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Inorganic Pyrophosphate: Energy Source for Sulfate-Reducing Bacteria of the Genus *Desulfotomaculum*

Abstract. *Sulfate-reducing bacteria belonging to the genus* Desulfotomaculum *utilized inorganic pyrophosphate as a source of energy for growth in the presence of fixed carbon (acetate and yeast extract) and sulfate. Pyrophosphate does not support the growth of* Desulfovibrio *under the same growth conditions. Over a limited range of concentrations, growth is proportional to pyrophosphate, and extracts of bacteria grown on pyrophosphate medium have enzymatic activities similar to extracts prepared from bacteria grown on medium containing lactate plus sulfate. The variety of cell types observed in crude anaerobic pyrophosphate-enrichment cultures from a marine environment suggests that this unique type of energy metabolism is not restricted to the sulfate-reducing bacteria of the genus* Desulfotomaculum.

Inorganic pyrophosphate (PP_i) has been proposed (*1*) as an evolutionary precursor of adenosine triphosphate (ATP) and more recently has been shown to be involved in a number of energy-yielding reactions (*2*). Liu and Peck (*3*) demonstrated that the bioenergetics of respiratory sulfate reduction by two of the described genera of sulfate-reducing bacteria, *Desulfovibrio* and *Desulfotomaculum*, are fundamentally different. In the case of *Desulfovibrio*, the PP_i produced during the formation of adenylyl sulfate (APS) from ATP and sulfate by ATP-sulfurylase (Eq. 1)

$$ATP + SO_4^{2-} \rightarrow APS + PP_i \quad (1)$$

in the first enzymatic step of respiratory sulfate reduction is hydrolyzed to orthophosphate (P_i) by inorganic pyrophosphatase (Eq. 2)

$$PP_i + H_2O \rightarrow 2P_i \quad (2)$$

Thus, the chemical energy in the anhydride bond of PP_i is not conserved and, in order to obtain a net yield of ATP during growth on lactate plus sulfate, *Desulfovibrio* must carry out electron transfer–coupled phosphorylation. In contrast, *Desulfotomaculum* is able to conserve the bond energy of the pyrophosphate produced by ATP-sulfurylase (Eq. 1) by means of the enzyme acetate: PP_i phosphotransferase (Eq. 3) (*4*).

$$\text{Acetate} + PP_i \rightarrow \text{acetyl phosphate} + P_i \quad (3)$$

Adenosine triphosphate can then be produced from acetyl phosphate and adenosine diphosphate (ADP) by acetate kinase (Eq. 4).

$$ADP + \text{acetyl phosphate} \rightarrow \text{acetate} + ATP \quad (4)$$

These two enzymatic reactions allow *Desulfotomaculum* to generate one high-energy phosphate by substrate-level phosphorylation per sulfate reduced to sulfide during growth with lactate, and it is not necessary for this microorganism to carry out electron transfer–coupled phosphorylation during growth with lactate and sulfate. These observations suggested the possibility that *Desulfotomaculum* might be capable of utilizing PP_i as a source of ATP for growth in the presence of fixed carbon. In this report, we describe the growth of *D. nigrificans*, *D. ruminis*, and *D. orientis* on a medium containing PP_i, acetate, yeast extract, sulfate, and salts. The results obtained with crude enrichment cultures from a marine spartina marsh suggest that PP_i respiration is a more general phenomenon among anaerobic microorganisms and is not restricted to the genus *Desulfotomaculum*.

The conditions for the anaerobic growth of *D. nigrificans* on PP_i are given in Table 1. The basal medium, containing acetate, yeast extract, sulfate, and salts, does not support growth of this bacterium. However, when the basal medium is supplemented with PP_i, growth is better than that obtained under usual growth conditions with lactate plus sulfate. On the basal medium, PP_i does not stimulate the growth of *Desulfovibrio vulgaris*, and

