ASSIMILATORY SULFUR METABOLISM IN MARINE MICROORGANISMS

by

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This thesis is dedicated to ROCK AND ROLL!!

"If you need statistics, you've done the wrong experiment."

Relayed by Ian Morris

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ASSIMILATORY SULFUR METABOLISM IN MARINE MICROORGANISMS

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RUSSELL LEE CUHEL

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ABSTRACT

The reductive assimilation of sulfate into cellular organic sulfur compounds was studied in aerobic marine bacteria, with emphasis on the relationship between sulfur metabolism and protein synthesis. The goal of the study was to develop and apply a method for the quantitative assay of total bacterial protein synthesis in aerobic ocean waters. The study consisted of four parts:

(1) The sulfate transport systems of two nutritionally different marine bacteria, Pseudomonas halodurans and Alteromonas luteo-violaceus, were characterized to provide information on environmental regulation of sulfate transport capacity. In common with terrestrial bacteria, the transport systems of both marine bacteria exhibit (a) size-selective competitive inhibition of sulfate uptake by sulfate analogs, (b) requirements for energy coupling, and (c) derepression of transport capacity as a result of sulfur starvation. Features which are unique to the marine bacteria include (a) a ten-fold lower affinity for sulfate (half-saturation constant ~200 μm), (b) derepression of transport capacity when grown with methionine as the sole source of sulfur, and (c) an inability to accumulate inorganic sulfate in excess of growth requirements. The different characteristics of the sulfate transport systems of the marine bacteria relative to terrestrial microorganisms are consistent with the saturating concentration of sulfate that is always present in their environment. Substantial differences also exist between the two marine bacteria, notably in the effect of thiosulfate on sulfate uptake. P. halodurans transports thiosulfate with a ten-fold higher affinity than sulfate. Sulfate and thiosulfate are mutually competitive inhibitors of transport, and the half-saturating concentration of thiosulfate for uptake also produces half-maximal inhibition of sulfate transport. Sulfate and thiosulfate transport systems both respond similarly to all inibitors. These facts implicate a common carrier for the two compounds. In contrast, sulfate transport in A. luteo-violaceus is relatively insensitive to thiosulfate. The effect of the sulfhydryl reagent pHMB is similarly much less pronounced than in P. halodurans. These and other differences indicate that the sulfate transport system of A. luteo-violaceus is unique among microorganisms.

(2)Growth experiments with P. halodurans and A. luteo-violaceus were carried out over a range of nutritional regimes. Biomass parameters (cell counts, bulk protein, particulate carbon and nitrogen), total uptake of radioactive sulfate, and the distribution of sulfur in major biochemical

components (low molecular weight [L.M.W.], alcohol soluble protein, lipid, hot TCA soluble material, and residue protein) were monitored to determine the variability in cellular composition as a function of the environment. Special emphasis was placed on the quantitative relationship between incorporation of sulfur into protein and bulk protein synthesis and conditions which might alter the sulfur content of protein. It was found that sulfur metabolism is restricted predominantly to the production and utilization of protein precursors. The protein synthesis inhibitor chloramphenicol caused an immediate halt to both bulk protein synthesis and sulfur incorporation into protein, accompanied by a rapid swelling of L.M.W. organic sulfur pools, in both bacteria. Incorporation of exogenous sulfur into protein was rapid due to the very small size of the L.M.W. pool. No significant deviation from the ratio of protein-S:bulk protein determined for unperturbed exponential growth was observed as a function of carbon limitation, nitrogen limitation, treatment with chloramphenicol, or during lag and stationary phases. However, the concentration of sulfate in the growth medium exerted a strong influence on the sulfur content of both whole cells and isolated protein. At concentrations less than 500 μM (P. halodurans) or 100 μM (A. luteo-violaceus) the weight % S in protein was proportional to the sulfate concentration in the medium. Since the sulfate concentration is invariably high in seawater (25mM), data from sulfur-limited growth were not included in the analysis of compositional variability. Under all the conditions examined, the incorporation of sulfur into protein provided the best measurement of protein synthesis and cell growth, with a very low coefficient of variation for the protein-S:bulk protein ratio (less than 16 %). The mean true weight % S in protein, 1.07 (P. halodurans) and 0.92 (A. luteo-violaceus) agrees well with the 1.1 % predicted from analyses of sequenced proteins.

