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Assimilatory Sulfur Metabolism in Marine Microorganisms: Considerations for the Application of Sulfate Incorporation into Protein as a Measurement of Natural Population Protein Synthesis†

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The sulfur content of residue protein was determined for pure cultures of *Nitrosococcus oceanus, Desulfovibrio salexigens*, 4 mixed populations of fermentative bacteria, 22 samples from mixed natural population enrichments, and 11 nutritionally and morphologically distinct isolates from enrichments of Sargasso Sea water. The average 1.09 ± 0.14 % (by weight) S in protein for 13 pure cultures agrees with the 1.1% calculated from average protein composition. An operational value encompassing all mixed population and pure culture measurements has a coefficient of variation of only 15.1% (n = 41). Short-term [^{35}S]sulfate incorporation kinetics by *Pseudomonas halodurans* and *Alteromonas luteoviolaceus* demonstrated a rapid appearance of ^{35}S in the residue protein fraction which was well modelled by a simple exponential uptake equation. This indicates that little error in protein synthesis determination results from isotope dilution by endogenous pools of sulfur-containing compounds. Methionine effectively competed with sulfate for protein synthesis in *P. halodurans* at high concentrations (10 μM), but had much less influence at 1 μM. Cystine competed less effectively with sulfate, and glutathione did not detectably reduce sulfate-S incorporation into protein. [^{35}S]sulfate incorporation was compared with [^{14}C]glucose assimilation in a eutrophic brackish-water environment. Both tracers yielded similar results for the first 8 h of incubation, but a secondary growth phase was observed only with ^{35}S. Redistribution of ^{14}C from low-molecular-weight materials into residue protein indicated additional protein synthesis. [^{35}S]sulfate incorporation into residue protein by marine bacteria can be used to quantitatively measure bacterial protein synthesis in unenriched mixed populations of marine bacteria.

The direct measurement of true bacterial growth rate or metabolic processes has been seriously hampered by the extreme complexity of aquatic environments in terms of both microbial community composition and the physical and chemical characteristics of the habitats. The result is that few quantitative short-term assessments of total bacterial growth exist.

Little attention has been paid to the utilization of inorganic nutrients by bacteria, although they require N, P, and S as do phytoplankton. Inorganic nutrient assimilation by marine bacteria may provide a much needed means of quantifying bacterial biomass production. For example, sulfate incorporation into bacterial protein has been shown to be an accurate measure of de novo protein synthesis in pure cultures of marine bacteria (7b, 8). In addition to its utility as a laboratory tool for pure culture studies, several features of the sulfate incorporation measurement recommend its use for measurement of natural bacterial population protein synthesis.

The high sulfate concentration of seawater can be readily measured and ensures that sulfur is never limiting microbial growth in marine environments. The concentration of dissolved sulfur-containing amino acids in seawater is in the undetectable-to-low nanomolar range (6, 11, 25, 29, 36), suggesting that sulfate is the only significant source of sulfur for bacterial protein synthesis, although measurements of S-amino acid turnover times need to be made to estimate their true contribution to bacterial sulfur uptake. Although reductive assimilation of sulfate into protein is restricted to microorganisms (i.e., bacteria, fungi, and green plants), it appears to be universal among them, thereby functionally

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differentiating the entire microbial community from protozoa and higher animals (30). The pool size of sulfur-containing protein precursors is also extremely small in bacteria (3, 7a, 7b, 34, 35), minimizing complications arising from label dilution by large endogenous pools (20).

The use of sulfate incorporation into bacterial protein as an assessment of natural population protein synthesis requires that the sulfur content of protein be relatively constant among bacteria. It is also necessary to determine whether sulfate incorporation measurements are significantly influenced by equilibration of endogenous pools (20), since sufficiently sensitive techniques for measurement of the intracellular specific activity of sulfur-containing amino acids in natural populations have not been developed. Finally, an understanding of competition for sulfate metabolism by reduced sulfur compounds is useful when measurements in natural waters are made near highly productive or anaerobic regions where reduced sulfur compounds exist.

This paper presents the results of a survey of the sulfur content of protein from natural population enrichments and pure cultures of marine bacteria representing diverse nutritional modes of metabolism. Short-term incorporation kinetics and competition with reduced sulfur compounds were investigated, and the method was applied to a highly eutrophic brackish-water environment as an initial field trial.