Table 1. Requirements for the growth of *Desulfotomaculum nigrificans*, with inorganic pyrophosphate as a source of energy. Growth was measured, in optical density (O.D.) units, at 580 nm in 1-cm cuvettes and expressed as averages of duplicate flasks (500 ml, containing 200 ml of medium) after 48 hours of incubation at 55°C under argon. A 5 percent inoculum of bacteria grown on the PP_i medium was used in all experiments. The basal medium contained per liter: sodium acetate, 3.3 g; Na_2SO_4, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MgCl_2 \cdot 6H_2O$, 1.8 g; K_2HPO_4, 0.5 g; $CaCl \cdot 2H_2O$, 0.2 g; Difco yeast extract, 2.0 g; $FeSO_4$, 10 mg; reducing agent (2.5 g of cysteine · HCl plus 2.5 g of $Na_2S \cdot 9H_2O$ per 200 ml of H_2O), 20 ml. KOH was used to adjust the *p*H to 7.2. Where indicated, the 0.05 percent PP_i (filter sterilized) was added. The lactate-sulfate medium contained per liter: sodium lactate (60 percent), 12.5 ml; NH_4Cl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; K_2HPO_4, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; Difco yeast extract, 1.0 g; and $Na_2S \cdot 9H_2O$, 0.25 g.

Additions and deletions	Growth (O.D.)
Basal medium	0.019
Plus PP_i	0.628
Plus PP_i; minus SO_4^{2-}	0.019
Plus PP_i; minus acetate	0.095
Plus PP_i; minus yeast extract	0.042
Plus PP_i; minus acetate and yeast extract	0.036
Lactate-sulfate medium	0.505

Table 2. Enzymatic activities in extracts of *Desulfotomaculum orientis* grown on either pyrophosphate medium or lactate-sulfate medium. For assay procedures, see text. Abbreviations: MV, methyl viologen; BV, benzyl viologen.

Enzymatic activity	Specific activity (nmole/min per mg protein)	
	Lactate-sulfate medium	PP_i medium
Bisulfite reductase ($MV^+ \rightarrow HSO_3^-$)	64.3	57.1
Nitrite reductase ($MV^+ \rightarrow NO_2^-$)	119.6	153.8
Thiosulfate reductase ($MV^+ \rightarrow S_2O_3^{2-}$)	23.7	21.5
Fumarate reductase ($MV^+ \rightarrow$ fumarate)	0	0
APS reductase [AMP + $SO_3^{2-} \rightarrow Fe(CN)_6^{3-}$]	397	385
Formate dehydrogenase (formate $\rightarrow BV^{2+}$)	64	87.1
Hydrogenase ($H_2 \rightarrow BV^{2+}$)	16.2	85.2
Pyruvate dehydrogenase (pyruvate $\rightarrow BV^{2+}$)	107	139
ATP-sulfurylase	140	151
PP_i: acetate kinase	820	1315
Inorganic pyrophosphatase	149	98

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P_i equivalent to the added PP_i does not support the growth of the three species of *Desulfotomaculum*. For optimal growth on PP_i, acetate, yeast extract, and sulfate are required; acetate and yeast extract probably meet the needs of *Desulfotomaculum* for cell carbon (*5*), and sulfate provides this microorganism with an electron sink with which to adjust the oxidation level of the supplied fixed carbon. The concentration of sulfate was only one-tenth of that in the usual lactate-sulfate medium, but the requirement for acetate was unexpectedly high, with little growth occurring below a concentration of 0.2 percent. The physiological basis for this high acetate requirement has not been investigated, but it may involve the bioenergetics of the permeation of acetate into the bacteria (*6*). The stimulatory effect of PP_i on growth does not appear to be due to the facilitation of anaerobic acetate oxidation (*7*) by PP_i, as the ratio of the disappearance of acetate to the production of sulfide was 3:14 rather than the expected ratio of 1:1. Similar growth responses to PP_i, acetate, yeast extract, and sulfate were found for the other two species of *Desulfotomaculum* (*D. ruminis* and *D. orientis*), and we conclude that PP_i serves as a source of energy for growth of these anaerobic sulfate-reducing bacteria.