(3)The method used for the analysis of sulfur incorporation into protein was tested with mixed natural populations of marine bacteria in enrichment culture and 13 isolates from the Sargasso Sea to establish the variability of the protein-S:bulk protein ratio among marine bacteria. The mean true weight % S in protein, 1.09, and the operational weight % S in protein, 0.93, have coefficients of variation of 13.1 and 15.1 %, respectively. The values are similar to those obtained with the two marine bacteria studied in detail and to that predicted from protein composition studies. Therefore sulfur incorporation into protein measures protein synthesis in marine bacteria within a small degree of error.

(4)The method was applied to unenriched natural populations of marine bacteria in waters of the continental shelf, slope, and Sargasso Sea. Time-course incorporation measurements revealed a long lag period at the shelf and slope stations, whereas incorporation of sulfur into protein began immediately in the Sargasso Sea. However, long term incubations confirmed that the potential for bacterial protein synthesis decreases in an off-shore transect. These observations were confirmed by simultaneous incorporation studies using labeled ammonia, phosphate, and organic carbon compounds.

The potential protein synthesis measured in the unenriched samples provides evidence suggesting that bacterial biomass may be an important contributor to marine food webs.

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Many long and often tedious times have been spent pulling this together. In spite of my pride at having done most or all of every facet of this work, the wonderful help and company of a large array of people have been extremely important. I have worked and learned with scientists, secretaries, draftsmen, photographers, and gardeners alike over the last five years, and I remain convinced that it is only co-operative effort that pays the final dividends.

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The calm and consistently testable approach to science embodied in Holger reminds me that I still am, and may always be, a student. Ah, what bliss. For to be a student is to be a scrounge. These circumstances often obscur the line between "us" and "them." They have been extremely good to me, in spite of the grief I've dealt out to them. But who are "they", anyway? They are Jake Peirson, Abbie Alvin, and the now New Alchemist Denise Backus, who have hustled and scrounged and smiled that crooked smile ("What do you want this time?) and altogether, made it work. More than just supporting the sordid journeys into financial hell as I try to raise the money for a major piece of equipment ("If I can get bucks from them, will you make up the difference? "), these fine folks have been instrumental in the maintenance of good attitude.

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CHAPTER 1

INTRODUCTION TO MICROBIOLOGICAL OCEANOGRAPHY

General Introduction

From the primary synthesis of dissolved and particulate organic matter to the regeneration of nutrients by mineralization of essentially the same material, microorganisms* are responsible for the generation of the marine food web. The increasing rate of human predation on this finite and largely unknown renewable resource has stimulated research on all aspects of marine biomass production, with the ultimate goal of developing effective models of marine ecosystems through which efficient management programs may be implemented. Within this context, bacteria have been regarded as mineralizers which provide the inorganic nutrients required by the primary producers, without appreciable impact on other aspects of the marine food web.

Our perspective of the potential contribution of bacteria to biomass production has been broadened by the discovery of bacterially-generated food webs in certain areas of the deep sea (Jannasch and Wirsen, 1979; Karl et al., 1980). On a global scale, the biomass production of the Galapagos Rift geothermal vent ecosystems may be of limited ecological significance; however, it provides unequivocal proof that bacteria can be nutritionally adequate to provide for the reproductive growth of higher organisms. Mounting evidence for the widespread distribution of potentially bactivorous microzooplankton and gelatinous zooplankton (Harbison

*Microorganisms and green plants are delineated by the unique ability to reductively assimilate nitrate and sulfate, a characteristic not found in the animal kingdom. In this context, the term "microorganism" will be used in reference to plants, algae, bacteria, and fungi. When explicit reference to bacteria is desired, it will be so designated.

and McAlister, 1979; Wiebe et al., 1979) makes clear the need for a quantitative assessment of bacterial growth and its contribution to the marine food web.