MATERIALS AND METHODS

Natural population enrichments. Enrichments of Sargasso Sea water were made on *R/V Oceanus* cruises 69 and 75 in the vicinity of 37°28.7′ N, 64°03.4′ W. Samples taken from below the euphotic zone (500 m) with EtOH-washed Niskin bottles were prefiltered through 28-μm-mesh Nitex net to remove particles, and 100-ml aliquots were dispensed into sterile flasks. Inorganic nutrients (12) and organic carbon compounds were added as concentrated stock solutions which had been sterilized by autoclaving or filtration through 0.2-μm filters as appropriate, and the flasks were shaken at 22 ± 2°C at 250 rpm on a Gyrotory shaker (New Brunswick Scientific Co., Edison, N.J.). Pure cultures were isolated by spreading dilutions of the enrichments on seawater medium containing inorganic nutrients and the growth substrate, solidified by 1.5% agar (Difco Laboratories, Detroit, Mich.). Colonies were picked and restreaked several times, and the isolates were maintained on slants of the same medium.

Seventy of the isolates obtained on *R/V Oceanus* cruise 75 were screened for nutritional capability by streaking onto a series of seawater plates, each containing one of 25 individual carbon compounds. Liquid cultures were used for nitrate and ethanol utilization. Based on colony morphology, microscopic observation, and the ability to grow on the various carbon and energy sources, 11 nutritionally and morphologically distinct strains were obtained.

Determination of the sulfur content of protein. (i) Natural populations. The enrichment series from *R/V Oceanus* cruise 69 was labeled with $Na_2{}^{35}SO_4{}^{2-}$ (10 μCi ml^{-1}; 0.8-dpm $pmol^{-1}$ final specific activity) for measurement of sulfate incorporation into protein. Paired samples (20 to 25 ml) were brought to a final concentration of 10% trichloroacetic acid at the first sign of turbidity and again when the culture was visibly dense (optical density of 420 nm, about 0.6). An aliquot was streaked onto solid medium of the same composition to check colony morphology and dominance at each sampling. The fixed samples were centrifuged (5,000 × *g*, 20 min), and the pellets were rinsed twice with 10% trichloroacetic acid. One sample from each pair was fractionated for the determination of radioactivity in the protein residue (7a), and the other was dissolved in 0.1 N NaOH for protein assay. Good growth was obtained on 12 different substrates.

Fermentative bacteria were enriched from an anoxic estuarine sediment sample (S. Henrichs, Ph.D. thesis, Woods Hole Oceanographic Institution, Woods Hole, Mass., 1980) in a similar manner except that the media were sparged with argon for 10 min and incubations were carried out in sealed tubes. Glucose, galactose, fructose, and mannitol were used as substrates at 20 mM final concentrations.

(ii) Pure cultures. *Nitrosococcus oceanus* was kindly provided by S. Watson, Woods Hole Oceanographic Institution. It was grown in RLC-water medium (7) containing 20 mM NH_4Cl, 1 mM $Na_2{}^{35}SO_4$ (2 dpm $pmol^{-1}$), and 0.1% phenol red. The pH was adjusted as necessary with 0.1 M K_2CO_3. The late-exponential-phase culture was harvested by centrifugation, the pellet was rinsed twice with RLC-water, and duplicate samples were taken for fractionation and protein assay.

Desulfovibrio salexigens (obtained from J. R. Postgate, University of Sussex, Sussex, U.K.) was grown anaerobically in RLC-water medium containing 10 mM lactic acid and 25 mM $Na_2{}^{35}SO_4$ (0.5 dpm $pmol^{-1}$). A total of 15 samples were taken for fractionation and protein assay during exponential- and early-stationary-phase growth in two separate experiments.

Cruise isolates were grown in RLC-water medium containing 1 mM Na_2SO_4 (2 dpm $pmol^{-1}$) and 10 mM sodium glutamate, sodium succinate, or glucose as the sole carbon and energy sources. The cultures were incubated aerobically with shaking and were sampled in the late exponential phase of growth. Samples for ^{35}S distribution were filtered onto Whatman GF/F filters and rinsed three times with 2 ml of 0.5 M NaCl. The punch funnel described previously (7) was used during filtration to reduce isotope background.

(iii) Other methods. Protein, dissolved in 0.1 N NaOH, was measured on the 10% trichloroacetic acid-insoluble material precipitated directly from the cell suspension and on the protein residue from the fractionation procedure by the method of Bradford (4), using bovine serum albumin as a standard. Direct counts were made by epifluorescence microscopy (9). The sulfate concentration of natural seawater was calculated from the chlorinity determined on a Buchler-Cotlove chloridometer (Buchler Instruments, Fort Lee, N.J.), using a sulfate/chloride ratio of 0.14 (28).

