would produce a large fuel supply for wildfire, and would in any event result in a flux of stored carbon into the atmosphere. The opening of the forest canopy would also increase the albedo of the landscape, leading to cooling of the land surface[3]. Either effect has serious implications for future climate change. Furthermore, although there is still some dispute over the potential migration rates of tree species in response to climate change, forest equilibrium is not only a matter of species presence, but also of forest structure[20,21]. Even with all relevant species and suitable soils present, it may nevertheless take several centuries for a forest to equilibrate with climate because of the lags inherent in seral succession; this has been simulated for northern Michigan mixed forest under future climate change scenarios[9].

In other regions, a succession such as this might not be observed in the pollen record. In southern Ontario, its prominence is due to several factors, including the high pollen representation of some of the species involved in the succession, the ecotonal nature of the region, the long lifespans of many of the tree species, and the infrequent prehistoric fire regime[22] which allowed a long seral succession to develop. Although a similar disequilibrium probably occurred in other regions, the absence of any one of these factors would render it nearly unobservable in the pollen record. □

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Nitrate respiration in the hydrothermal vent tubeworm *Riftia pachyptila*

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THE vestimentiferan tubeworm *Riftia pachyptila* is found around hydrothermal vent areas in the deep sea. Intracellular bacterial chemoautotrophic symbionts use the oxidation of sulphide from the effluent of the vents as an energy source for CO_2 fixation. They apparently provide most or all of the nutritional requirements for their gutless hosts[1–5]. This kind of symbiosis has since been found in many other species from various other phyla from other habitats[6–9]. Here we present results that the bacteria of *R. pachyptila* may cover a significant fraction of their respiratory needs by the use of nitrate in addition to oxygen. Nitrate is reduced to nitrite, which may be the end product (nitrate respiration)[10] or it may be further reduced to nitrogen gas (denitrification)[11]. This metabolic trait may have an important role in the colonization of hypoxic habitats in general by animals with this kind of symbiosis.

Because the symbionts cannot be cultured[12] we used physically purified bacteria[13] for our experiments which were incubated under anaerobic conditions in the presence of nitrate. Nitrite appeared in the medium at a linear rate (0.91 ± 0.07 nmol per mg protein per min, $n = 3$) for at least 90 min (Fig. 1) and was independent of the nitrate concentration between 50 μM and 1 mM. Nitrite was not formed without nitrate ($n = 3$), with plume tissue containing no symbionts ($n = 1$), when the bacteria were boiled ($n = 3$), or when cyanide was added ($n = 2$). When the concentration of nitrate dropped below 20 μM (Fig. 1), nitrite was reduced as well, indicating that nitrite can be respired further. There was no indication that the symbionts can respire nitrate to ammonium. Nitrate respiration was stimulated by sulphide with maximal rates at 500 μM sulphide (Fig. 2), but not by thiosulphate. This supports previous results[14,15] that the symbionts are sulphide specialists.

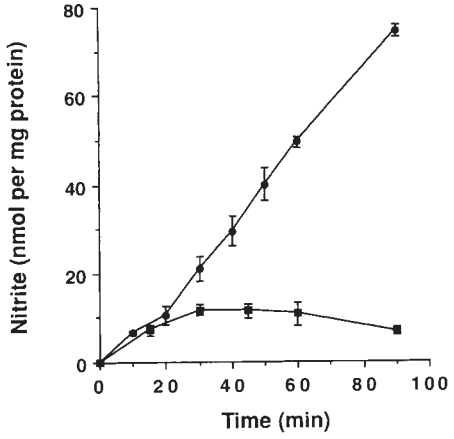

FIG. 1 Production of nitrite by purified symbionts from *R. pachyptila*. Live tubeworms were collected in temperature-insulated containers from the Genesis vent site at 2,638 m depth at 12° 48.675′ N, 103°56.386′ W on the East Pacific Rise. Symbionts were purified under anaerobic conditions[13] from crude homogenates of trophosome[17], the tissue containing the bacteria. Isolated symbionts were incubated anaerobically in 10 ml *Riftia* saline[24] containing 500 μM sulphide and 1 mM (●) or 50 μM nitrate (■). The concentration was adjusted to about 2 mg symbiont protein ml^{-1}. The incubations were done at 15 °C and stirred continuously. To maintain anaerobic conditions, each incubation chamber was closed with a rubber septum and a nitrogen gas stream was introduced through a needle 15 min before and during the experiment. At 10 min intervals, 1.5 ml aliquots were drawn through a syringe and centrifuged for 5 min in a Fisher table-top centrifuge. The concentration of nitrite in the supernatant of the samples (nmol per mg symbiont protein) was determined after removal of sulphide[25]. Data points represent means ± s.e., $n = 3$.