Both open ocean and inshore pelagic bacterial activity is dominated by aerobic heterotrophic and autotrophic processes. Heterotrophic activity, an oxidative mode of metabolism, results in the release of carbon dioxide and inorganic forms of nitrogen and phosphorus for reutilization by photosynthetic organisms. The nutrient regeneration provided through bacterial mineralization of animal excretory products and dead organisms is often considered to be the only contribution of bacteria to the marine food web.

An inevitable result of mineralization processes is bacterial growth. From a microbial perspective, the oxidation of organic and inorganic compounds is only a means of obtaining the elemental constituents and energy necessary for the production of new cells. The production of biomass by bacteria requires carbon, nitrogen, sulfur, and phosphorus in proportions similar to those required by plants; hence the composition of the dissolved and particulate organic matter available for mineralization is of great importance when considering potential nutrient regeneration. In surface waters with high concentrations of dissolved organic carbon relative to nitrogen and/or phosphorus, bacteria may actually compete with plants for some of the very nutrients they are supposed to produce (Parker et al., 1975).

The combination of allochthonous inputs such as terrestrial runoff, ocean dumping, and fallout of airborne particulates, plus the autochthonous contributions of decaying organisms and excretion products results in

a very complex mixture of organic compounds with varying degrees of biological lability. The diversity of metabolically useful compounds is matched by the number of bacterial processes which have evolved to use them. In an evolutionary history longer than that of any other kingdom, bacteria have developed the ability to extract from nearly every imaginable source the energy required to carry out life processes. The principal issue for consideration of mineralization and nutrient regeneration is therefore one of rates. The constituents of decaying organisms, for example, are highly susceptible to bacterial degradation and are expected to be rapidly utilized. Consistent with this, the maximum potential for heterotrophic utilization of representative monomeric compounds such as amino acids (Williams et al., 1976) and glucose (Takahashi and Ichimura, 1971) occurs in surface waters.

In addition to the readily degraded compounds are materials of biological value which require longer periods of time for utilization due to physical or biochemical limitations. Examples include cellulose, chitin, deoxyribonucleic acid, and the cell walls of many algae and bacteria. The rate of hydrolysis and assimilation of polymeric materials by bacteria is dependent on their composition (Gunnison and Alexander, 1975), resulting in a preference for the most labile polymers. The action of extracellular hydrolytic enzymes is required for breakdown into assimilable components; both time and a means of reducing diffusive loss of monomeric components are necessary for their utilization. Bacterial attachment to particles is often characterized by webbing (Paerl, 1973) which produces microenvironments for containment of dissolved materials released by polymer hydrolysis. Utilization of complex compounds is postponed when more readily as-

similable dissolved organic compounds are simultaneously present (Reichardt, 1975), indicating that low organic nutrient conditions favor the degradation of complex polymers.

A third class of organic compounds frequently available to marine bacteria includes highly refractory substances with extremely slow or incomplete degradation. Included in this category may be the mucopolysaccharide matrices of fish slimes and gelatinous zooplankton, as well as complex petroleum hydrocarbons from natural and humanly-mediated seepages. Much of the organic carbon contained in these materials may be deposited in sediments for subsequent anaerobic decomposition or burial.

If most of the organic carbon were introduced into the oceans in the euphotic zone (> 1 % light penetration) and no factors other than concentration and composition governed its bacterial transformation, it could be imagined that the rate of bacterial activity and biomass production would decline rapidly with depth. The quantity of material descending from the surface input would decrease and the quality would change due to the selective removal of the most labile components. Considering the effects of pressure (c.f. Wirsen and Jannasch, 1975) and temperature (c.f. Ingraham and Bailey, 1959) on the growth of bacteria, it is little wonder that bacterial activity in the bulk of the deep sea is very slow (c.f. Jannasch and Wirsen, 1973). Furthermore, it appears that labile substrates such as sugars and amino acids, which are turned over rapidly in surface waters, are likely to be poor indicators of microbial activity at greater depths, where metabolism of more slowly degraded polymers can be important.