Calculation of the sulfur content of protein. The subcellular fractionation procedure used in this study

TABLE 1. Total protein, total sulfur, and weight percent sulfur in protein of marine bacteria[a]

Expt	Per 10^8 cells		S (wt % in protein)	
	Total protein (μg)	Total sulfur (ng)	Operational[b]	True
Mixed-population enrichments	ND[c]	ND	0.94 ± 0.13 (22)	ND
Fermentative enrichments	ND	ND	0.92 ± 0.15 (4)	ND
Enrichment isolates	16.7 ± 10.3 (8)	274 ± 142 (8)	0.91 ± 0.15 (12)	1.10 ± 0.14 (9)
N. oceanus	142.7	2,122	0.78	1.31
D. salexigens	9.6	125	1.10 ± 0.15 (15)	1.00 ± 0.06 (6)
P. halodurans	23.8 ± 11.6 (162)	292 ± 129 (110)	0.86 ± 0.12 (80)	1.07 ± 0.17 (60)
A. luteo-violaceus	12.9 ± 4.9 (79)	106 ± 32 (80)	0.72 ± 0.11 (45)	0.92 ± 0.15 (10)
Overall mean			0.93 ± 0.14 (42)	1.09 ± 0.14 (13)

[a] Experimental procedures are given in the text. Data for *P. halodurans* and *A. luteo-violaceus* are taken from Cuhel et al., (8). The mean ± 1 SD is given for the number of determinations in parentheses. Overall means are for the indicated number of individual organisms and populations.

[b] As defined in the text.

[c] ND, Not determined.

permits the determination of the amount of ^{35}S incorporated into residue protein in natural population samples. However, about 10 to 15% of both the total cellular protein (by assay) and macromolecular sulfur are solubilized in other fractions, principally the alcohol-soluble, ether-insoluble component ("alcohol-soluble protein") (7a, 27). Thus, it is necessary to define an operational relationship between the residue protein-S and total protein synthesis as well as to determine the true sulfur content of protein. The operational weight percent S used in this work is (residue protein-S/total protein) × 100. When sufficient residue protein was available for assay in pure culture studies, the true weight percent sulfur in protein could be calculated as (residue protein-S/residue protein) × 100.

Radiochemicals. [*U*-^{14}C]glucose (255 mCi mmol^{-1}) and $Na_2{}^{35}SO_4$ (carrier-free) were obtained from Amersham Corp. (Chicago, Ill.).

Other chemicals. Reduced glutathione was obtained from Sigma Chemical Co. (St. Louis, Mo.). Aquasol was purchased from New England Nuclear Corp. (Boston, Mass.). All other chemicals were reagent grade.

RESULTS

Survey of the sulfur content of protein in marine bacteria. Enrichment of Sargasso Sea water with a variety of individual carbon and energy sources was performed to ascertain the protein-sulfur/total protein relationship for organisms responding to different growth substrates. Streaking of the cultures on solid medium gave rise to numerous colony morphologies in most flasks, indicating that the residue protein-S/total protein ratio was not dominated by protein from single species.

To further verify the applicability of the protein-S analysis to natural populations, isolates from a later enrichment series (*R/V Oceanus* cruise 75) were grown in pure culture, and the cell number, sulfur, and protein relationships were determined under completely defined conditions. These parameters were also measured for an anaerobic sulfate-reducing bacterium, *D. salexigens*, a marine nitrifying bacterium, *N. oceanus*, and four mixed populations of fermentative bacteria enriched from a mud sample from the Pettaquamscutt River estuary (Kingston, R.I.) during cooperative experiments with other laboratories at Woods Hole. The results of these analyses are summarized in Table 1. The great variability in cellular protein content of the isolates was accompanied by a similar range of protein-S, resulting in a relatively constant sulfur content of protein (coefficient of variation, 13.1%).

A key difference between bacterial and algal sulfate assimilation is the synthesis of sulfolipid by plants (cf. Busby and Benson [5]); hence, knowledge of the distribution of sulfur in the major biochemical fractions is of interest. Table 2 shows the averages for cruise isolates, *Alteromonas luteo-violaceus, N. oceanus*, and *Pseudomonas halodurans*. One cruise isolate, no. 50, stood out from the others by virtue of its high proportion of inorganic sulfate and low-molecular-weight (LMW) organic sulfur. Only in *N. oceanus*, however, was there a significant amount of lipid-S. The four populations of fermentative bacteria displayed an unusually high proportion of protein-S (>90% of the total ^{35}S).

The weight percent S in protein and sulfur distribution for each isolate or enrichment are tabulated in a thesis (R. L. Cuhel, Ph.D. thesis, Woods Hole Oceanographic Institution, Woods Hole, Mass., 1981).