Because denitrification is usually a trait found in bacteria from anaerobic environments, the influence of oxygen on the reduction of nitrate by the symbionts had to be assessed. The only measurements of oxygen consumption by purified *R. pachyptila*

symbionts have been made at oxygen saturation (260 μM)[15]. But these measurements appear to be unrealistic, because the levels of free oxygen in the bacteriocytes, the cells containing the symbionts, will be much lower. They can be roughly estimated from the physiology of the haemoglobin dissolved in the blood. Oxygen is delivered from the plume to the trophosome through the vascular blood in a closed circulatory system. The trophosome itself is completely submerged in the coelomic fluid filling the coelomic cavity. Both, the vascular and coelomic haemoglobin molecules have an extremely high affinity for oxygen (P_{50} = 0.1–0.3 Torr at pH 7 (ref. 16) equivalent to about 2 μM). Because the concentration of free oxygen inside the tissue is determined by the PO_2 of the blood, its concentration in the bacteriocytes should be maximally around 2 μM (ref. 17). This is a conservative estimate, because factors such as oxygen consumption in the cytosol or diffusion barriers could lower the free oxygen levels even more. The large pool of oxygen bound to haemoglobin[18] should not increase the concentration of free oxygen in the bacteriocyte cell but only maintain it at a low level. It has been proposed[6,17] that one of the major roles of the two haemoglobins is to decrease the concentration of free oxygen in the immediate vicinity of the symbionts possibly resulting in a reduction of the oxygenase reaction of the symbionts' ribulose 1,5-bisphosphate carboxylase, the key enzyme of the Calvin–Benson cycle.

Although oxygen respiration decreases with decreasing oxygen concentrations, nitrate respiration increases (Fig. 3). At 100% saturation, oxygen respiration is 20 times higher than nitrate respiration. At the lowest oxygen concentration (below 10% saturation equal to 26 μM or less) the rates are statistically equivalent. We did not determine oxygen consumption at lower levels because they could not be measured reliably with the techniques available to us. We can calculate that under estimated *in vivo* conditions (at a free oxygen concentration of 2 μM or less and availability of nitrate in a concentration range similar to that of sea water) at least half of the oxidative capacity of the symbionts can be provided by nitrate. This is assuming no enhancement in respiration or increase in oxygen concentration in the bacteriocytes by the blood binding capacity for oxygen[18].

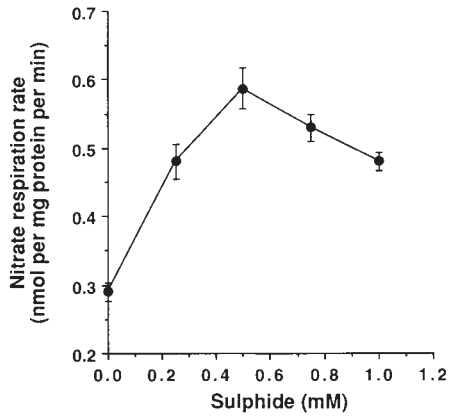

FIG. 2 Influence of sulphide on nitrate respiration in purified symbionts. Incubations were done in *Riftia* saline[24] with sulphide concentrations between 0 and 1 mM nitrate. At timepoints, the concentrations of nitrite were determined and the respiration rate (nmol nitrite mg^{-1} min^{-1}) calculated. Data points represent means ± s.e., $n = 4$.

This use of nitrate for respiration allows the oxygen around the symbionts to be maintained at extremely low concentrations yet energy can still be gained through respiratory pathways.