The heterogeneity of organic and inorganic compounds in seawater leads to a similarly heterogeneous assemblage of microbial species. Variability in potential uptake rates, substrate affinity, and other factors results in a variety of different bacteria growing at different rates (Jannasch and Jones, 1959). A sample of open ocean water can yield hundreds of nutritionally distinct bacteria with similar population densities; dominance by one or few bacterial species may only occur where a large discharge of a single organic compound occurs. The diversity of aquatic bacterial populations has posed serious problems to the measurement of total microbial community growth.

Determination of Microbial Numbers and Activity

The following section is a synopsis of the currently available means of analysis of pertinent standing crop and rate parameters. No single method will resolve the complex nature of natural microbial community growth. It is necessary that a coordinated suite of measurements be made, with inclusion of both standing crop and several different rate parameters in order that microbial growth may be more fully understood. Many approaches have been used for the measurement of microbial biomass and growth in marine ecosystems, falling into three basic categories: (1) analysis of standing crops, which estimates the amount of biomass present, but provides no information on growth, (2) measurement of standing crop parameters from which growth may be inferred, and (3) true rate measurements.

(1) Analysis of Standing Crops

Standing crop measurements assay the amount of a characteristic material which may be related to microbial biomass. Analyses of such parameters as particulate organic carbon (POC), particulate organic nitrogen (PON), protein, DNA, and carbohydrate are often performed in conjuction with photosynthesis measurements (Antia et al., 1963; Strickland et al., 1969) but are rarely used in field studies of bacterial distribution and growth (but see Hobbie et al., 1972). An underlying assumption in this type of analysis is that only microorganisms are sampled and subsequently trapped on a filter. It must be borne in mind that all organisms, living and dead, as well as detritus, contain these components.

In an effort to distinguish living from detrital material, HolmHansen and Booth (1966) proposed the use of adenosine-5'-triphosphate (ATP) analysis as an indicator of living biomass. In addition to this important differentiation between living and non-living microorganisms, the sensitivity of the assay permits the detection of probable microbial population maxima at low densities. Nevertheless, the measurement includes the contribution of microzooplankton as well as microorganisms, and a single copepod can often completely obscur the microbial biomass component.

Estimation of bacterial standing crops has been advanced by the development of several analyses specific for bacterial components. Among the oldest is the plate count, in which bacterial colonies growing on various solid media are used to estimate the number of viable cells in a sample. The method is subject to severe quantitative artifacts, as illus-

trated by Jannasch and Jones (1959) but has use in determining potentially metabolizable substrates in a given sample and other qualitative applications. Direct counts of bacteria, aided by stains to help differentiate bacteria from detritus, have increased our awareness of the potential food resource available as bacterial biomass. Acridine orange epifluorescence microscopy has been refined to permit rapid and reproducible direct counts of natural bacterial populations (Daley and Hobbie, 1975), thereby stimulating surveys of bacterial concentrations in many habitats (Ferguson and Rublee, 1976; LaRock et al., 1979; Wiebe and Pomeroy, 1972).

Additional assays specific for bacteria have been developed that are especially useful where direct counts are difficult due to high concentrations of detrital particles, as well as providing corroborative data in conjunction with other measurements. The lipopolysaccharide assay of Watson et al. (1977) measures the amount of Gram-negative cell envelope present in a sample and can be related to cell numbers and biomass within broad bounds. Following a similar premise, King and White (1977) described an assay for bacterial cell wall muramic acid, found in all bacteria except extreme halophiles and methanogenic bacteria. Although quite specific for bacteria, these assays cannot differentiate between living and dead cells.