Effects of low concentrations of compounds containing reduced sulfur on sulfate incorporation by *P. halodurans*. A consideration in the use of sulfate incorporation into protein as a measurement of marine microbial protein synthesis is the possible effect of other sulfur-containing compounds (e.g., cysteine, methionine, glutathione, and thiosulfate). Sulfate must be reduced before entering into the protein bio-

TABLE 2. Distribution of ^{35}S in biochemical fractions of marine bacteria in pure culture[a]

Organism	% of total radioactivity					
	SO_4^{2-}	LMW organic	Alcohol soluble	Lipid	Hot TCA[b] soluble	Residue protein
N. oceanus	2.0	19.4	8.3	4.3	13.4	52.6
P. halodurans	0.9	13.3	4.1	1.7	8.6	71.4
A. luteo-violaceus	3.2	17.9	3.0	0.9	4.9	70.1
Cruise isolates	2.7	18.5	11.7	1.7	7.4	59.6
Isolate 50	5.6	44.8	6.7	0.8	5.9	36.2

[a] The mean distribution for cruise isolates is shown. Data for *P. halodurans* and *A. luteo-violaceus* were taken from previous work (8).

[b] TCA, Trichloroacetic acid.

synthetic pathway, and preferential utilization of naturally occurring sulfur compounds would lower apparent sulfur incorporation measured with sulfate. It is therefore desirable to know at what concentration reduced sulfur compounds affect the incorporation of sulfate sulfur into protein.

P. halodurans was grown in complete medium containing 1 mM sulfate supplemented with low concentrations of cystine (1 and 4.5 μM, equal to 2 and 9 μM S, respectively), methionine (1 and 10 μM), glutathione (1 and 10 μM), thiosulfate (1 and 10 μM), and a control without added sulfur. The supplements had no effect on the growth rate. During late-exponential-phase growth (ca. 5×10^7 cells ml^{-1}), methionine and cystine clearly affected the amount of sulfate-S appearing in LMW organic pools and residue protein-S (Table 3). Methionine caused a redistribution of sulfur from residue protein-S to the LMW organic-S fraction, and the effect was proportional to the concentration of added sulfur. In contrast, cystine suppressed incorporation of sulfate into the LMW organic-S pool, thus increasing the proportion of sulfate-S in residue protein. A similar pattern of sulfate-sulfur distribution was observed in the early stationary phase (ca. 2×10^8 cells ml^{-1}), but only at the higher level of added sulfur. Thiosulfate and glutathione were without appreciable effect at these concentrations.

The effect of cystine and methionine observed in the distribution of sulfur was amplified by analysis of the sulfate-sulfur content of the total and residue protein. Table 3 emphasizes the effect of methionine on sulfate incorporation; at the 10 μM concentration over two-thirds of the residue protein sulfur was derived from methionine early in growth, and the effect remained, although damped, at the higher cell density. A proportional reduction in the residue protein-S/residue protein ratio was observed. The lower concentration of methionine exerted a barely detectable effect. The effect of cystine at 1 μM was more pronounced than the effect of methionine at the same concentration.

Short-term sulfate incorporation kinetics. Application of sulfate incorporation into protein as a method for measuring bacterial protein synthesis requires that there be little lag in the incorporation of sulfate into protein due to the equilibration of radiosulfate with endogenous pools (cf. Karl [20]). Data illustrating this to be the case for *P. halodurans* and *A. luteo-violaceus* are shown in Table 4. The experiments are based upon the fact that cells in a balanced state of growth are of constant cellular composition in time and are characterized by constant specific rates of polymer synthesis. Expression of these properties in terms of isotope incorporation into polymers,

TABLE 3. Effects of methionine and cystine on the incorporation of sulfate into LMW organic compounds and residue protein by *P. halodurans*[a]

Supplement	Level (μm)	% of total radioactivity		Protein = S (wt %)
		LMW organic	Residue protein	
None		11.9	78.5	0.75
Methionine	1	16.1	72.8	0.71
	10	35.9	57.1	0.23
Cystine	1	9.6	78.8	0.65
	4.5	8.4	82.4	0.59

[a] Complete medium containing 10 mM sodium glutamate and 1 mM $Na_2{}^{35}SO_4$ (2 dpm $pmol^{-1}$) was inoculated with late-exponential-phase cells to a final density of 3×10^4 cells ml^{-1}. Aliquots were transferred to sterile flasks and supplemented with filter-sterilized sulfur sources at the indicated concentration. During the mid- to late-exponential phase and again in the early stationary phase of growth samples were taken for total protein and biochemical fractionation. Data are expressed as radioactivity derived from sulfate.