Nitrate respiration can also be measured in the intact symbiosis. In preliminary experiments small animals (1–4 cm long) were incubated under pressure in closed vials in sea water containing 1 mM nitrate. They excreted nitrite at rates of up to 250 nmol per g worm fresh weight per h. These rates are corrected for activities of epibiotic bacteria on the tubes by removing the worm from its tube at the end of the incubation and continuing to measure nitrite production by the empty tube. But during these experiments the animals were stressed. The result is, therefore, only indicative of their ability to reduce nitrate, not of their *in situ* metabolic rate.

In the hydrothermal vent environment, both the presence of

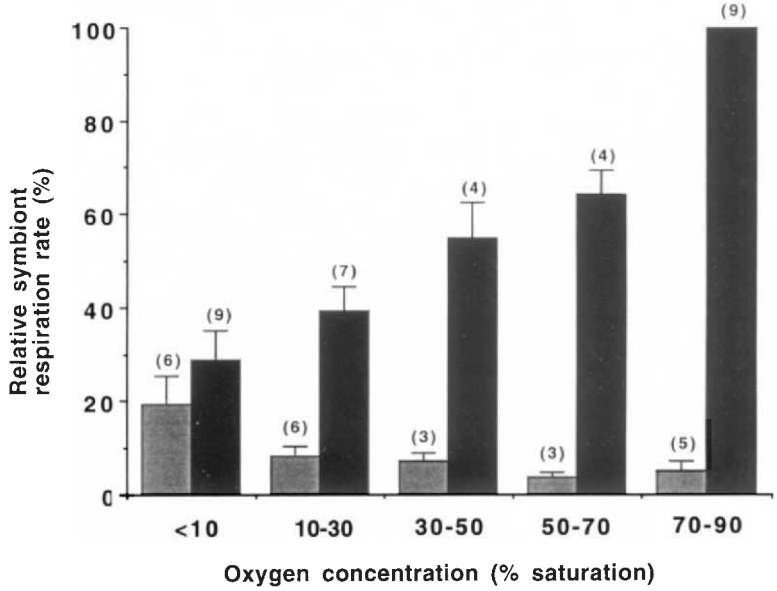

FIG. 3 Oxygen and nitrate respiration rates at different oxygen concentrations. Black bars represent oxygen respiration rates, grey bars are nitrate respiration rates (mean ± s.e., *n* given above each bar).

METHODS. Symbiont oxygen respiration was measured with a modified Clarke-type oxygen electrode[26]. Before every incubation, nitrogen gas was bubbled in the incubation mixture through small-diameter tubing inserted into the side groove of the electrode setup until the desired oxygen concentration was established. Symbiont suspension was added to a final concentration of 1–2 mg ml^{-1} in 1.9 ml *Riftia* saline[24] and allowed to equilibrate for several min. The oxygen consumption rate under these conditions was defined as the baseline. Then sulphide was added to a final concentration of 200 μM and the sulphide-stimulated oxygen consumption rate measured. After a few minutes oxygen was reintroduced to reestablish the concentration when sulphide had been added. Then azide was added (final concentration 50 μM) as a respiratory inhibitor. The azide-insensitive oxygen consumption rate was measured and found to be largely independent of the sulphide or oxygen concentrations in the range used. To avoid the introduction of artefacts due to the order in which the rates were measured we changed the oxygen concentrations randomly before we measured nitrate and oxygen consumption, the results were identical. The oxygen respiration rate at 90–70% saturation was measured at the beginning and at the end of each preparation to ensure the viability of the symbiont suspension. They remained constant for several hours, when the symbiont suspension was kept on ice. Nitrate respiration rates were measured after addition of 1 mM nitrate. After 15 min, an aliquot was taken, centrifuged for 5 min and the concentration of nitrite determined in the supernatant. The rates are expressed relative to the sulphide-stimulated oxygen respiration rate at oxygen saturation.

nitrate and its consumption near tubeworm clusters have been demonstrated[19]. The rate of nitrate removal was five times higher than necessary if it were due to nitrate assimilation. In this area, characterized by silicate concentrations of 500–600 μM, the animals can be exposed to conditions where the concentration of oxygen is only double that of nitrate (around 30 μM). The sulphide consumption rate of tubeworms *in situ* was highest under these conditions[19].

In addition, initial data indicate that the symbionts of the clam *Lucinoma aequizonata*, collected in a hypoxic basin from the coast of Santa Barbara (CA), may also have the ability to use nitrate respiration to supplement the lack of oxygen[20].