Various methods for the enumeration of bacterial standing crops have been tested simultaneously in field trials and tend to demonstrate coincident maxima (Hobbie et al., 1972; Watson, 1978), although in some more unusual environments, such as the Orca Brine (LaRock et al., 1979), direct counts and ATP measurements do not follow the same pattern.

The methods described above yield information about the amount of particulate matter in the microbial size fraction but give no data on rates of growth or metabolism. However, they are useful measurements when taken in conjunction with other parameters.

(2) Indirect Methods for Estimating Bacterial Growth

The complex, heterogeneous composition of organic and inorganic compounds in seawater and the attendant diverse microbial populations have made direct measurement of mineralization and microbial growth difficult in the extreme. Attempts have been made, therefore, to find an accurate, indirect method for measuring growth rates through the investigation of the relationships among standing crop parameters. Progress in this area is largely due to the ability to concentrate microorganisms from large volumes of water by filtration, enabling the detection of low concentrations of cells and their components in dilute environments. It is, however, necessary that a predictable relationship exists between the measured index and the growth of microorganisms.

One of the first indirect methods for estimating microbial growth stemmed from the ATP assay of Holm-Hansen and Booth (1966). The ATP assay is a good method for measuring total living material, but becomes a more powerful tool when measured together with the other adenine nucleotides adenosine-5'-monophosphate (AMP) and adenosine-5'-diphosphate (ADP). The ratio of these nucleotides, known as the energy charge, relates to the state of growth of a population (Atkinson and Walton, 1967; Atkinson, 1968). Valuable information regarding the growth potential may be obtain-

ed through this measurement, as demonstrated by the sharp increase in both ATP and the energy charge in the vicinity of the $H_2S:O_2$ interface in the Black Sea (Karl, 1978).

Refinement of techniques for separating nucleotides from extracts of filtered water samples has allowed even closer approximations to growth rate measurement through the analysis of standing crops. Due to the central nature of guanosine-5'-triphosphate (GTP) to the energy requirement for protein synthesis, Karl (1978) proposed that the GTP:ATP ratio is an index of the growth rate. He found a linear relationship between the growth rate and the GTP:ATP ratio for a pure culture grown in a chemostat over a range of growth rates. If the relationship is constant over a variety of growth conditions as well as rates, and similar among microorganisms, the assay of the GTP:ATP ratio along with the estimation of viable biomass from ATP analysis will be a valuable means of delineating regions of rapid microbial growth (c.f. Karl et al., 1980).

It must again be borne in mind that the assay of nucleotides and their ratios is not specific for microorganisms. Ciliates and other microzooplankton will be included in the measurement, and the extent of their contributions is currently unknown.

(3) Direct Measurement of Bacterial Rate Processes

The direct measurement of true bacterial metabolic or growth rate 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 no quantitative assessment of total bacterial growth yet exists. The reasons for this may be more clearly understood by considering the basic criteria which must be satisfied in order to achieve a true, quantitative rate measurement for the entire bacterial assemblage in a water sample. Such criteria include:

(1) The substrate concentration must be measurable. Without meeting this primary criterion, all measurements will be qualitative and incomparable from sample to sample.

(2) The addition of sufficient quantity of labeled material must not alter the ambient concentration of substrate significantly nor affect the chemistry of the sample;

(3) There must be no competing compounds which interfere with the uptake of the tracer or replace it in metabolic processes;

(4) The tracer must measure a process that is mediated solely by bacteria. Equilibration of inorganic compounds with the tracer by microzooplankton must be avoided for whole cell uptake studies, and certainly any incorporation of the tracer into organic compounds by protozoa and animals is unacceptable;

(5) The tracer must measure a process which, while unique to bacteria, is universal among them. Ideally all the different nutritional types of bacteria present should metabolise the tracer in a similar manner.

(6) the uptake or incorporation of the substrate must be interpretable in terms of bacterial growth under the range of conditions likely to be found in the natural environment.

