APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1988, p. 386–396
0099-2240/88/020386-11$02.00/0
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Vol. 54, No. 2

Effect of Phosphate on the Corrosion of Carbon Steel and on the Composition of Corrosion Products in Two-Stage Continuous Cultures of *Desulfovibrio desulfuricans*†

PAUL J. WEIMER,* MARGARET J. VAN KAVELAAR, CHARLES B. MICHEL, AND THOMAS K. NG‡

Central Research and Development Department, Experimental Station, E. I. du Pont de Nemours & Co., Inc., Wilmington, Delaware 19898

Received 8 September 1987/Accepted 20 November 1987

A field isolate of *Desulfovibrio desulfuricans* was grown in defined medium in a two-stage continuous culture apparatus with different concentrations of phosphate in the feed medium. The first stage (V1) was operated as a conventional chemostat ($D = 0.045\ h^{-1}$) that was limited in energy source (lactate) or phosphate. The second stage (V2) received effluent from V1 but no additional nutrients, and contained a healthy population of transiently starved or resting cells. An increase in the concentration of phosphate in the medium fed to V1 resulted in increased corrosion rates of carbon steel in both V1 and V2. Despite the more rapid corrosion observed in growing cultures relative to that in resting cultures, corrosion products that were isolated under strictly anaerobic conditions from the two culture modes had similar bulk compositions which varied with the phosphate content of the medium. Crystalline mackinawite (Fe_9S_8), vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$], and goethite [FeO(OH)] were detected in amounts which varied with the culture conditions. Chemical analyses indicated that the S in the corrosion product was almost exclusively in the form of sulfides, while the P was present both as phosphate and as unidentified components, possibly reduced P species. Some differential localization of S and P was observed in intact corrosion products. Cells from lactate-limited, but not from phosphate-limited, cultures contained intracellular granules that were enriched in P and Fe. The results are discussed in terms of several proposed mechanisms of microbiologically influenced corrosion.

Anaerobic corrosion of iron and ferroalloys is a serious problem which causes severe economic losses because of destruction of industrial and process equipment, devaluation of industrial products (e.g., oil and natural gas), and downtime of processes (14, 25). A considerable fraction of this corrosion is thought to be mediated by microorganisms (6, 14, 25). Under anaerobic conditions, the chief biological agents are thought to be the dissimilatory sulfate-reducing bacteria (SRB). The mechanisms by which these organisms cause or accelerate corrosion has been a matter of controversy for decades (see reference 14 for a recent review). The most widely accepted mechanistic model is the cathodic depolarization theory, which was first proposed by Von Wolzogen Kuhr and Van der Vlugt in 1934 (28). This model, which was subsequently modified by other workers to account for new experimental observations (9, 14, 21), remains the most widely accepted mechanism for anaerobic biocorrosion. An alternative model descried by Iverson (16, 17) and Iverson and Olson (18), in which the corrosive activity of sulfate reducers is ascribed to the production of highly corrosive reduced phosphorous compounds, is supported by only limited experimental evidence and has not been widely accepted.

The possible importance of reduced P-containing compounds in anaerobic biocorrosion led us to examine the effects of their likely ultimate precursor, P_i, on the corrosion process. We report here the results of studies on the biocorrosion of carbon steel by continuous, axenic cultures of SRB; these results demonstrate the importance of phosphate availability on the rate and the extent of the corrosion process.

MATERIALS AND METHODS

Organism. *Desulfovibrio desulfuricans* G100A was isolated from a producing oil well in Ventura County, Calif. The organism was isolated as a single colony on an agar plate streaked from an end dilution series of enrichment tubes of BTZ-4 medium (see below) containing lactate as the electron donor and sulfate as the electron acceptor. Standard anaerobic (Hungate) techniques as modified by Balch and Wolfe (5) were used for the enrichment, isolation, and maintenance of cultures.

Chemostats and culture medium. The two-stage continuous culture vessels used in this study have been described previously (P. J. Weimer and T. K. Ng, Proc. Annu. Meet. Natl. Assoc. Corrosion Engineers, in press). This apparatus contained two culture vessels linked in series. The first vessel (designated V1) received culture medium, while the second vessel (designated V2) received spent culture medium (including bacterial cells) which overflowed from V1, but V2 received no additional nutrients. A dilution rate (D) of $0.045\ h^{-1}$ was used for all experiments. Culture vessels were maintained at ambient temperature (19 to 24°C); the temperature within the vessels was recorded at 6-h intervals from immersed thermocouple probes.

A mineral salts medium containing sodium lactate (energy source) and sodium acetate (supplementary carbon source) but lacking yeast extract, vitamins, or other metabolizable carbon sources was used for all experiments. The composition and preparation of this medium has been described previously (Weimer and Ng, in press).

Analysis of cultures. Samples were removed at 1- to 4-day

* Corresponding author.

† This is contribution no. 4516 of the Central Research and Development Department.

‡ Present address: Medical Products Department, E. I. du Pont de Nemours & Co., Inc., Glasgow Site, Wilmington, DE 19898.

intervals and analyzed for pH and cell density. For cell density analysis, a portion of the culture was suspended in an equal volume of glycerol and examined with a phase-contrast microscope and a Petroff-Hausser counting chamber. A portion of each sample was also clarified by centrifugation, and the supernatant was analyzed for residual lactate (by using a lactate dehydrogenase assay coupled to an autoanalyzer [Technicon]), phosphate (by using the ammonium molybdate-stannous chloride method described by the American Public Health Association [3]), and total soluble sulfides (by the methylene blue method described by Siegel [26]).

Corrosion measurements. Generalized corrosion of carbon steel was measured by determining weight loss by using 5.08 by 2.54 by 0.16 cm (1 by 2 by 1/16 in) 1020 carbon steel (mild steel) coupons (Metal Samples, Munford, Ala.) polished with a 120-grit belt. The coupons were hung (two per vessel) in the reactors by their center holes in a vertical position from J-shaped, 0.16-cm (1/8 in)-outer-diameter stainless steel rods that were held to the headplate by bored-through fittings (Swagelock; Crawford Fitting Co., Solon, Ohio). With coupons of this type, a weight loss of 1 g over a 60-day run period corresponded to an average corrosion rate of 0.274 mm per year (10.8 milli-inches per year, or 61 mg/dm^2 per day). Corrosion was also monitored at 6-h intervals with electrical resistance (ER) probes (type T10; Rohrbach/Cosasco, Santa Fe Springs, Calif.) connected to a Corrosometer (model 4208; Rohrbach).

