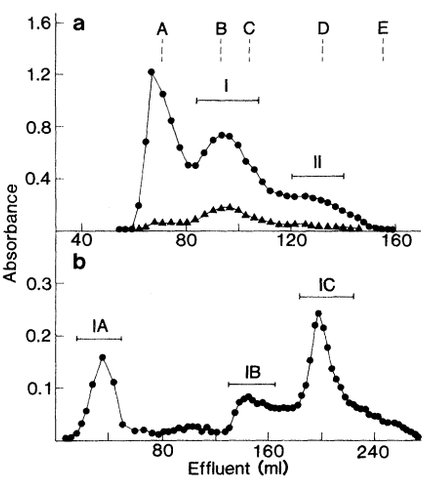

Fig. 1. (a) Chromatography of hemoglobin on a column (1.8 by 78 cm) of Sephadex G-100-120 in equilibrium with 0.05*M* tris-HCl (*p*H 7.5) and 0.1*M* NaCl. The calibrants were *A*, blue dextran; *B*, serum albumin (M_r, 68,000); *C*, ovalbumin (M_r, 43,000); *D*, α-chymotrypsinogen A (M_r, 25,100); and *E*, sperm whale metmyoglobin (M_r, 17,800). The peak associated with the excluded volume showed some turbidity; thus, the measured absorbance of this peak at 540 nm does not correspond to heme. (●) Absorbance at 280 nm; (▲) absorbance at 540 nm. (b) Ion exchange chromatography of the Sephadex-purified sample was carried out on a DEAE-cellulose column (1.8 by 18 cm) in equilibrium with 0.01*M* ammonium bicarbonate. The sample had been dialyzed exhaustively against this solution before chromatography. The elution gradient was 250 ml of 0.01*M* ammonium bicarbonate and 250 ml of 0.01*M* ammonium bicarbonate, 0.5*M* in NaCl. All purification procedures were performed at 0° to 4°C. (●) Absorbance at 422 nm.

major bands by regular gel electrophoresis at *p*H 8.9. This electrophoretic pattern correlates well with the three major peaks, fractions IA, IB, and IC, resolved by DEAE-cellulose chromatography (Fig. 1b). Sodium dodecyl sulfate gel electrophoresis of fraction IA shows one band (M_r, 14,200); fraction IB has two components (M_r, 14,200 and 13,400) in approximately equal amounts as assessed by band staining intensity; and fraction IC has one major component (M_r, 13,400), with a trace of the 14,200 molecular weight material (lanes D to F, in Fig. 2a). Urea gel electrophoresis of the DEAE fractions (Fig. 2b) shows that fraction IA has two major bands in approximately equal concentration (bands 1 and 2), fraction IB has three major components present in equal amounts (bands 1, 2, and 3), and fraction IC has one major component (band 3) with minor contamination of bands 1 and 2. When the fractions IA, IB, and IC were together subjected to electrophoresis in urea, the bands from the different DEAE-cellulose fractions migrated together. Thus, hemoglobin obtained from clams collected from the East Pacific Rise appears to contain three subunits. The distribution of these subunits among the three hemoglobin fractions isolated by DEAE-cellulose chromatography allows the following interpretation. Fraction IA could be an $\alpha_2\beta_2$ tetramer; fraction IB could contain $\alpha_2\beta_2$, $\beta_2\gamma_2$, or $\alpha_2\gamma_2$ tetramers; and fraction IC could be a γ_4 tetramer. The observed heterogeneity may be a precise description of native *C. magnifica* hemoglobin or may be the result of dissociation or even rearrangement of subunits caused by freezing or storing the blood. The electrophoretic differences between fractions IA and IC, and therefore subunits 1 and 2 versus subunit 3, are substantiated by differences in amino acid composition of fractions IA and IC. The amino acid compositions of both tetramers were compared to those of other clam hemoglobins by the method of Harris and Teller (*3*). By this comparison, *C. magnifica* hemoglobin subunits are as similar to one another as are human β and γ chains, which show sequence homology as high as 75 percent. It is likely that the sequences of the *C. magnifica* hemoglobin subunits resemble one another, and these chains probably evolved from a common myoglobin-like protein, as has been proposed for the vertebrate hemoglobins. However, neither fraction IA nor fraction IC of *C. magnifica* hemoglobin appears to resemble closely any of the other bivalve hemoglobins so far investigated. In contrast, the hemoglobins from two different

but closely allied bivalve families, the Astartidae and the Carditidae, show very low compositional divergences when compared by this method. The observation that *C. magnifica* hemoglobin chains do not closely resemble those of any other bivalve, at least with respect to amino acid composition, is consistent with a systematic interpretation based on the morphology of this animal.