Violation of any of these requirements results in a deviation of the observed metabolism from that which actually occurs in the closest physical approximation to the natural state the experimenter can devise. Assuming that a close approximation to the natural environment can be obtained (which is for argument's sake alone), an analysis of the metabolic consequences of these criteria with respect to current technology for measuring bacterial rate processes is in order.

Major Elements of Biomass as Tracers of Bacterial Activity and Growth

The major components of microbial cells (i.e. protein, nucleic acids, lipids, carbohydrates, cell wall polymers, and soluble intermediates) contain 6 elements of practical value in terms of potential for use as a tracer of bacterial growth: C, H, O, N, P, and S. The sources of these elements available to microorganisms in natural environments are diverse. Exchange reactions of hydrogen and oxygen with the highly mobile molecule H_2O virtually eliminate the use of either stable or radioactive isotopes of these elements in field situations, except for ^3H-labeled organic compounds labeled at positions which are not subject to significant exchange. A consideration of the other elements, their sources and products will be discussed below.

Carbon

A great deal of effort has been expended to develop means of estimating total microbial carbon production because of attempts to create a world carbon budget for modelling purposes. Reduced carbon is necessary for both biosynthesis of cell constituents and production of energy via respiratory or fermentative processes. Carbon is a major constituent of all macromolecules except for polyphosphates, and therefore is distributed in all cellular compartments. It is available to bacteria in a myriad of forms: the lysis of dead organisms and excretion of extracellular products by algae (c.f. Hellebust, 1965) and bacteria (Dunstall

and Nalewajko, 1975) release a wide variety of dissolved organic carbon compounds into the aquatic environement. In addition, insoluble cell wall polymers (e.g. chitin, cellulose, muramic acid, etc.) and mucopolysaccharide slimes often associated with fish and gelatinous zooplankton contribute potentially degradable and metabolizable carbon and energy sources for microbial growth. The diversity of these compounds and the difficulty of their quantitative analysis, especially in seawater, makes the estimation of total carbon metabolism in aquatic environments difficult if not impossible. The ability of microorganisms to use numerous compounds simultaneously is well known to microbial physiologists (Roberts et al., 1963; Boffi, 1969; Neidhardt and Magasanik, 1960; Law and Button, 1977). The practical problems posed by this phenomenon in field studies of heterotrophic uptake of organic carbon compounds were addressed in the introductory methodology paper on this subject (Parsons and Strickland, 1961).

The absence of sufficiently sensitive methods for the measurement of concentrations of specific organic carbon compounds in aquatic environments resulted in emphasis on the determination of the maximum velocity of uptake, turnover time, and other kinetic parameters (Hamilton and Preslan, 1970; Wright and Hobbie, 1966) for specific compounds. Although the meaning of these measurements with respect to microbial growth remains obscure in light of the variable uptake kinetics of various natural populations (Vaccaro and Jannasch, 1967; Williams, 1973), the work stimulated advances in methodology which partially relieved the restraints of items (1) and (2) outlined above. The use of tritiated substrates of high specific activity (Azam and Holm-Hansen, 1973; Dietz et

al., 1977) permitted the measurement of heterotrophic uptake rates at near ambient substrate concentrations, an important advance in organic carbon uptake measurements. However, advances in the measurement of actual concentrations of glucose (Hicks and Carey, 1968) and concommittant measurement of heterotrophic uptake of glucose (Vaccaro et al., 1968) revealed very low rates of uptake of this supposedly representative carbon compound. The application of amino acid analysis to aquatic samples with simultaneous measurement of uptake and respiration of these ubiquitous compounds (Williams et al., 1976) emphasized the abilities of natural populations to take up a wide variety of organic carbon compounds rapidly, and the frequently short turnover times suggest that analyses of the concentrations of individual compounds may be severely biased by microbial metabolism during sample collection and processing.