Recovery of coupons and corrosion products. Coupons (with their adherent corrosion products) were removed from the chemostats after 60 days of exposure and were immediately dropped into wide-mouth bottles containing 0.0002% resazurin that was reduced with a trace of sodium dithionite. The sealed bottles were transferred to an anaerobic glove bag (Coy Laboratory Products, Ann Arbor, Mich.). The corrosion products were dislodged from the coupons with a spatula and separated from the liquid by filtration through 3-μm-pore-size polycarbonate membranes. The coupons were scraped with a fine-bristled toothbrush and then dipped for a few seconds in Clarke's solution (4) to remove any remaining acid-labile corrosion products. The corrosion products recovered by filtration were washed with anaerobic water and dried over silica gel in a dessicator in the glove bag (to prevent oxidation of the samples) and then gently ground to a fine powder with a spatula.

Surface measurements. The surface geometry of the coupons were determined with a profilometer (Sloan-Dektak II) by using a slow scan speed over a series of 1-mm-wide tracks perpendicular to the grinding lines which paralleled the long axis of the coupons.

Analysis of bulk corrosion products. Fe, other metals, P, and S contents were determined by inductively coupled plasma atomic emission spectroscopy after dissolution of dried corrosion products in aqua regia. Organic C and Kjeldahl N were analyzed by Micro-Analysis, Inc. (Wilmington, Del.). Some of the dried corrosion products were also analyzed by powder X-ray diffraction with a Norelco diffractometer (40 kV of radiation energy through a diffraction scanning range of 2 to 60 degrees). The components were identified by using a multipeak analysis library maintained at the National Bureau of Standards.

Compositional mapping of intact corrosion products. Chunks of corrosion products were recovered intact and dried under strictly anaerobic conditions for several days over silica gel in a dessicator that was placed in an anaerobic glove bag. The intact material was removed from the glove bag and placed in a plastic beaker. Loctite 290 penetrating sealant (Loctite Corp., Newington, Conn.) was added to completely cover the corrosion product, and the beaker was placed in a vacuum dessicator for 1 h to facilitate penetration of the sealant by removal of entrapped gases from the corrosion product. The sealant was next allowed to harden by placing it in an anaerobic glove bag for 2 weeks. The resulting block was removed from the beaker, and the excess sealant was trimmed away with a razor blade. The corrosion product, which by this time was sufficiently immobilized to permit handling, was embedded in a polystyrene resin. After the samples were sectioned in various directions and polished with diamond powder, they were subjected to energy-dispersive X-ray analysis by using a microprobe (JEOL JXA-35) operated at 25 kV; a Tracor-Northern 5600 microanalysis system was used for digital analysis of the X-ray beam.

Electron microscopy of bacterial cells. Samples (80 ml) were removed from chemostats and were filtered through 3 μm-pore-diameter polycarbonate membranes (Nuclepore Corp., Pleasanton, Calif.) to remove most of the suspended inorganic material. The filtrates were centrifuged at 37,000 × *g* for 60 min, and the pellet was suspended in 10 mM morpholinoethanesulfonate (MES) buffer (pH 6.5) supplemented with potassium phosphate at the same concentration as that in the original feed medium in the chemostat. The suspension was centrifuged again, and the pellet was fixed overnight in 5% glutaraldehyde. Subsequent sample preparation procedures were those described by Jones and Chambers (19). Thin sections were visualized with a transmission electron microscope (Hitachi 600) at an accelerating voltage of 100 kV.

RESULTS

Characteristics of strain G100A. The organism was a strictly anaerobic, non-sporeforming, actively motile vibrio, approximately 2.5 by 0.5 μm in size. Growth by sulfate respiration was observed by using H_2, lactate, pyruvate, choline, or glycerol as electron donors. Methanol or acetate did not serve as electron donors. Growth with lactate as the electron donor was observed with sulfate, sulfite, or thiosulfate (but not nitrate or elemental sulfur) as the electron acceptors. Pyruvate and choline were actively fermented with the formation of H_2. Cultures also grew by dismutation of fumarate in the absence of sulfate.

Growth on lactate-sulfate was optimal at 34°C and was completely inhibited by chlorhexidine (hibitane) at 25 mg/liter (but not at 10 mg/liter). Hydrogen was detected in large amounts in the headspace above batch cultures grown on lactate-sulfate.

Although no vitamins or complex nutrients were required for growth, the addition of 0.1% yeast extract to the basal medium decreased the doubling time in batch (test tube) cultures from 5.1 to 3.7 h, and increased the growth yield by ~40%. A defined vitamin mixture (29) had no significant effect on the growth rate or the growth yield.

Whole cells yielded a positive test for desulfoviridin. Antibodies to the adenosine-5′-phosphosulfate reductase purified from cell extracts of strain G100A cross-reacted strongly with cell extracts of *Desulfovibrio* strains and, to a lesser extent, with cell extracts of other genera of dissimilatory sulfate-reducing bacteria (J. M. Odom, Abstr. Annu. Meet Am. Soc. Microbiol. 1987, K77, p. 215). On the basis of the physiological and biochemical characteristics described above, strain G100A was classified as a strain of *D. desulfuricans*.

Continuous culture parameters. *D. desulfuricans* G100A was grown in continuous culture at a dilution rate of 0.045 to 0.048 h^{-1} and at phosphate concentrations of 0.3, 1.8, 4.0, and 10 mM. Average values for various culture parameters are given in Table 1. With 11 mM lactate as the growth-limiting nutrient, the organism consistently produced cell densities in the range of 1.8×10^8 to 3.0×10^8/ml in the growing-cell vessel (V1); cell concentrations in the resting-cell vessel (V2), as determined by direct cell counts, averaged 8 to 23% lower. Despite the lower cell densities, these resting cells were quite viable, as evidenced by (i) their uniform phase-dark appearance under phase-contrast microscopy, and (ii) their rapid growth (with a <2-h lag) in batch cultures on transfer to fresh medium containing 11 mM lactate. The residual lactate concentration in both vessels was in the range of 0.05 to 0.13 mM, and did not differ significantly between the two vessels. These cultures were considered to be lactate limited and high in phosphate.

Continuous cultures grown in the same medium but with only 0.05 mM phosphate added to the feed medium initially displayed signs of phosphate limitation, including (i) significant amounts of residual lactate in the medium; (ii) culture pH values ~0.2 units lower than those obtained in chemostats run at higher phosphate concentrations (due to less sulfate reduction and, thus, less proton consumption); and (iii) lower cell density (due to less lactate oxidation). Within a week, however, the culture parameters (pH, cell density, residual lactate concentration) changed to resemble those obtained under conditions of lactate limitation. This culture was thus considered to be lactate limited but low in phosphate.