A tetrameric hemoglobin has never been found in erythrocytes of a heterodont clam. Although intracellular, circulating hemoglobins are abundant in other invertebrates (*4*), among the mollusks, they are usually found only in pteriomorph bivalves, clams traditionally considered to represent the more primitive condition. A diversity of intracellular hemoglobin structures is found in the pteriomorph bivalves. For example, some arcid clams (*Noetia ponderosa*) have multiple dimeric intracellular hemoglobins (*5*). Others such as *Anadara* have both tetrameric and dimeric intracellular hemoglobins (*6*). The arcid clam *Barbatia reeveana* has not only a tetrameric hemoglobin (M_r, 68,000) but also a very large (M_r, 430,000) polymeric hemoglobin in its erythrocytes (*7*). The more advanced heterodont clams generally lack erythrocytes. However, numerous species do contain intracellular myoglobins (*4*), and members of the heterodont families Astartidae and Carditidae have most unusual extracellular hemoglobins dissolved in their hemolymph. These hemoglobins are huge (M_r, 8×10^6 to 12×10^6) and have very large subunits (M_r, 3×10^5 to 4×10^5) (*8*). One hypothesis that might describe hemoglobin distribution among bivalves is that the intracellular dimeric and tetrameric hemoglobins represent a primitive condition, whereas the large extracellular hemoglobins represent a more advanced condition. The pteriomorph *B. reeveana*, with both tetrameric and polymeric hemoglobins within erythrocytes, may represent an intermediate stage. The subunit of *B. reeveana* tetramer is similar to that of other pteriomorph bivalves, whereas the subunit of its polymeric hemoglobin has more than one heme group per polypeptide chain and therefore shares some similarities with the large subunit of the heterodont clam hemoglobins. The presence of a tetrameric intracellular hemoglobin in *C. magnifica* suggests that the tetrameric hemoglobins may have persisted in this heterodont clam. Alternatively, hemoglobins may have evolved independently in at least this one bivalve group.

A thorough interpretation of the function of hemoglobin in *C. magnifica* must await more extensive oxygen equilibrium studies of its hemoglobin as well as a better understanding of the animal's oxygen needs. However, preliminary oxygen-binding studies, carried out as described (*9*), indicate that *C. magnifica* hemoglobin within cells has a relatively high oxygen affinity. The partial pressure of oxygen at which hemoglobin is half-saturated (P_{50}) was 7.6 ± 0.23 (standard deviation) at *p*H 6.78 and 10.1°C for seven animals, and the Hill coefficient was 1.24; both of these values appear to vary little with temperature between 2° and 10°C. These values and the oxygen-carrying capacity of the blood of this clam (4.2 ± 0.92 volume percent for three animals) are similar to values determined for other clams (for example, *Noetia*) (*10*) and therefore are not unusual; even the temperature independence of P_{50} has been seen in some other clam hemoglobins (*11*). As in *Noetia*, this hemoglobin probably transports oxygen under the variable temperature and ambient O_2 conditions found near the thermal vents. The large vestimentiferan worm *Riftia pachyptila*, another conspicuous thermal rift organism, has abundant hemoglobin with temperature-insensitive high oxygen affinity (*12*). It has been proposed that the vestimentiferan worm (*13*, *14*) and possibly the bivalves (*14*) utilize chemoautotrophic bacteria to fix carbon. This process is an aerobic phenomenon, and it has been suggested that the hemoglobin of *R. pachyptila* may aid the transport of oxygen to fulfill this demand. Perhaps the hemoglobins of *C. magnifica* also help transport oxygen to the site of carbon fixation under variable environmental conditions.