A further complication in the use of organic carbon compounds as the sole index of microbial growth is that incorporation, transformation, and respiration of organic carbon compounds can occur in the absence of reproductive growth (Antoine and Tepper, 1969; Belaich et al., 1972; Herbert, 1961; Neidhardt and Magasanik, 1960). Interpretation of whole cell uptake studies is not affected by such uncoupling of growth and metabolism when considered in terms of total carbon metabolism, but growth estimates can be in error substantially as a result of the accumulation of storage products or the uncoupled respiration and metabolism of organic compounds under stress.

Thus, in the best case, a quantitative assessment of the utilization of a small per cent of the total available organic carbon can be made. This does yield a minimum value for microbial carbon metabolism, but the

amount of unmeasured carbon metabolism and the proportion of total actively metabolizing bacteria measured with a given compound or compounds remains unknown. Nonetheless, valuable information pertinent to the understanding of in situ microbial growth can be obtained. In fact, the very disadvantages of the method, as outlined above, work to the advantage of the experimenter when cautions on interpretation are exercized and appropriate supplementary measurements are made. The widespread distribution of carbon in cellular components can facilitate determination of the nutritional state of a population. When disproportionately high rates of incorporation of carbon into key macromolecular fractions such as carbohydrate occur in the absence of other indications of reproductive growth as discussed below, one can suspect that a nutrient or physical stress has thrown the population into unbalanced or uncoupled growth.

The use of an organic carbon compound may provide a semi-quantitative estimate of total bacterial growth in one case. The uptake and incorporation of ^{3}H-labeled adenine by a pure culture of Serratia marinorubra was shown to be a sensitive indicator of RNA synthesis (Karl, 1979) and has potential for predicting protein synthesis rates by natural bacterial populations. The ubiquity of uptake of adenine at ambient concentrations by bacteria needs to be ascertained, as well as the magnitude of errors associated with non-bacterial adenine nucleotides in the particulate fraction. However, this method has great promise in regions devoid of both phytoplankton and microzooplankton, if they exist.

Nitrogen

The metabolism of nitrogen is more closely related to reproductive growth than is that of carbon. In bacteria, nitrogen occurs predominantly in proteins (about 17 % N by weight) and nucleic acids (about 9 % N by weight), with a small portion of the total N in lipids (less than 2% N by weight) and cell wall polymers. Nitrogen may be stored in excess of growth requirements as inorganic compounds in phytoplankton (Bhovichitra and Swift, 1977) or, in bacteria, as free amino acids (Tempest et al., 1970) and possibly soluble polyamines (Cohen, 1971). The major sources of nitrogen in aquatic environments include inorganic N_2, NO_3^-, NO_2^-, and NH_4^+; dissolved free amino acids (Lee and Bada, 1977; Williams et al., 1976); urea (Carpenter et al., 1972); and nucleic acid bases (Hodson and Azam, 1977), as well as less readily available macromolecular forms found in lipids, chitin, bacterial cell walls, etc. Methods are available for analysis of the concentrations of virtually all of these nitrogen sources.

Major problems associated with measurement of microbial nitrogen metabolism are altering the ambient substrate concentrations and achieving sufficient sensitivity to detect microbial metabolism. The stable isotope ^{15}N is frequently used as a tracer of nitrogen uptake by phytoplankton (Eppley and Rogers, 1970; Goering and Dugdale, 1964; McCarthy and Goldman, 1979), but substantial amounts must be added in order to detect metabolism. In nutrient-depleted waters, this addition can greatly stimulate the rate of nitrogen uptake (Eppley et al., 1973), an especially impor-

tant consideration because phytoplankton can engage in "luxury uptake", a phenomenon whereby the assimilation of nitrogenous compounds is far in excess of growth requirements (McCarthy and Goldman, 1979). The application of ^{15}N uptake to natural bacterial populations has not been reported. A radioactive isotope of nitrogen, ^{13}N, has been used for bacterial denitrification studies (Gersberg et al., 1976) but its 10 minute half-life makes its use impractical in most field situations.