Cultures were also grown in the same medium but with 0.01 mM phosphate. A considerable elevation in the residual lactate content of V1 was observed, along with some consumption of the excess lactate in V2. It appeared that in both vessels the cultures were phosphate limited, although the additional consumption of lactate in V2 suggested that the cells in this vessel adapted to more efficient phosphate utilization.

With minor exceptions, good agreement was obtained for each measured reactor parameter (cell density, pH, temperature, and concentrations of lactate or free sulfide) between V1 and V2 within an individual experiment, suggesting that the vessels were similar with respect to the important chemical components influencing corrosion and differed only with respect to the physiological state of the cells. Good agreement was also observed for these parameters among different experimental runs at different phosphate concentrations.

Although considerable amounts of H_2 were evolved during growth of the organism on lactate in batch culture, H_2 was not detected on periodic testing of the headspace of the chemostat cultures during either lactate-limited or phosphate-limited growth.

Characteristics of the corroded metal. The metal coupons and probes that were removed from the reactors after 60 days of exposure were encrusted with a delicate black granular corrosion product which was easily dislodged in chunks from the residual metal surfaces.

Examination of corroded coupons under low-power magnification after removal of the corrosion product revealed a conversion of the grit-polished flat surface of the coupon to an irregular surface with a relatively uniform, etched appearance (Fig. 1A). Flaking or exfoliation of metal from the coupon surface was occasionally observed, but no pitting of the metal surface was detected. Coupons viewed along their edges, however, had a severely pitted appearance that is characteristic of end-grain corrosion (Fig. 1B).

A more quantitative measurement of this surface roughness was obtained by using a profilometer, which employs a diamond stylus passed along the coupon to provide a high-resolution map of the metal surface, along with a direct measurement of average surface roughness. Typical scans perpendicular to the original grit-polished surface revealed that unexposed coupons contained depressions (produced in the grinding process) of up to 8 μm in depth. Coupons exposed to the bacterial cultures for 60 days showed even more uneven surfaces, with depressions of up to 23 μm in

TABLE 1. Culture conditions in chemostat vessels at different feed phosphate concentrations[a]

Feed PO_4^{3-} (mM)	Vessel[b]	D (h^{-1}) (n = 42 to 56[c])	Cell density, 10^8/ml (n = 25 to 28)	pH (n = 25 to 28)	Temp (°C) (n = 240)	Total free sulfide (mM) (n = 6 to 18)	Residual lactate (mM) (n = 25 to 28)	Residual phosphate (mM) (n = 3 to 12)
0.01	V1	0.0472	1.66	6.58	24.0	1.8	1.61	<0.01
	V2		1.32	6.63	23.1	2.3	0.32	<0.01
0.05	V1	0.0464	2.25	6.67	27.6	0.9	0.25–0.07[d]	<0.02
	V2		1.85	6.68	27.2	0.8	0.05	<0.02
0.3	V1	0.0460	2.95	6.69	24.7	1.5	0.19	NT[e]
	V2		2.71	6.73	23.6	1.5	0.04	NT
1.8	V1	0.0463	2.42	6.59	27.5	1.1	0.08	1.8
	V2		1.93	6.60	26.9	0.9	0.08	1.8
4.0	V1	0.0479	2.55	6.61	26.6	0.9	0.13	3.7
	V2		2.01	6.61	26.6	0.8	0.10	3.8
10.0	V1	0.0465	2.29	6.54	28.1	1.2	0.11	9.8
	V2		1.77	6.56	26.8	0.2	0.08	10.1

[a] All values are means for each culture over the 60-day run periods. All cultures were lactate limited, except for the culture fed 0.01 mM phosphate, which was phosphate limited.
[b] V1, Growing cell vessel; V2, resting cell vessel.
[c] n is the number of determinations during a 60-day run period for each given parameter.
[d] Decreased from 0.25 to 0.07 mM during the first 7 days of the run period.
[e] NT, Not tested.