The growth response of *D. ruminis* is proportional to PP_i concentrations up to 0.04 percent and growth is accompanied by the hydrolysis of PP_i to P_i (*3*). In the absence of growth, there is little hydrolysis of added PP_i. Above 0.05 percent PP_i, there appears to be inhibition of growth that may be due to alkalization of the medium (*p*H 8.5). This aspect of growth has not been investigated further; however, similar results were obtained with *D. nigrificans* and *D. orientis*.

It was of interest to determine whether the enzymatic complement of cells grown on the PP_i medium differed significantly from that of cells grown on the lactate-sulfate medium. The specific activities of various enzymes found in PP_i and in cells of *D. orientis* grown on lactate-sulfate medium are shown in Table 2. The reductases of respiratory sulfate reduction—APS reductase (*8*), thiosulfate reductase (*9*), bisulfite reductase (*10*), and ATP-sulfurylase (*11*)—have about the same levels of activity in each cell preparation. Fumarate reductase (*9*) is absent in both preparations, and nitrite reductase (*12*), formate dehydrogenase (*9*), PP_i:acetate kinase (*3*), pyrophosphatase (*3*), and pyruvate dehydrogenase (*9*) are present at similar specific activities. The reason for the significantly higher hydrogenase activity in cells grown on PP_i medium is not known, but it may be due to difficulties with the assay procedure. These observations (also confirmed for *D. ruminis* and *D. nigrificans*) indicate that the bacteria growing on PP_i and lactate-sulfate media exhibit no basic changes in their enzymatic patterns, as has been reported for *Desulfovibrio vulgaris* when grown on different media (*13*).

There is no a priori reason to believe that the utilization of PP_i as a source of energy for growth is limited to the genus *Desulfotomaculum*. We have, therefore, obtained mud samples from a salt water spartina marsh and used them to inoculate the basal medium supplemented with 2.5 percent NaCl, with and without PP_i, under aerobic and anaerobic conditions. Within 24 hours at 37°C only the anaerobic enrichments containing PP_i showed extensive microbial growth and contained an unexpected number of morphological types of microorganisms that persisted through as many as five serial transfers. Similar results have been obtained with PP_i enrichments from freshwater anaerobic environments (*14*). The observed morphotypes were both Gram-negative and Gram-positive and included motile and nonmotile rods of different sizes, with and without spores, and large and small vibrios and cocci. The diversity of cell types strongly suggests that PP_i respiration is not restricted to *Desulfotomaculum*, which are characterized as spore-forming rods. Five new genera of sulfate-reducing bacteria have recently been described by Widdel (*15*), and the diversity of bacterial types found in these enrichment cultures may be due to growth of these new microorganisms on PP_i; however, some initial isolates from these enrichments do not appear to be sulfate-reducing bacteria. We have been unable to find any reports on the existence of PP_i in marine environments, but the formation of polyphosphates is common in bacteria (*16*) and might supply PP_i for the growth of the sulfate-reducing and possibly other bacteria.

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N-β-Alanyldopamine: Major Role in Insect Cuticle Tanning

Abstract. N-β-*Alanyldopamine is the major tyrosine metabolite in the hemolymph and cuticle during pupal tanning in the tobacco hornworm,* Manduca sexta *L. Its concentration in hemolymph increases over 800-fold above larval levels by the start of tanning and decreases as the pupal cuticle darkens and hardens. It is a major catechol in species representing several insect orders and is the preferred substrate for pupal cuticular* o*-diphenol oxidase. In insects,* N-β-*alanyldopamine appears to be the main precursor for tanning chemicals at certain developmental stages.*

N-Acetyldopamine has been considered the principal catecholamine metabolite in sclerotization or tanning of insect cuticle (*1*). We now report that *N*-β-alanyldopamine (NBAD) is the major catecholamine metabolite in hemolymph and cuticle during tanning of pupal cuticle in the tobacco hornworm, *Manduca sexta*, and that *N*-acetyldopamine is a relatively minor component.

Hemolymph was extracted from *M. sexta*, and the catechols were analyzed by liquid chromatography with electrochemical detection (LCEC) (*2*). The major oxidizable substance in hemolymph extracts from pharate or newly ecdysed pupae was an unknown compound with a longer retention time than dopa, dopamine, or *N*-acetyldopamine (compound V in Fig. 1). Labeled compound V was

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