approximate 30 percent relaxation. The muscle bath medium was changed and serotonin ($4.5 \times 10^{-8}M$) and PDGF ($33 \times 10^{-12}M$) were added. After contraction reached maximal force, acetylcholine ($5.5 \times 10^{-7}M$) was again added and again induced a similar 30 percent decrease in tension.
13. Rat aortic strips were contracted by EC_{30} doses of either serotonin or $PGF_{2\alpha}$ which resulted in approximately 400-mg contractile tension. The muscle bath medium was changed, and after relaxation, an EC_{30} dose of the above agonists was added simultaneously with PDGF ($33 \times 10^{-12}M$). At maximal contraction, tension generation was approximately 460 mg of force, which represents summation of the effects of both agonists (Figs. 1 and 2).
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24. Partially purified TGF-β (prepared from human sources with estimated 80 percent purity) and cationic FGF (prepared from bovine pituitary with estimated 5 percent purity) were obtained from Biomedical Technologies Inc., Cambridge, MA. Helical strips of rat aorta were contracted as described in Fig. 1. Experimental values are mean ± SEM of four observations. EC_{50} values were estimated as described in Fig. 2. Based on a value of 5 percent for the purity of the cationic FGF fraction, the EC_{50} for FGF contraction would be 134 pg/ml, which is close to the EC_{50} value of 60 pg/ml reported for mitosis in VSMC in response to FGF [F. Esch *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6507 (1985)].
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Extended Oxygen Delivery from the Nerve Hemoglobin of *Tellina alternata* (Bivalvia)

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An oxygen-binding hemoglobin localized in the nerves of *Tellina alternata* (Bivalvia) required 30 minutes to unload oxygen when excised nerves were exposed to pure nitrogen. Neural excitability under these conditions could be sustained only until deoxygenation of the hemoglobin was complete. When the oxygen-combining function of the hemoglobin was abolished with carbon monoxide, the neural excitability ceased within a few minutes of oxygen removal, a response identical to that of hemoglobinless homologous nerves of other bivalves. These results demonstrate that aerobic activity can be supported by the oxygen stored on hemoglobin in microscopic tissues for a considerable time under anoxic conditions.

IF PIGMENTS CARRYING O_2 FUNCTION mainly to increase the availability of O_2 to respiring mitochondria, the molecular characteristics of the pigment should be matched to the magnitude and duration of O_2 delivery required by the tissue. As an example, myoglobin rapidly dissociates O_2 (*1*), exhibits high intracellular mobility (*2*), and has an O_2 affinity intermediate between those of hemoglobin and cytochrome oxidase (*3*). These properties enable myoglobin to facilitate O_2 diffusion during steady-state O_2 consumption (*4*) and to function as a short-term O_2 store (for a few seconds) at the onset of muscular contraction (*5*). Hemoglobins that slowly dissociate O_2 could not function in facilitated O_2 diffusion (*6*) or short-term storage. Instead, they could function as a long-term supply of O_2 if matched to a similar demand (*7*).

A few studies relating hemoglobin characteristics to animal behavior have indirectly demonstrated hemoglobin participation in extended O_2 supply (*8*). On the basis of O_2 unloading rates from the hemoglobin, body geometry, and O_2 consumption rates, we proposed that hemoglobin could be used for long-term O_2 storage in a microscopic animal, *Neodasys* (Gastrotricha) (*7*). By using continued nervous function as evidence for the presence of O_2 (*9*), we have found more direct evidence that the oxygen-carrying hemoglobin located in the ganglia and all major nerves of *Tellina alternata*, a deposit-feeding bivalve, is well suited to act as a long-term O_2 storage center. Under anoxic conditions the propagation of externally stimulated action potentials by the nerves continued until deoxygenation of the hemoglobin was complete (~30 minutes). If the hemoglobin was made nonfunctional with CO, the action potentials ceased shortly after ambient O_2 was removed (5 minutes), a response identical to that of hemoglobinless homologous nerves from a sympatric filter-feeding bivalve, *Tagelus plebeius*.