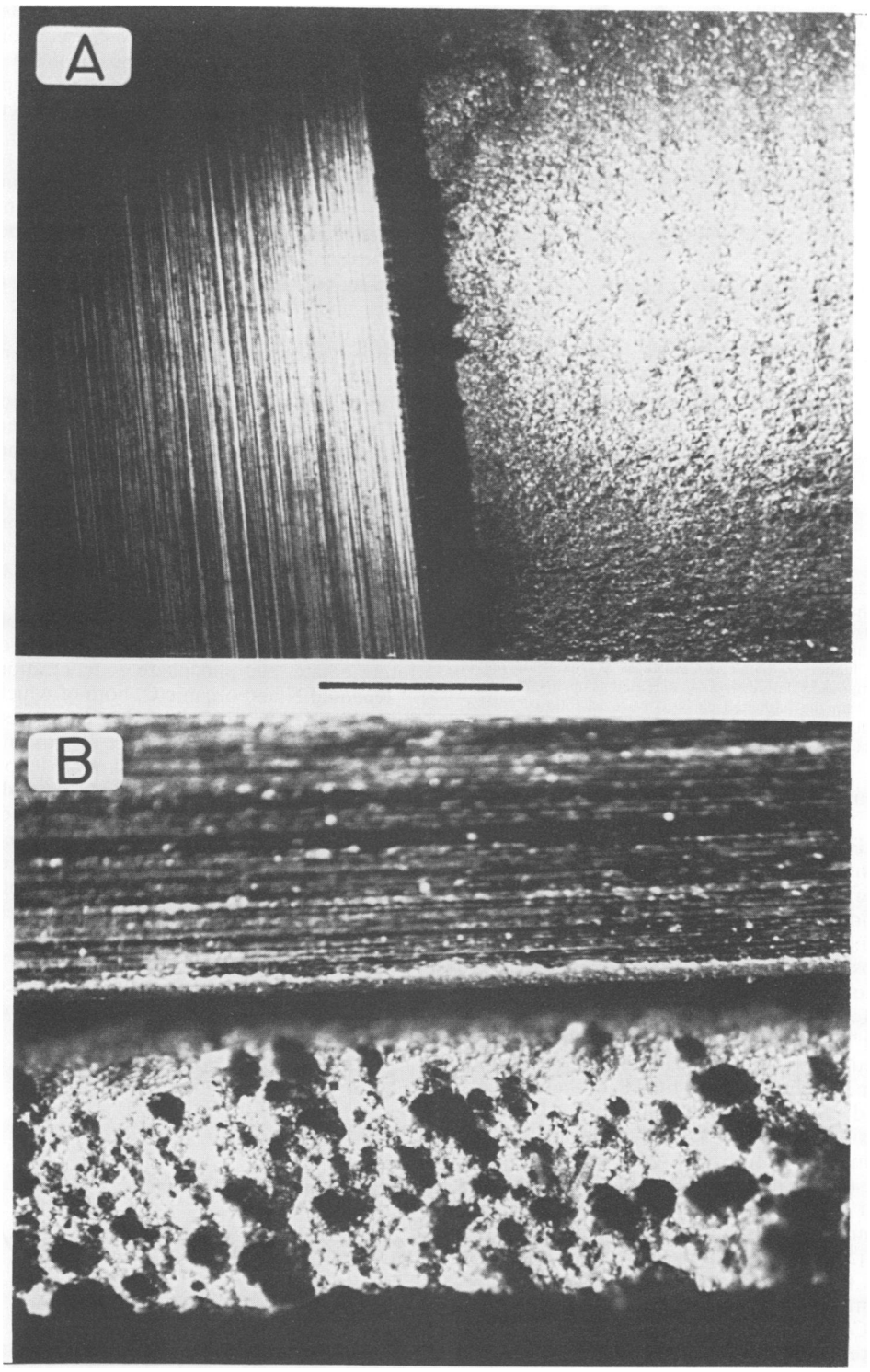

FIG. 1. Photomicrographs of carbon steel coupons not exposed to bacterial culture or exposed for 60 days to lactate-limited chemostat cultures of *D. desulfuricans* that contained 4 mM phosphate in the feed medium. (A) Flat surface of coupon. (B) Edge of coupon. Bar, 1 mm.

depth (data not shown). Because of the very large variability that was encountered in the profiles obtained from the scanning of different portions of the same coupon, it was determined that a good measure of surface roughness and its variability could be provided by two values: a mean of several average roughness values and the relative error in the average roughness of all scans made on the same coupon. Data expressed in this manner (Table 2) revealed that (i) coupons that were exposed to the bacterial cultures displayed consistently more roughness and surface variability than did unexposed coupons, and (ii) there was no significant difference in the roughness of the coupons that

TABLE 2. Surface roughness of unexposed and exposed coupons

Coupon	Average surface roughness (μm)[a]	Relative error (%)[b]
Not exposed	1.581	8.1
	1.523	15.0
Exposed to the following cultures:		
0.05 mM PO_4^{3-}		
V1	4.342	20.0
	3.596	29.7
V2	3.984	38.8
	3.111	57.0
1.8 mM PO_4^{3-}		
V1	3.694	15.4
	5.788	65.5
V2	3.962	37.8
	2.251	25.4
10 mm PO_4^{3-}		
V1	4.190	23.6
	4.846	56.2
V2	1.929	17.1
	5.258	57.0

[a] Values are means from the scanning of five randomly selected areas perpendicular to the long axis of separate coupons by using a profilometer.

[b] Relative error is (standard deviation of surface roughness/mean surface roughness) × 100%. A higher relative error indicates a greater degree of surface topography. Composite relative errors (calculated as means of relative errors from coupons within the indicated classes) were as follows: all V1, 35.1%; all V2, 38.9%; all 0.05 mM, 36.4%; all 1.8 mM, 36.0%; all 10 mM, 38.5%; all unexposed coupons, 11.6%.

were exposed to cultures maintained under different conditions.

Corrosion data. For each separate 60-day run, the corrosion rate of carbon steel (as determined by the ER probe) exhibited a similar time course. Corrosion gradually increased over a period of several weeks before it stabilized at a relatively constant value that was characteristic of each culture. A clear positive correlation was evidenced between the rate of corrosion of carbon steel and the availability of phosphate in the feed medium. With increasing phosphate concentration, the lactate-limited, high-phosphate cultures exhibited increased corrosion rates, as measured both by weight loss from metal coupons and by ER probes (Fig. 2). The lactate-limited, low-phosphate culture displayed no measurable corrosion (by the ER probe) during its first week, at which time the culture was apparently phosphate limited; however, once this culture adapted to the lower phosphate content and became lactate limited, corrosion increased to measurable levels which stabilized at low levels (0.13 mm per year [5 milli-inches per year]) for the remainder of the experiment. Corrosion rates in the phosphate-limited (0.01 mM phosphate) culture remained very low throughout the experiment. Corrosion rates measured with the ER probe were consistently below those obtained by weight-loss methods, although good correlation ($r = +0.82$) was obtained between the two methods by linear regression analysis.

In all cases, the corrosion rates in the growing-cell vessels were significantly greater than were those of the resting-cell cultures. One striking observation was the virtually insignificant rates of corrosion (<0.03 mm per year [<1 milli-inches per year]) observed in resting-cell cultures at low phosphate concentrations, even at relatively high cell densities and significant sulfide concentrations.

Characteristics of the corrosion products. The amount of corrosion products adhering to the coupons roughly correlated with the extent of corrosion of the underlying metal coupons, although the gross physical properties of the corrosion products were independent of the culture conditions. The wet granular corrosion product slowly oxidized to a rust color if it was left exposed to air, but it remained black in color if it was held under anaerobic conditions. Material dried under anaerobic conditions did not display gross evidence of oxidation on subsequent exposure to air, with one exception. One of the corrosion products from the growing-cell chemostat (V1) maintained at 0.3 mM phosphate underwent a dramatic exothermic oxidation on removal from the glove bag, and it ignited its glassine paper holder. This combustion resulted in a change in the color of the solid product from black to brown and was accompanied by a release of acrid vapors of unknown composition.

The bulk compositions of the powdered corrosion products recovered from chemostats maintained under different steady-state conditions are shown in Fig. 3. The products contained approximately 50% Fe on a dry weight basis, regardless of the phosphate concentration of the feed medium. The P content of the products increased with increasing phosphate concentration in the feed and reached unexpectedly high levels (7% of dry weight). S and carbonate contents were considerable in all samples and exhibited maxima (~26 and ~7%, respectively, of dry weight) at intermediate feed phosphate concentrations.

Kjeldahl N and organic C, both of which are indicators of microbial cell material, together composed approximately 1 to 3% of the dry weight of the corrosion product (Fig. 4). Examination by phase-contrast microscopy of freshly recovered product, which was suitably diluted in sterile culture media, indicated that the corrosion product contained viable cells, but that cell populations in this adherent material were not much larger than those observed in the bulk liquid phase of the chemostats themselves. Attempts to analyze the corrosion products for total carbohydrates (15) were unsuccessful due to interferences cause by the release of ferrous iron on acid treatment.