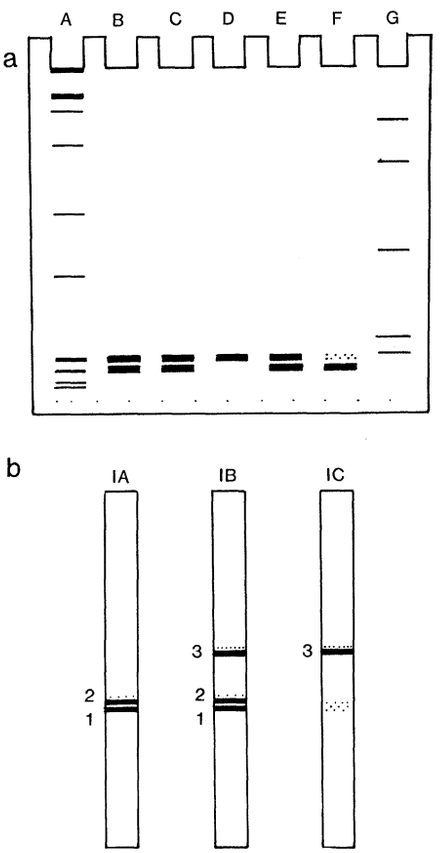

Fig. 2. (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in 1.5-mm slab gels with a discontinuous buffer system (*15*). Gel concentrations of 10 and 12.5 percent were used with a constant ratio of acrylamide to bisacrylamide of 30:0.8. The samples were first denatured in boiling incubation buffer containing 2 percent sodium dodecyl sulfate, 5 percent 2-mercaptoethanol, and 1 m*M* phenylmethylsulfonyl fluoride for 1.5 minutes at 100°C. Calibrants were bovine serum albumin, ovalbumin, α-chymotrypsinogen A, sperm whale myoglobin, and lysozyme (Sigma). The gels were stained with Coomassie blue R (*16*); (lane A) excluded peak, G-100; (lane B) fraction 1, G-100; (lane C) fraction II, G-100; (lane D) fraction IA, DEAE; (lane E) fraction IB, DEAE; (lane F) fraction IC, DEAE; and (lane G) calibrants. (b) Gel electrophoresis at *p*H 2.2 was carried out on globin in the presence of deionized 6.25*M* urea (*17*). Electrophoresis of the gels and electrophoresis of the sample were carried out at 2 mA per tube. The gels were stained with Coomassie blue R and destained with acetic acid. IA, IB, and IC are DEAE fractions.

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Tumor Cells Secrete a Vascular Permeability Factor That Promotes Accumulation of Ascites Fluid

Abstract. *Tumor ascites fluids from guinea pigs, hamsters, and mice contain activity that rapidly increases microvascular permeability. Similiar activity is also secreted by these tumor cells and a variety of other tumor cell lines in vitro. The permeability-increasing activity purified from either the culture medium or ascites fluid of one tumor, the guinea pig line 10 hepatocarcinoma, is a 34,000- to 42,000-dalton protein distinct from other known permeability factors.*

Abnormal accumulation of fluid commonly accompanies solid and particularly ascites tumor growth (*1*). To investigate the mechanism of tumor ascites formation, we measured the rates of influx and efflux of ^{125}I-labeled human serum albumin (HSA) at various times after the implantation of tumor cells in the peritoneal cavities of guinea pigs. We detected a markedly increased influx of HSA as early as 1 hour after intraperitoneal injection of guinea pig line 10 hepatocarcinoma cells, which provoke a substantial accumulation of ascites fluid (Table 1). In contrast, efflux of HSA from the peritoneal cavities of animals bearing line 10 tumors did not change significantly, even with progressive tumor growth (*2*).

To establish whether the increased influx of fluid induced by tumor cells reflects an alteration in vessel permeability, we injected animals intravenously with colloidal carbon. Examination of the peritoneal cavities of strain 2 guinea pigs, Syrian hamsters, and A/Jax mice bearing syngeneic ascites tumors (line 10, HSV-NIL8, and TA3-St, respectively) revealed that many venules of the peritoneal wall, diaphragm, mesentery, and gastrointestinal serosal surfaces were heavily labeled with colloidal carbon, indicating increased permeability; comparable vessels in control animals were not labeled.