If nitrate respiration were to be of major significance for the tubeworms then a means to transport nitrate to the symbionts inside the trophosome has to be provided. The concentration of nitrate in the blood could not be determined accurately, because it coprecipitated on denaturation of the blood proteins. Several methods used for the determination of nitrate in mammalian blood gave erroneous results in *R. pachyptila* blood[21,22]. Nitrite, by contrast, was measured both in the vascular (31.6 ± 6.5 μM, $n = 11$) and the coelomic blood (3.4 ± 1 μM, $n = 12$) of freshly collected animals. Because the concentration of nitrite in the ambient water around the vent sites is, as commonly found in sea water, below 1 μM (K. Johnson, personal communication), it must originate from the symbionts' respiration. This result appears to be in contrast with others showing an even distribution of small molecules between vascular and coelomic fluid[23]. In our experiments, the vascular blood samples used for nitrite determination were taken from the dorsal trunk vessel, the vessel carrying blood from the trophosome to the plume. It is, therefore, feasable that nitrite is released into the vascular blood stream in the trophosome and efficiently excreted in the plume.

Although the average concentration of nitrite in the vascular blood was 31.6 μM, three samples had much higher nitrite concentrations of 89 μM, 138 μM and 218 μM (values not included in the average). Apparently, certain conditions promote higher concentrations of nitrite and presumably nitrate in the blood. Further studies are necessary to determine how nitrate is transported, whether specific uptake systems exist, and whether nitrate can be concentrated and bound in the blood. □

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The retinoid ligand 4-oxo-retinoic acid is a highly active modulator of positional specification

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RETINOIDS (vitamin A and its metabolites) are suspected of regulating diverse aspects of growth, differentiation, and patterning during embryogenesis[1], but many questions remain about the identities and functions of the endogenous active retinoids involved. The pleiotropic effects of retinoids may be explained by the existence of complex signal transduction pathways involving diverse nuclear receptors of the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families, and at least two types of cellular retinoic acid binding proteins (CRABP-I and -II)[2]. The different RARs, RXRs, and CRABPs have different expression patterns during vertebrate embryogenesis[2,3], suggesting that they each have particular functions. Another level at which fine tuning of retinoid action could occur is the metabolism of vitamin A to active metabolites, which may include all-*trans*-retinoic acid[4–7], all-*trans*-3,4-didehydroretinoic acid[8], 9-*cis*-retinoic acid[9,10], and 14-hydroxy-4,14-retroretinol[11]. Formation of the metabolite all-*trans*-4-oxo-retinoic acid from retinoic acid was considered to be an inactivation pathway during growth and differentiation[12–14]. We report here that, in contrast, 4-oxo-retinoic acid is a highly active metabolite which can modulate positional specification in early embryos. We also show that this retinoid binds avidly to and activates RARβ, and that it is available in early embryos. The different activities of 4-oxo-retinoic acid and retinoic acid in modulating positional specification on the one hand, and growth and differentiation on the other, interest us in the possibility that specific retinoid ligands regulate different physiological processes *in vivo*.

Treating *Xenopus laevis* embryos with retinoic acid (RA) during gastrulation causes a dose-dependent loss of anterior structures, leading to microcephaly (Fig. 1*a*, *b*; refs 6, 15, 16). We found that also 4-oxo-retinoic acid (4-oxo-RA) (see Fig. 1*e* for structural formula) is very active in causing microcephaly in *Xenopus* embryos, much more, in fact, than RA (Fig. 1*a*, *b*). The effects of 4-oxo-RA and RA on morphology appear qualitatively indistinguishable. The most sensitive stages for 4-oxo-RA treatment are the gastrula till mid-neurula stages (when the anteroposterior axis is specified), the same sensitive period reported previously for RA[6] (not shown). Next, we tested whether 4-oxo-RA can induce the expression of Hox genes (class 1 homeobox containing genes), which are believed to confer anteroposterior positional information during vertebrate embryogenesis[17–19], and are inducible by RA[18,19]. *Xenopus* embryos were treated with retinoids as in Fig. 1*a*, and expression of two *Xenopus* Hoxb genes, Hoxb-4 and Hoxb-9, was measured at several develop-