Colorimetric assays of acid-hydrolyzed corrosion products indicated that virtually all of the S was in the form of

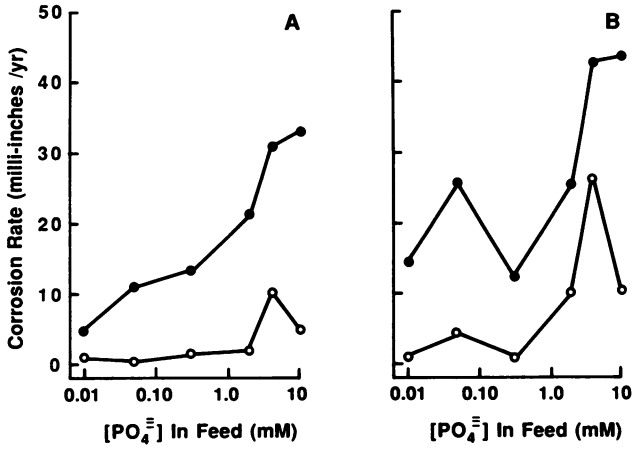

FIG. 2. Corrosion rates of carbon steel by continuous cultures of *D. desulfuricans* G100A. (A) Average of 240 corrosion rates (collected at 6-h intervals over 60-day runs) from electrical resistance probes. (B) Average corrosion rates over the same 60-day runs described for panel A, calculated from the weight loss from paired coupons. Symbols: ●, V1; ○, V2.

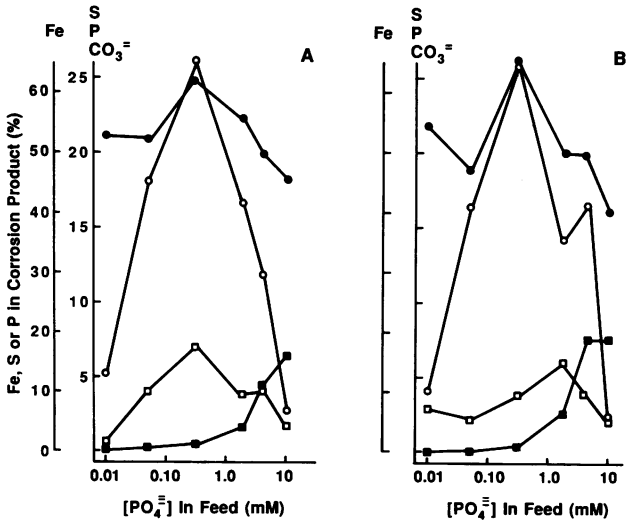

FIG. 3. Compositions of corrosion products recovered from coupon surfaces after immersion for 60 days in culture vessels fed media with different amounts of phosphate. (A) Fe (●), S (○), P (■), and carbonate (□) contents of products from growing (V1) cultures. (B) The same elements described for panel A from resting (V2) cultures.

mineral sulfides, presumably ferrous sulfides. Acidification of the corrosion products and subsequent qualitative assays for phosphine (11) gave equivocal results, suggesting that any phosphides in the corrosion products were present in only small amounts. However, treatment of the corrosion products with more dilute acid (0.1 or 1 N HCl), which should have dissolved mineral phosphates (27), resulted in incomplete hydrolysis of the samples and recovery of only 24 to 93% of the total P as phosphate.

Within an individual experiment, the corrosion products from coupons in V1 and V2 were similar in bulk composition, despite the considerable (1.6- to 22-fold) differences in corrosion rates observed in the two vessels.

Examination of powdered corrosion products by X-ray diffraction identified mackinawite (Fe_9S_8) as the primary crystalline mineral. At low feed phosphate concentrations, some goethite [FeO(OH)] was observed. At higher feed phosphate concentrations, significant amounts of vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$] were detected. In all cases, the extent of peak broadening suggests that the crystallites were relatively small or had deformed lattices or other structural defects (23).

Compositional mapping of the corrosion products. To determine the three-dimensional distribution of the elements in the corrosion product, samples of intact corrosion product from growing chemostat cultures (V1) were studied by energy-dispersive X-ray analysis. Examples of the resulting elemental maps are shown in Fig. 5. All sections contained obvious cavities. The corrosion product from the phosphate-limited culture displayed a reasonably uniform distribution of Fe and S and contained very little P. The corrosion product from the lactate-limited culture grown at a high (10 mM) feed phosphate concentration displayed distinct zones where P and S predominated. In general, these zones were of variable shape and were distributed randomly throughout the product. In at least one section of the corrosion product, however, definite bands of Fe and S were observed both adjacent to the coupon surface and at the outer edge of the product adjacent to the culture medium (Fig. 5 row D).

Analysis of cells. Transmission electron microscopy of thin sections (Fig. 6A and B) indicated that cells from lactate-limited chemostat cultures contained electron-dense intracytoplasmic granules. Cells from actively growing cultures (V1) contained more granular material than did those from resting-cell cultures (V2), although a small minority of cells from each culture lacked the granules. Attempts to isolate the intracytoplasmic granules for subsequent chemical analysis were unsuccessful, since it was not possible to separate either intact cells or lysed cell fractions from the large amounts of precipitated mineral sulfides and phosphates in the vessels. Electron probe microanalysis of the granules in thin sections (Fig. 7) revealed that the granular material was enriched in P and Fe relative to the rest of the cytoplasm. The extent of this enrichment was highly variable, however, suggesting the presence of other elements, most likely those of $z < 10$ that would not be detected by this method. No polyphosphates were detected in granule-containing cells by either direct staining (19) or wet chemical methods (12).

Cells cultured under phosphate-limited conditions at the same concentration of Fe^{2+} (0.5 mM) did not produce electron-dense granules. Instead, they contained a different type of granular inclusion which resisted heavy metal staining (Fig. 6C and D). Microprobe analysis of this material revealed a lack of significant amounts of Fe, P, or any other element with $z > 10$. The availability of phosphate was not by itself sufficient for the formation of intracellular electron-dense granules, as cells grown under conditions of lactate limitation in a separate chemostat at low (0.05 mM) Fe^{2+} concentrations also lacked these granules.

DISCUSSION

In all of the chemostat experiments, the corrosion rate of carbon steel increased over time until a relatively stable plateau value (which varied with the individual culture) was reached. Because the bacterial culture was allowed to reach a steady state prior to the insertion of probes and coupons, the increased corrosion rate over time was probably due at least in part to the establishment of electrochemical cells following the deposition of ferrous sulfide on the metal

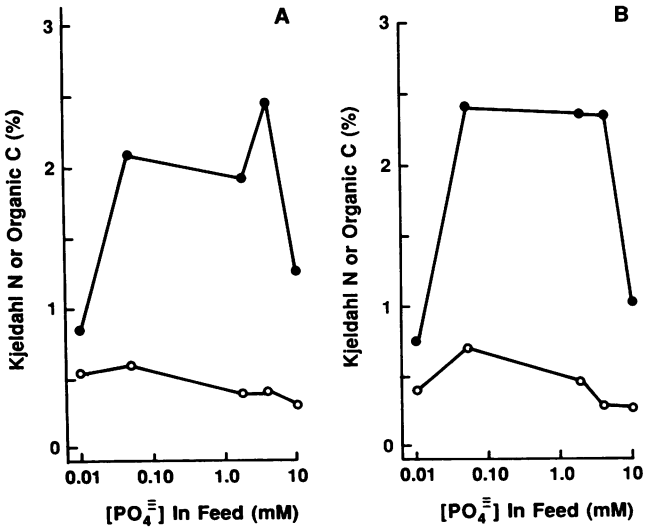

FIG. 4. Organic C (●) and Kjeldahl N (○) contents of corrosion products recovered from coupon surfaces after immersion for 60 days in culture vessels fed media with different amounts of phosphate. (A) Growing (V1) cultures. (B) Resting (V2) cultures.