The nerve connecting the cerebral and visceral ganglia (the c-v nerve, which is typically 2 to 4 cm long, 0.012 to 0.016 cm in diameter, and surrounded by a tough, nonremovable collagenous sheath 0.0003 cm thick) was used for this study because it is relatively large and unbranched. The c-v nerve is densely packed with glial cells (red-pigmented in *T. alternata*) and slowly conducting axons (*10*), 0.1 to 0.3 μm in diameter (*11*). Hemoglobin characteristics in the excised nerve were determined on a microspectrophotometer with a specially constructed gas slide (*7*). A functional hemoglo-

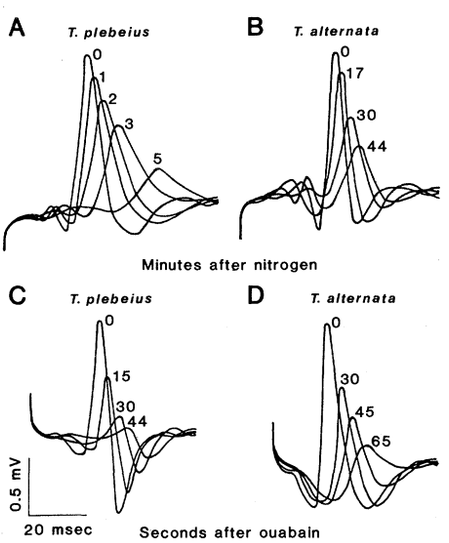

Fig. 1. Time course changes in action potential appearance under different conditions. Action potentials retraced from sequential photographs show a decrease in amplitude and conduction velocity until indistinguishable from baseline. A supramaximal stimulus of 4 to 7 V and 0.5 to 1.0 msec at 2 pulse/sec was used in (A) and (B) and 1 pulse/sec in (C) and (D); stimulus artifact is a vertical line at the left of the trace. The number at each peak indicates the time at which that trace was recorded after the treatment began.

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bin was identified through the recording of characteristic changes in absorption spectra corresponding to successive deoxygenation and reoxygenation of the tissue with humidifed N_2 and air, respectively (*12*). The affinity for O_2 was calculated with the Hill equation from changes in fractional saturation of the hemoglobin as a function of stepwise increases in O_2 partial pressure (PO_2). The PO_2 that half-saturated the hemoglobin, P_{50}, was 1.1 ± 0.2 mmHg PO_2, and the measure of heme-heme interaction, the Hill number, was 4.8 ± 0.8 (n = 8), at 20°C and pH 7.8, which indicates that the hemoglobin exhibited a high affinity for O_2 and a high degree of cooperativity (*13*).

The significance of these affinity measures becomes apparent when the PO_2 at which the hemoglobin begins to deliver O_2 is compared with the PO_2 at which O_2 supplied by diffusion becomes limited. At a PO_2 of 2.1 mmHg, this hemoglobin is 95 percent saturated, as computed from the Hill equation, which suggests that O_2 delivery will start at a PO_2 value slightly higher than 2.1 mmHg. The PO_2 value at which O_2 supply becomes limited by diffusion was estimated from Harvey's equation for a long cylinder (*14*):

$$C_o = (Ar^2)/(4D)$$

where C_o is the oxygen partial pressure necessary to supply a given O_2 consumption rate A for a cylinder of radius r with a diffusion coefficient D. For the *T. alternata* c-v nerve, A was approximately 150 μl of O_2 per gram per hour, r averaged 0.007 cm, and D was conservatively estimated to be 1.1×10^{-5} ml cm^{-2} min^{-1} $(atm/cm)^{-1}$ (at standard temperature and pressure, dry) (*15*). The value of C_o is 0.0028 atm or 2.13 mmHg PO_2, almost exactly the PO_2 at which the hemoglobin begins to unload. Continuously monitoring transmitted light intensity at a specified wavelength with an X-Y recorder or by recording absorption spectra at intervals of 3 to 5 minutes while flushing the gas slide with humidified 99.999 percent N_2 revealed that it took 29.8 ± 6.4 minutes (n = 9) for 99 percent of the hemoglobin to unload O_2 (*16*). Hemoglobin was oxygenated within 20 seconds of being exposed to air.