TABLE 4. Proportion of total ^{35}S in residue protein in short-term incubations[a]

Time (min)	% of total ^{35}S	
	P. halodurans	*A. luteo-violaceus*
5	67.2	50.7
15	74.3	63.1
30	75.8	69.8
120	75.8	71.8
480	63.7	73.1

[a] Experimental details are given in the legend to Fig. 1.

however, will be manifest only when the LMW precursor pools are in a state of isotope equilibrium. In *P. halodurans* over 67% of the total newly incorporated S was found in the protein residue in 5 min. After 15 min of incubation the proportion of sulfur in the residue protein, 74.3%, was essentially constant in time, indicating isotopic equilibration of the precursor pools within approximately 0.25 generation. The decreased proportion of ^{35}S-labeled protein at 480 min reflects a condition of imbalanced growth of *P. halodurans* and resulted from the abrupt decrease in the rate of protein synthesis after approximately 2.5 h of incubation (Fig. 1).

A similar pattern of incorporation was observed for *A. luteo-violaceus*, where in 30 min (approximately 0.25 generation) the proportion of ^{35}S in residue protein was within 6% of the maximum observed.

Under conditions of balanced growth and rapid precursor equilibrium the accumulation of isotope into specific cellular substituents (I) may be described by the equation (27):

$$I = \frac{Q_0}{k \ln 2} (e^{k \ln 2 t} - 1)$$

Q_0 is the initial rate of isotope incorporation and k is the reciprocal doubling time. A semilog plot of isotope accumulation versus time (t) is convex, and asymptotically approaches a straight line of slope k ln2. The degree of curvature is directly related to k and can be used to determine the growth rate of exponentially growing pure cultures (32, 33). Examined in this manner, the isotope uptake data in Fig. 1 are well described by equation 1 throughout the exponential period of growth. For comparison, total protein and direct cell counts are also shown.

For *P. halodurans* growth rate constants computed from total uptake of ^{35}S and incorporation into residue protein was 0.97 ± 0.01 and 1.01 ± 0.02 h^{-1}, respectively, over the time period of 0 to 2.5 h. The values for k obtained from cell counts and total protein were 1.03 and 1.29 h^{-1}, respectively. Although for *A. luteo-violaceus* the growth rate determined for direct counts (0.48 h^{-1}) was less than that for total protein (0.57 h^{-1}), there was no deviation from exponentiality for nearly 8 h of growth. In this case the best-fit k values for all of the isotope incorporation data from 5 min to 8 h were determined to be 0.59 ± 0.01 h^{-1} for total ^{35}S uptake and 0.63 ± 0.01 h^{-1} for ^{35}S incorporation into residue protein.

Sulfate incorporation in a eutrophic marine system. The first field trial of this method was conducted in a highly eutrophic natural seawater system, a holding tank for the estuarine killifish, *Fundulus heteroclitus*. This environment offered two advantages for initial attempts to measure natural population protein synthesis rates. First, the environment of the *Fundulus* is estuarine; hence, the holding tank was kept at half-strength seawater. The reduced sulfate concentration (14

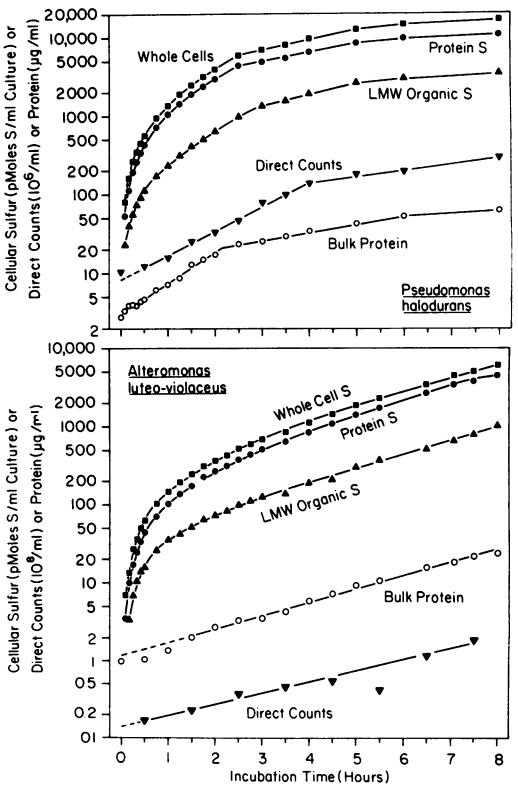

FIG. 1. Direct counts, total protein, and $^{35}SO_4^{2-}$ uptake and incorporation kinetics for exponentially growing cultures of *P. halodurans* and *A. luteo-violaceus*. Complete medium containing 10 mM glutamate and 1 mM sulfate was inoculated with the appropriate organism at about 2 × 10^4 cells ml^{-1}. When the cell density was near 10^7 cells ml^{-1}, $Na_2{}^{35}SO_4{}^{2-}$ was added to a final activity of 4 × 10^6 dpm ml^{-1} (*P. halodurans*) or 3 × 10^6 dpm ml^{-1} (*A. luteo-violaceus*), and aliquots were sampled for radioisotope uptake at short intervals.