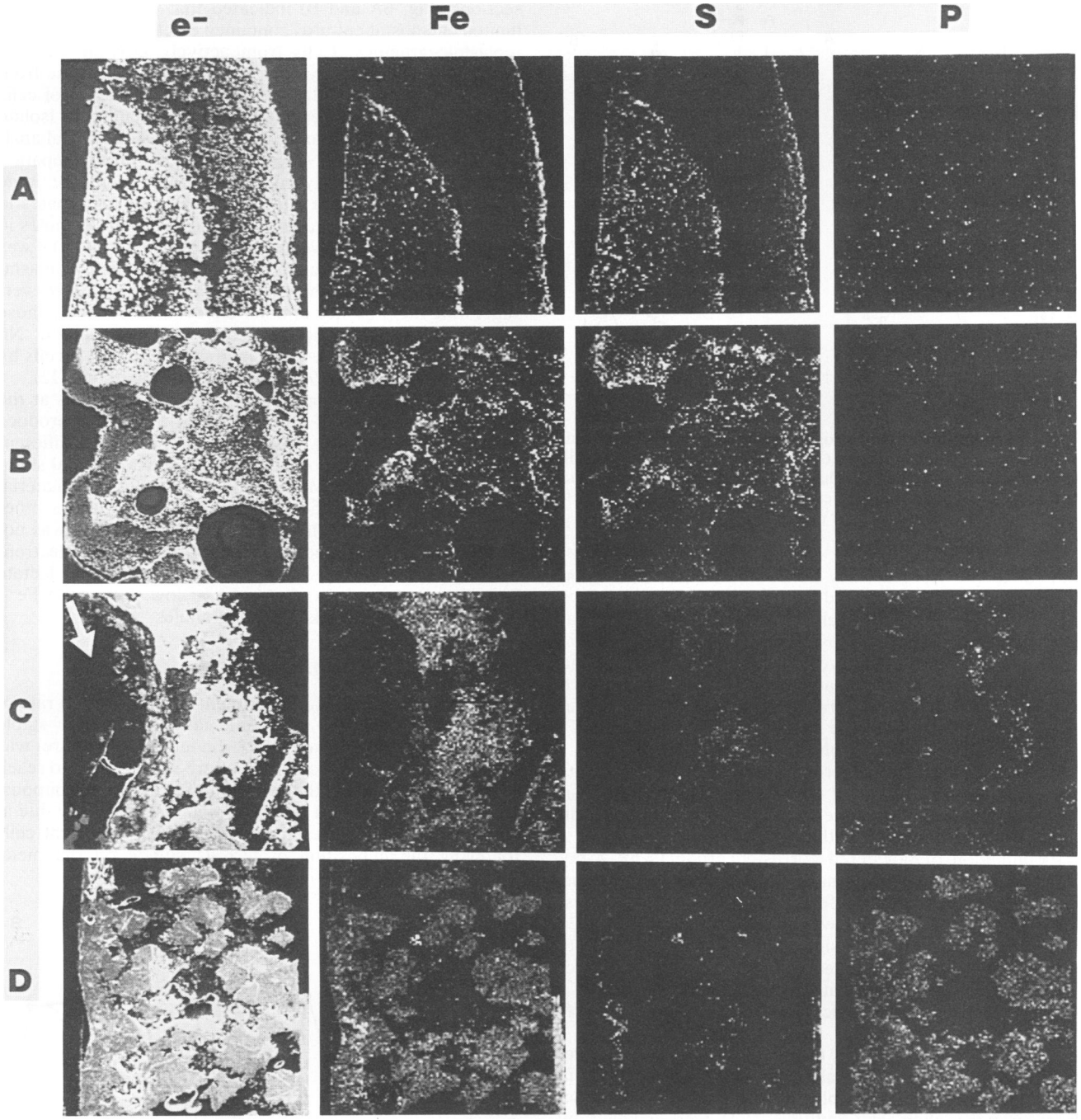

FIG. 5. Examples of elemental mapping (Fe, S, and P) of intact sections of corrosion products by energy-dispersive X-ray analysis. The back-scattered electron images (column labeled e^- in each row) are provided to give a visual image of the morphology of each section. In all cases the surface adjacent to the metal coupon is at the left of each picture, except in the case of row C, in which the product is oriented at an angle to show the corrosion product wrapped over the edge of the coupon; in this case, the outline left by the coupon can be readily observed (arrow). Magnifications are ×25 (rows A, C, and D) and ×7.5 (row B). Rows A and B, Corrosion product from phosphate-limited V1 culture (feed phosphate concentration, 0.01 mM; bulk corrosion product, 53.0% Fe, 5.2% S, <0.01% P); rows C and D, corrosion product from lactate-limited V1 culture (feed phosphate concentration, 10 mM; bulk corrosion product, 45.3% Fe, 2.6% S, 6.4% P).

surface (20–22). The rates of corrosion in these continuous culture experiments were considerably higher than those reported in batch culture biocorrosion experiments (for a summary, see reference 22) and were similar to those reported by Booth et al. (7) for continuous cultures of different strains of *Desulfovibrio*.