These observations suggest that tumor ascites may be attributable to alterations in the permeability of vessels lining the peritoneum. To investigate the basis for this increased permeability, we used the Miles assay (*3*) to test ascites fluid for the presence of factors that increase vascular permeability (Table 2 and Fig. 1). Ascites fluid from line 10 guinea pig and TA3-St mouse carcinomas and the HSV-NIL8 hamster sarcoma all markedly increased local cutaneous vascular permeability. The increase was evident after 1 minute and maximal within 5 to 10 minutes. By contrast, platelet-poor plasma samples from the same species (Table 2 and Fig. 1) and oil-induced peritoneal exudate fluids (*4*) had little or no activity. The tumor ascites permeability-increasing activity was not inhibited by soybean trypsin inhibitor (1000 μg/ml); therefore, it is not PF/dil (*5*), a permeability factor unmasked when serum is diluted ≥ 1:100 (*6*).

We previously reported that line 10 tumor cells release a vascular permeability–increasing activity in serum-free culture (*7*). This activity is not inhibited by soybean trypsin inhibitor (200 μg/ml), and its production by cells in vitro requires protein synthesis (complete inhibition by 20 μg of cycloheximide per milliliter). Many other tumor cell lines also release permeability-increasing activity in serum-free culture, including guinea pig 104 C1 fibrosarcoma, hamster HSV-NIL8 sarcoma, rat sarcomas B77 Rat 1 and RR 1022, and mouse TA3-St carcinoma, MOPC 21 myeloma, and polyoma BALB/c 3T3 sarcoma. Line 1 guinea pig hepatocarcinoma cells release one-fourth the activity released by line 10 cells, a finding that may explain the relative ability of these cells to promote HSA influx (Table 1) and ascites fluid accumulation (the volume of line 1 ascites fluid was routinely one-fourth that of line 10). Oil-induced guinea pig peritoneal exudate cells (> 70 percent macrophages) neither increase the influx of HSA into the peritoneum (Table 1) nor secrete detectable permeability-increasing activity in vitro. Guinea pig fibroblasts and smooth muscle cells release approximately one-eighth the activity released by comparable numbers of line 10 cells (*8*).

Table 1. Peritoneal vessel permeability. Guinea pigs (400 g) were injected intraperitoneally with 3×10^7 line 1 or line 10 tumor cells (*17*) or with peritoneal exudate cells in Hanks balanced salt solution (HBSS) and immediately thereafter were injected intravenously with ^{125}I-labeled HSA (5×10^6 dis/min). One hour later the animals were exsanguinated under ether anesthesia, and peritoneal fluid was collected following intraperitoneal injection of 20 ml of heparinized (10 U/ml) HBSS. For each animal total radioactivity in the ascites fluid was determined and normalized for that in the blood: influx of HSA was computed as the ratio of total disintegrations per minute in peritoneal fluid to those per milliliter of blood. Net influx was determined by subtracting influx values for control animals. Values are means ± standard errors ($N = 4$).

Type of cells injected intraperitoneally	Net peritoneal influx of HSA
Line 1	0.09 ± 0.04
Line 10	0.41 ± 0.08
Line 10 + immune IgG (2 mg)	0.11 ± 0.03
Peritoneal exudate	0

We next purified both the ascites and tissue culture permeability factors from a single tumor, the line 10 guinea pig carcinoma. Permeability-increasing activities from both sources chromatographed identically as single peaks on columns containing Sephadex G-150, heparin-Sepharose, or hydroxylapatite (*9*) and electrophoresed as a single peak with an apparent molecular weight of 34,000 to 42,000 on sodium dodecyl sulfate–polyacrylamide gels (Fig. 2). Using the heparin-Sepharose, hydroxylapatite, and electrophoretic steps in tandem, we purified the permeability-increasing activity approximately 1200-fold from serum-free conditioned medium and approximately 10,000-fold from ascites fluid. As little as 200 ng (5×10^{-12} mole) of the purified material increased vascular permeability to a degree equivalent to that induced by 1.25 μg (4×10^{-9} mole) of histamine.

Rabbits immunized with the purified permeability factor secreted by line 10 cells in vitro produced an immunoglobulin G (IgG) that bound and neutralized virtually all the permeability-increasing activity in undiluted line 10 and line 1 tumor ascites fluids (Table 2) and in line 10 and line 1 culture media. This antibody also blocked the peritoneal influx that follows intraperitoneal injection of line 10 tumor cells (Table 1). In every