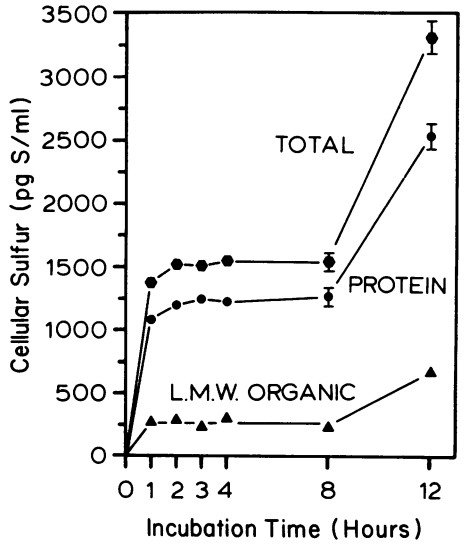

FIG. 2. Time course of sulfate assimilation by natural bacterial populations in a brackish-water fish tank. A 2-liter sample from a well-aerated half-strength seawater (chlorinity, 9.97‰) holding tank for *Fundulus* was rapidly filtered through 28-μm Nitex net to remove coarse particles. One liter was poured into a large syringe made from a chromatography column (6 by 38 cm; Bellco Glass Co., Vineland, N.J.) fitted with a polycarbonate end cap and piston with rubber O-rings. A 10-mCi amount of carrier-free $Na_2{}^{35}SO_4{}^{2-}$ (final specific activity, 2 dpm $pmol^{-1}$) was added and mixed by inversion of the syringe. Subsamples were removed through a Luer-lock fitting in the end cap after flushing about 5 ml of the sample through the port. Duplicate 50-ml samples were filtered through Whatman GF/F filters. Error bars are shown when larger than the symbol. The initial cell density was 4.4×10^6 ml^{-1}, and the sample and incubation temperature was 22°C.

mM) was almost 100 times the concentration at which sulfate limitation occurs in marine algae (24) or bacteria (7a) but gave a twofold-greater sensitivity to the sulfate incorporation measurement. Second, the use of high-food-value fish feed combined with the excretion of organic and inorganic compounds by the fish resulted in an environment highly conducive to healthy bacterial growth, verified by the observation of a relatively high cell density (4.4×10^6 cells ml^{-1}) of large, curved, rod-shaped bacteria about 2 by 5 μm in dimensions. Incoming seawater flushed the tank about three times per day, providing a semicontinuous culture regime.

The time course of sulfate uptake by the bacterial population in the fish tank was characterized by rapid incorporation into protein and LMW organic-S components during the first hour followed by an extended plateau (Fig. 2). At 8 to 12 h of incubation, incorporation of sulfate doubled in both fractions. LMW organic-S accounted for 20% of the total ^{35}S.

The total uptake of glucose did not give any indication of the second growth phase between 8 and 12 h of incubation (Fig. 3). Glucose assimilation increased during the first 3 h to a plateau which remained unchanged for the remainder of the experiment. Production of CO_2 continued to increase rapidly for 1 h after the cessation of incorporation of glucose into cell material (data not shown) and then continued at a slow rate for the duration of the experiment to achieve a final maximum utilization (incorporation plus respiration) of 63.5% of the total glucose.

Fractionation of the ^{14}C-labeled bacteria into major biochemical components provided indications of the secondary growth phase, even though total incorporation had ceased. Figure 3 shows a rapid increase in all fractions during the first 3 h, with protein as the major end product of glucose assimilation. During the stationary period (as determined by whole-cell ^{14}C), movement

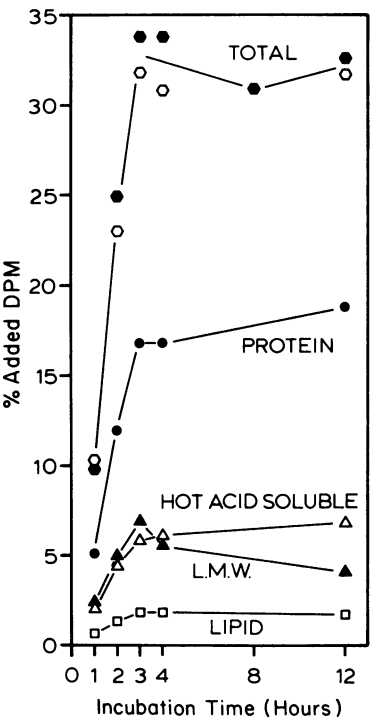

FIG. 3. Total uptake of [*U*-^{14}C]glucose and its distribution in major biochemical fractions of bacteria from a brackish-water fish tank. Sampling and incubation procedures are described in the legend to Fig. 2. [*U*-^{14}C]glucose (250 mCi $mmol^{-1}$) was added at a final activity of 3.7 μCi $liter^{-1}$, and 10-ml samples were filtered for total uptake (0.2-μm membrane filters) and fractionation into biochemical components (GF/F filters). Symbols: ○, sum of the activity in each fraction; ●, whole-cell unprocessed controls.