The two-stage continuous culture system used in these experiments allowed us to compare corrosion rates in cultures which had essentially identical chemical compositions and cell densities but which differed in the physiological state of the cells. Actively growing cultures displayed a more rapid corrosion of carbon steel than did resting cell cultures,

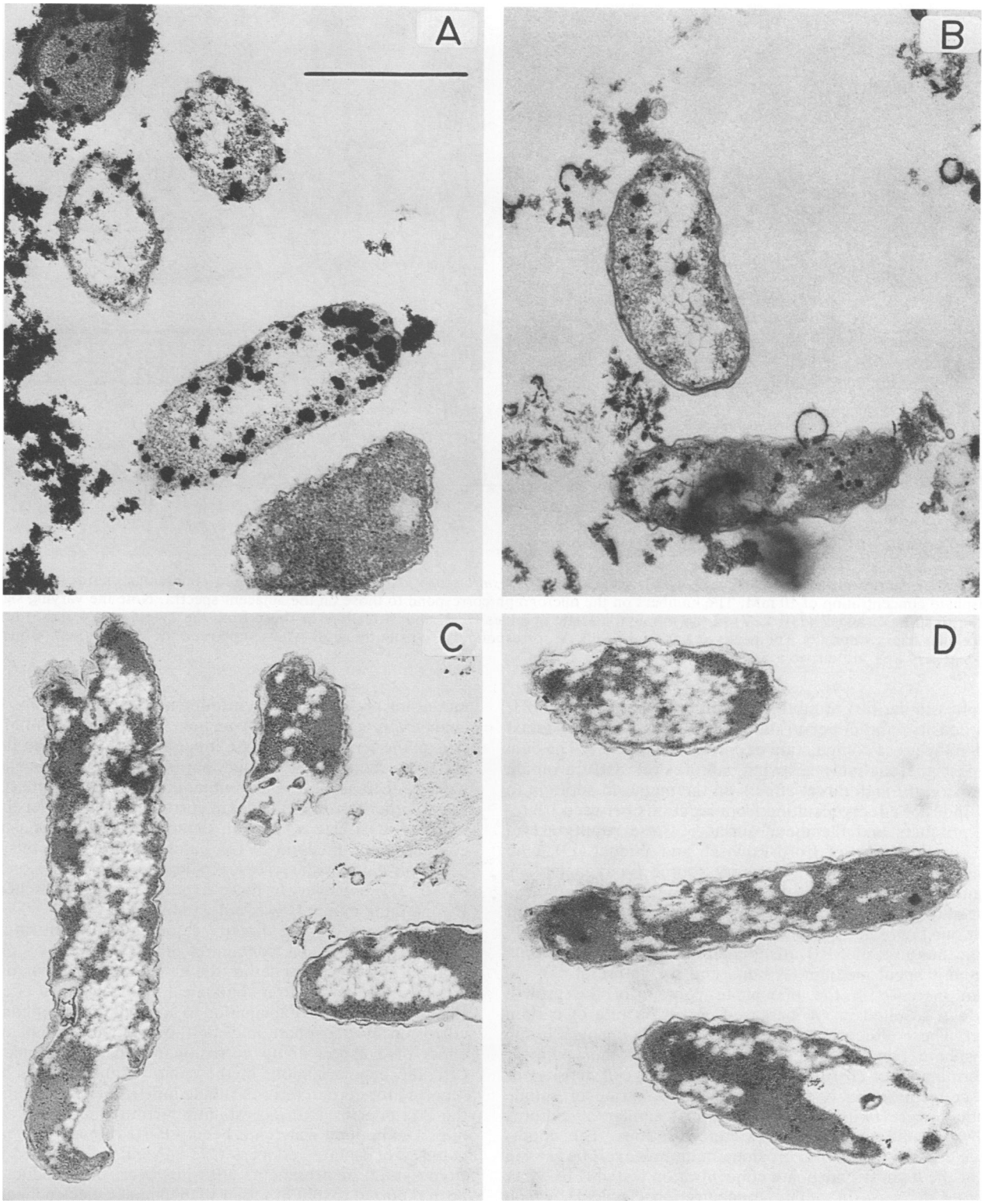

FIG. 6. Representative photomicrographs of thin sections of *D. desulfuricans* grown in chemostat culture under conditions of lactate limitation (phosphate excess) and phosphate limitation. (A) Growing cells (V1); 10 mM feed phosphate. (B) Resting cells (V2); 10 mM feed phosphate. (C) Growing cells; 0.01 mM feed phosphate. (D) Resting cells; 0.01 mM feed phosphate. Bar, 1 μm.