We recorded in situ electrical activity and simultaneous fractional saturation of the hemoglobin from entire c-v nerves by using another gas slide containing a miniature nerve chamber having 12 miniature platinum electrodes. Chamber gas tensions were controlled through gas ports, and temperature was controlled with internal fluid coils. A light beam was passed through the nerve to monitor heme saturation while the nerve was positioned on the electrodes for external

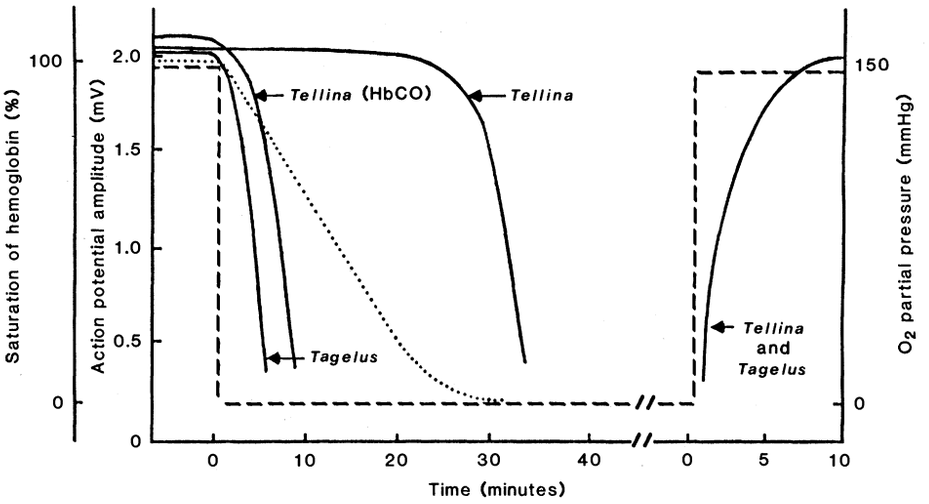

Fig. 2. Changes in action potential amplitude dependent on ambient O_2 or O_2 stored on the hemoglobin. Action potential amplitudes, solid lines; saturation of hemoglobin, dotted line; PO_2 in the chamber, dashed line. The action potential trace for *Tellina* is accompanied by a trace of the hemoglobin saturation for that experiment. The other two action potential amplitude traces of *Tellina* HbCO and *Tagelus* are similar because the hemoglobin was not functional or was not present. Nerve chamber volume was 0.1 ml, and gas flow was 70 ml/min, so PO_2 change was immediate. Carboxyheme (HbCO) was formed by exposing the nerve to 2 to 3 mmHg in air for 30 minutes, after which a carboxyheme spectrum was recorded (P_{50} for CO = 0.4 mmHg). During this CO exposure, c-v nerves from both clams showed no change in electrical excitability.

stimulation and recording of action potentials. Action potentials from *T. alternata* c-v nerves were monitored with a Tektronix 5103N oscilloscope, photographed with a Coleman Oscillotron, and compared with the action potentials from the hemoglobinless homologous c-v nerves from *T. plebeius*. In air, externally recorded action potentials from c-v nerves of both animals were 2 to 3 mV in amplitude, were propagated at 20 to 40 cm/sec, and exhibited refractory periods of 100 to 150 seconds at 20°C. These values agree with those of action potentials recorded from slow fibers in other bivalves (*10*). Freshly extracted nerves from both clams propagated action potentials at 1 to 3 pulses per second for more than 2 hours in humidified air.

Representative action potentials recorded sequentially during four experiments when the nerves were exposed to either pure N_2 or ouabain are overlaid and presented in Fig. 1. When the nerve chamber was flushed with N_2, action potential amplitude in c-v nerves from *T. plebeius* immediately began to decline, and propagation ceased in 7.0 ± 3.7 minutes (n = 8) (Fig. 1A). In contrast, action potentials in the *T. alternata* nerve were propagated for 36.3 ± 9.6 minutes (n = 6) after N_2 flushing began. Amplitude did not decline by more than 10 percent for at least 15 to 20 minutes (Fig. 1B). When stimulated at 1 pulse/sec in air with a drop of 1 m*M* ouabain in seawater placed on the nerve between the stimulating and recording electrodes, the ability to propagate action potentials beyond that point ceased in ~60 seconds in both nerves (Fig. 1, C and D). These data indicate that the nerves from both clams depend on operational Na^+-K^+ pumps to maintain membrane potentials and that, under anoxic conditions, *T. alternata* nerves can remain active five to six times as long as those from *T. plebeius*. Figure 2 illustrates changes in action potential amplitude as dependent on either ambient O_2 or O_2 stored on the hemoglobin. When the nerve chamber was flushed with N_2, the action potentials from *T. plebeius* ceased in about 5 minutes, but those from *T. alternata* ceased only after the hemoglobin was deoxygenated. However, when more than 95 percent of the hemoglobin was bound with CO to eliminate the O_2-binding capability, and then the nerve chamber was flushed with N_2, action potentials ceased in 5 to 10 minutes, a response similar to that of the *T. plebeius* c-v nerve under anoxic conditions. When the nerves were reaerated, action potentials from both *T. alternata* and *T. plebeius* returned to their initial amplitudes at similar rates.