of label from the labile LMW pool into residue protein (and to a lesser extent hot trichloroacetic acid-soluble material) was evident. The ^{14}C content of lipid, a more structurally related fraction, remained constant during this time.

DISCUSSION

The results of the survey of the residue protein-S/total protein ratio for mixed natural populations in enrichment culture and isolates from similar enrichments in pure culture corroborate the validity of measuring marine bacterial protein synthesis by using sulfur incorporation into protein. A wide variety of microorganisms were studied, including a chemolithotroph (*N. oceanus*), a sulfate-reducing bacterium (*D. salexigens*), mixed populations of fermentative and heterotrophic bacteria, and pure cultures of heterotrophic bacteria with varying degrees of nutritional versatility. Few organisms among them deviated seriously from the mean ratio, and the coefficient of variation of the residue protein-S/total protein ratio for the cruise isolates, 16.8%, is much less than that for either the protein (61.8%) or the total sulfur (51.7%) per cell.

The agreement of the true weight percent sulfur in protein (i.e., [residue protein-S/residue protein] × 100) determined for 13 different bacteria, 1.09%, with the 1.1% calculated from the Jukes et al. (19) average protein, is reassuring. The variation in individual protein composition summarized by Holmquist (17) is indeed averaged out in total protein. In view of the consistency of the residue protein-S/total protein ratio among bacteria and in an individual organism under a variety of nutritional regimes, it is proposed that the measurement of sulfate incorporation into residue protein is a quantitative assay for marine bacterial protein synthesis.

The small absolute size of the LMW organic sulfur pool and the negligible contribution of inorganic sulfate to the total cellular sulfur for two marine bacteria (7a) permit extremely rapid incorporation of $^{35}SO_4^{2-}$ into residue protein (Table 4). Much of the sulfur amino acid requirement for protein synthesis can be satisfied by direct reduction of sulfate rather than utilization of preexisting precursors. Studies of the molecular organization of the cysteine synthetase system (22) support this concept, finding that a single, multimeric complex in *Salmonella typhimurium* performed both *O*-acetylation of serine and sulfurylation of the product *O*-acetylserine with S^{2-} to yield cysteine. Likewise, the reduction of adenosine 3′-phosphosulfate to the level of sulfide occurs in a single six-electron step while bound to an LMW protein carrier (1, 31); the authors suggest that the entire sulfate reduction pathway occurs while enzyme bound. The fast reaction kinetics of such systems due to increased substrate concentrations resulting from proximity to reaction centers while enzyme bound allows the maintenance of small precursor pools. Further reactions of cysteine during methionine biosynthesis may be similarly coordinated, since the intermediates homocysteine and cystathionine are also found at very low concentration (15). The slowly equilibrating LMW component may be primarily glutathione, which is well known to be a sulfur reserve in bacteria (2, 10, 13). Glutathione may serve as an emergency sulfur source and oxidation-reduction buffer without substantial involvement as a source of cysteine for amino acid biosynthesis, since it derepresses components of the cysteine biosynthetic pathway effectively (21).

The measurement of sulfate incorporation into the protein residue therefore represents de novo protein synthesis within a small degree of error in growing cells containing a normal complement of sulfur in the LMW pool. This is especially valuable because it minimizes problems arising from dilution of label by large endogenous pools of protein precursors (20) and permits the direct interpretation of sulfate incorporation studies in mixed natural populations.

The specificity of the incorporation of sulfur into residue protein for protein synthesis is emphasized by the good fit of incorporation data to the model derived from elementary considerations of isotope uptake kinetics. For both *P. halodurans* and *A. luteo-violaceus*, determined values of k from total sulfur uptake and incorporation into residue protein agreed within an experimental error of ±5%. These values compared within ±25% of values obtained from independent cell count or total protein measurements, which in themselves agreed within a similar magnitude of error. Thus, within the limits discussed earlier for use of the model, studies of the dynamics of isotope incorporation can, in the absence of any further ancillary information, provide an estimate of the growth rate of the population under study. The approach is particularly attractive in instances in which classic measures of cellular growth (e.g., biomass, cell density) are not possible or feasible. For the appropriate implementation of the technique, incubation should extend for a minimum of 1.0 to 1.5 generations. This derives from the fact that data described by equation 1 must possess sufficient curvature for the accurate determination of k by nonlinear regression techniques.