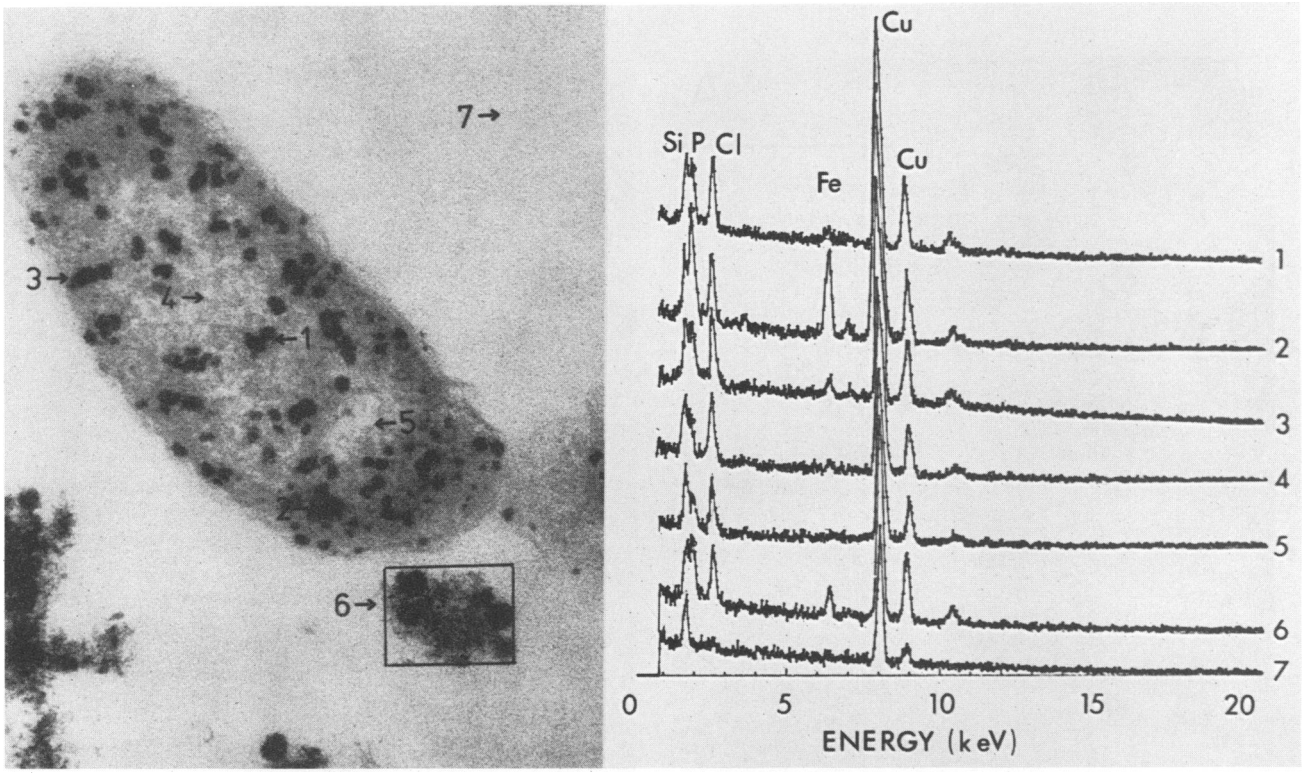

FIG. 7. Electron probe microanalysis of thin sections of *D. desulfuricans* grown in a lactate-limited continuous culture with a feed phosphate concentration of 10 mM. The numbers on the micrograph correspond to those on the adjacent spectra. Note the varying signals for Fe (6.40 keV) and P (2.01 keV) in the intracytoplasmic granules (1, 2, and 3) relative to those from the cytoplasm (4 and 5) or the noncellular background (7). The peaks at 8.05 and 8.90 keV correspond to Cu from the grids which supported the thin sections, while the peaks at 1.74 keV are due to a Si contaminant.

despite similarities in culture conditions (temperature, pH, cell density, and concentration of substrates and products) and in the gross composition of corrosion products. The data suggest that actively growing cultures of SRB promote corrosion through direct effects on the metal, in addition to any indirect effects resulting from reactions between bacterial products and the metal surface. These results are in accord with those of Cord-Ruwisch and Widdel (13), who reported that an auxiliary energy source (viz., lactate) is required for coupling spontaneously generated cathodic hydrogen to growth of the SRB. The data are also in agreement with our previous report that the corrosion rates in slightly acidic cultures of SRB are greater than those in a sterile synthetic spent medium (Weimer and Ng, in press).

An increase in the phosphate content of the growth medium resulted in an increased corrosion rate of carbon steel. These effects were specifically due to changes in the phosphate content of the medium, since other putative determinants of corrosion rate such as pH, cell density, or the concentrations of residual lactate substrate or sulfide product were (with minor exceptions) similar in cultures grown on different phosphate concentrations. The importance of free sulfide on corrosion could, however, be seen in V2 at the highest phosphate concentration tested (Fig. 2). A fivefold decrease in sulfide concentration (Table 1), which was apparently due to a slight leak in the fermentor headplate, was accompanied by a low corrosion rate, even in the presence of 10 mM phosphate.

Analysis of the corrosion products revealed that the P content of the corrosion product increased dramatically with increasing phosphate concentration in the feed medium. This change was accompanied by an increase in the amount of the crystalline ferrous phosphate mineral vivianite in the product and a reduction in the amount of goethite and mackinawite. A decrease in the predominance of mackinawite (relative to other ferrous sulfides) in corrosion products has been reported (20, 22) to occur with an increase in the ferrous iron content in the medium of fed batch cultures, and this has been correlated with increases in the observed corrosion rate (8, 20, 22); however, in these experiments the presence of P-containing minerals was not examined.

Because we did not directly examine corrosion rates in cell-free spent media containing different levels of phosphate, it is not clear whether the enhancement of corrosion by phosphate is due to its biological or its chemical effects. In fact, one possible explanation to account for the enhanced corrosion by phosphate may be the direct electrochemical effect of a P-containing corrosion product in stimulating cathodic depolarization, in the same manner as has been reported for certain ferrous sulfide minerals (9, 21). Some of the P is present as the crystalline ferrous phosphate vivianite, a compound which has been reported to occur in batch cultures of sulfate reducers (17, 24) and in corroded iron from natural environments (10); however, this product has been reported to inhibit rather than enhance electrochemical corrosion (17). The incomplete recovery of P on mild acidification of the corrosion products suggests that more reduced P-containing compounds may be present in these products. In particular, ferrous phosphides (suggested by Iverson [16, 17] and Iverson and Olson [18] as likely reduced

P compounds in cultures of sulfate reducers) are particularly resistant to chemical degradation and are thus extremely difficult to detect qualitatively or quantitatively (1). We regard the pyrophoric behavior of one of the isolated corrosion products as evidence for the existence of a highly reactive chemical species (possibly containing P) that is capable of being produced in cultures of SRB. The fortuitous sequestering of this reactive agent by other components of the corrosion product probably accounts for the anomalously low corrosion rate observed on the coupons from this culture.

A reactive P-containing species, if produced in these cultures (either biogenically or from chemical reactions between medium components and bacterial metabolites), might contribute significantly to the corrosion of ferrous metals. If such a compound were produced only in growing cultures and reacted with any available metal in the growth vessel before it could pass to the resting cell vessel, the considerable difference in corrosion rates between the two vessels might be explained.

In lactate-limited, high-phosphate cultures, the presence of ferrous sulfide-enriched bands on the corrosion product immediately adjacent to the coupon surface suggests that ferrous sulfide is initially formed on immersion of the metal in the sulfide-containing culture. This observation is in agreement with results of reports by King et al. (20, 22), who noted that the protective ferrous sulfide mackinawite is the first product deposited from fed batch cultures of sulfate-reducing bacteria. Our observation that Fe and S can also be enriched at the leading edge of the growing corrosion product (adjacent to the culture medium) suggests that ferrous sulfides are responsible for the growth of the corrosion product (even in high-phosphate media), but that subsequent reactions may alter the composition and structure of these products.

While phosphate clearly accelerated the rate of corrosion in pure cultures of planktonic SRB, the involvement of phosphate in enhancing corrosion in natural or process environments containing mixed microbial populations and complex biofilms is far from clear. In addition, many of these environments contain very low (perhaps growth-limiting) amounts of phosphate; for example, the total P content of seawater is 1 to 100 μg/kg (2), which is equivalent to 0.03 to 3 μM total P.

D. desulfuricans G100A produced electron-dense granules which contained various amounts of Fe and P when cells were grown in media which facilitated metal corrosion (i.e., when grown in media that were high in phosphate and ferrous iron). The P in these granules was apparently not in the form of polyphosphate, which is a common storage form of P in procaryotes. The exact composition of these granules and their physiological role, including a possible association with corrosion processes, remains to be elucidated. One attractive hypothesis is that these granules may represent an intracellular reserve of various elements (such as Fe and P), which may otherwise be available only in growth-limiting quantities in some natural anoxic environments.

ACKNOWLEDGMENTS

We thank Frederick B. Cooling III, Thalia Harris, and Roger Hesser for excellent technical assistance; Catherine Foris for X-ray diffraction; and Frances Yoder for inductively coupled plasma atomic emission spectroscopic analysis. We also thank James M. Odom, Emeric Schultz, Robert E. Tatnall, and Michael L. Van Kavelaar for helpful discussions.

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