The small diameter axons in these nerves seem to depend on aerobic metabolism to maintain membrane potentials, probably as a result of the high ratio of surface area to volume (*17*), and the high-affinity, slowly unloading hemoglobin in the *T. alternata* c-v nerve seems to begin to release O_2 just when diffusion becomes insufficient, providing at least enough O_2 during deoxygenation to support axon function.

The criteria necessary in the design of this particular O_2 storage system probably include: (i) a high-affinity hemoglobin that slowly dissociates and rapidly reassociates O_2 to reduce the amount of free O_2 diffusing out of the nerve and that, combined with high cooperativity, restricts O_2 unloading to a very low and narrow PO_2 range so that the diffusion gradient is always small; (ii) diffusion distances, which are smaller from glial cells to axons than from glial cells across the collagen sheath to the outside, that favor O_2 consumption by axon bundles; and (iii) O_2 consumption rates of active nerves that are equivalent to the O_2 unloading rates. A measure of how much of the unloaded O_2 is actually consumed by the nerve as opposed to how much is lost could indicate how efficiently the hemoglobin functions in storage. Preliminary calculations based on O_2 unloading rates, O_2 consumption rates, heme concentration, and the anatomy of the nerve suggest that more than 80 percent of the unloading O_2 could be consumed by the nerve during high activity, demonstrating an appropriate match between hemoglobin O_2 supply and neural O_2 demand. The molecular phenomena influencing the long unloading time and a model relating the important variables to long-term O_2 supply by hemoglobin will be discussed elsewhere (*18*).

Tellina alternata, unlike the filter-feeding *T. plebeius*, does not inhabit a permanent burrow, because its mode of deposit feeding quickly depletes the surrounding region of food and it must continually burrow through anoxic sediment to new locations. When laterally burrowing several centimeters below the sediment surface, it does not maintain siphonal contact with the surface and therefore does not seem to have access to a source of O_2. It seems reasonable that its slowly unloading neural O_2 stores may enable continued nervous function and burrowing activity even in anoxic sediments for an extended period of time, allowing it to reach fresh deposits.

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Nitrogen Fixation by *Azotobacter vinelandii* Strains Having Deletions in Structural Genes for Nitrogenase

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Phenotypic reversal of Nif$^-$ mutant strains to Nif$^+$ under molybdenum-deficient conditions has been cited as evidence that *Azotobacter vinelandii* possesses two nitrogen fixation systems: the conventional molybdenum-enzyme system and an alternative nitrogen-fixation system. Since explanations other than the existence of an alternative system were possible, deletion strains of *A. vinelandii* lacking the structural genes for conventional nitrogenase (nifHDK) were constructed. These strains were found to grow in molybdenum-deficient nitrogen-free media, reduce acetylene (at low rates), and incorporate molecular nitrogen labeled with nitrogen-15. Thus it can be concluded that the phenotypic reversal phenomenon cannot be due to altered phenotypic expression of *nif* mutations under molybdenum-deficient conditions, but is due to the existence of an alternative nitrogen-fixation system in *A. vinelandii* as originally proposed.

SEVERAL YEARS AGO WE REPORTED that Nif$^-$ mutant strains of *Azotobacter vinelandii* containing mutational lesions in the structural genes for nitrogenase underwent phenotypic reversal to Nif$^+$ when cultured in molybdenum-deficient nitrogen-free media (*1, 2*). To account for this phenotypic reversal, we proposed the existence of an alternative N_2 fixation system expressed under conditions of molybdenum starvation and repressed in the presence of molybdenum (*1*). It can be argued, however, that phenotypic reversal of Nif$^-$ mutants under conditions of molybdenum starvation is due to increased leakiness of the mutant phenotypes and not to derepression of an alternative N_2-fixation system. Mutant strains containing deletions in the structural genes for nitrogenase (*nifHDK*) should distinguish between these two explanations since mutational alterations caused by deletions should preclude leaky mutant phenotypes. Here we report N_2 fixation by Nif$^-$ mutant strains containing deletions in *nifHDK*.

Deletions in *nifHDK* were constructed by removing internal restriction fragments from cloned DNA's containing these genes.

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