The competition for sulfate incorporation into protein by methionine and cystine can pose a problem in the interpretation of protein synthesis measurements when the organic compounds are present at moderate levels (ca. 1 μM). Methionine is especially effective at replacing sul-

fate-sulfur in protein in *P. halodurans*, with characteristics identical to those observed for *Escherichia coli* (27). The results of competition with methionine and cystine also suggest that a large percentage of the LMW organic sulfur pool is glutathione. The replacement with methionine of part of the residue protein-S normally derived from sulfate would reduce the amount of sulfate-^{35}S incorporated into this fraction. However, the lower growth rate of *P. halodurans* with methionine as the sole source of sulfur relative to cysteine or glutathione-grown cells indicates that the reverse reaction of methionine to cysteine is unfavorable (7). Therefore, intracellular cysteine and glutathione are most likely synthesized from sulfate. Since much of the protein-S is derived from methionine in its presence, a disproportionately high percentage of the total sulfate-^{35}S would be in LMW organic-S compounds (cysteine plus glutathione), as observed. The results of cystine competition are consistent with this mechanism, since exogenous cystine should replace sulfate in intracellular pools of both cysteine and the cysteine-containing peptide. A detectable decrease in the LMW organic-S derived from sulfate was observed, but the effect was small. Grown with cystine as the sole source of sulfur, *P. halodurans* achieves only half the growth rate obtained with sulfate (7), so cystine transport may be too difficult to provide enough sulfur to replace sulfate for metabolism at low cystine concentrations. The absence of a detectable effect of glutathione on the sulfate-^{35}S distribution or incorporation into residue protein indicates that glutathione is not acting as a reservoir of cysteine for protein synthesis.

The failure to detect sulfur-containing amino acids in seawater (6, 11, 25, 29, 36), even in coastal waters, means that competition for sulfate uptake will be negligible in isolated water samples. However, an investigation of the turnover times and flux of regenerated LMW organic sulfur compounds into microbial cells is warranted.

The initial application of sulfate incorporation into protein, using an organic-rich seawater system with a well-developed microbial flora, demonstrated the value of using an inorganic tracer of bacterial activity. Assimilation of glucose and that of sulfate were very similar during the first few hours of the experiment, but nearly complete mineralization of glucose indicated the preferential utilization of dissolved LMW material in the early stages of the incubation. Sulfate incorporation into protein was sensitive to the secondary growth period because it is never utilized to a significant extent as a percentage of the total label and is incorporated in direct relation to growth. The change in distribution of ^{14}C after the plateau in total uptake suggests that the glucose-assimilating portion of the population also experienced a secondary growth phase, during which time label previously incorporated into precursor pools was chased into end products by a new source of carbon and energy. The fish food used in the tank consisted primarily of polymeric materials (crude protein and plant fibers), and several hours could be required for the induction, synthesis, and action of hydrolytic enzymes required for the degradation of proteinaceous and cellulosic polymers. The monomers thus provided could stimulate continued growth which would not be observed by glucose assimilation.

In addition to having observed the secondary growth phase in the bacterial population from the fish tank, the ratio of residue protein-S/total protein (Table 1) can be used to calculate the amount of bacterial protein synthesized during the incubation period. The operational value, 0.93% by weight, indicates the synthesis of 123 μg of protein $liter^{-1}$ during the first hour, a very healthy amount of growth. Since protein synthesis values for other natural populations are not available for comparison, an assumption of 50% carbon by weight in protein gives a little over 60 μg of protein-C $liter^{-1}$ h^{-1}, the minimum carbon production (since carbon is contained in many nonprotein cellular constituents). Values for natural bacterial population growth in a rich coastal inlet in British Columbia (14) determined by cell counts and thymidine incorporation into DNA range from 0.7 to 70 μg of C $liter^{-1}$ day^{-1}. The fish tank population was therefore more than 25 times as productive as the most rapid growth observed in their study.

In summary, the relatively constant sulfur content of protein among marine bacteria and its independence of growth conditions make sulfate incorporation into protein a reliable measure of net bacterial protein production. The major limitation to its use in seawater is the high activity necessary to detect bacterial metabolism (10 to 20 μCi ml^{-1}). However, the use of appropriate filtration and sample processing methods (8) yields low and reproducible blanks, as well as providing a more readily interpretable result than whole-cell sulfate uptake.

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