The measurement of uptake of the suite of nitrogen compounds generally available to microorganisms may therefore be of use in determining the potential assimilation capability of natural populations, but obtaining quantitative rates at in situ concentrations is constrained by many of the same kinetic and metabolic arguments considered for the measurement of carbon uptake.

Phosphorus

Further specificity for macromolecular synthesis is found in the metabolism of phosphate. Predominant cellular compounds containing phosphate include only lipids, nucleic acids, polyphosphates, and soluble intermediates. The incorporation of phosphate into lipids and nucleic acids should be an excellent indicator of reproductive microbial growth. Phosphate is subject to luxury uptake similarly to nitrogen (Lean, 1973), but carrier free radioactive isotopes (^{32}P and ^{33}P) are available and uptake studies may be performed at virtually in situ nutrient concentrations. Furthermore, only two sources of phosphate are available: inorganic orthophosphate and organic phosphate esters. Phosphate esters are

frequently found in significant concentration, but the enzymes necessary to metabolize them are usually under genetic regulation such that phosphatase activities are not expressed until inorganic phosphate becomes limiting (Berman, 1970; Karl and Craven, 1980; Patni et al., 1977; Perry, 1972). The uptake and incorporation patterns of phosphate should be very informative in the assessment of aquatic microbial growth. Essentially all the criteria of a quantitative rate measurement can be fulfilled, yet surprisingly little work has been done in this area, especially in marine systems.

Sulfur

The distribution of sulfur in microorganisms is of even higher macromolecular specificity than that of phosphorus. The major sulfur-containing compounds of bacteria are soluble intermediates and protein. For most microorganisms, over 80% of the total cellular sulfur is found in protein (Roberts et al., 1963; Datko et al., 1978). S-containing RNA has been found in bacteria (Lipsett, 1965; Carbon et al., 1965), but comprises only a small portion of the total cellular sulfur. One group of bacteria produces sulfonolipids (Godchaux and Leadbetter, 1980), but there is no evidence of widespread production of these compounds. The metabolism of sulfur is therefore likely to be very closely related to protein synthesis.

Because of the very narrow range of sulfur-containing compounds in microorganisms, the relatively small pool sizes of sulfur-containing intermediates (Tempest et al., 1970; Tindall et al., 1977), and the fail-

ure to detect sulfur amino acids in marine environments (Schell, 1974; Lee, Mague, personal communications), the measurement of sulfate incorporation into protein presents itself very favorably as a potential quantitative assay for total microbial biosynthesis. Considering the criteria outlined above: (1) Numerous methods are available for the analysis of the sulfate concentration; a constant relationship exists between sulfate concentration and chlorinity (Rosenbauer et al., 1979) and the simple analysis of chlorinity may therefore be used to determine the sulfate concentration of ocean waters removed from zones of dissimilatory sulfate reduction. (2) The high sulfate concentration of seawater, over 25mM, eliminates the possibility of growth stimulation by increased nutrient concentration, and the radioactive isotope of sulfur, ^{35}S, can be obtained carrier free, allowing truly trace additions. (3) As previously mentioned, dissolved sulfur containing amino acids are not detected in seawater, and the sulfate esterases capable of releasing free sulfate are repressed in the presence of sulfate (Fitzgerald, 1976), eliminating the problems of competition for metabolism. (4) The reductive assimilation of sulfate is restricted to microorganisms (Schiff and Hodson, 1970), although sulfate esters are common among animals. (5) The absence of sulfur-containing compounds other than sulfate in seawater suggests that all microorganisms in the ocean must use sulfate as the sole source of sulfur.

The present work will demonstrate that sulfur metabolism can be related to reproductive growth by using the appropriate measurement technique, thus fulfilling the sixth and final requirement for a quantitative measurement of bacterial growth. Because there is complete absence of

sulfur metabolism literature for marine microorganisms and only scanty literature on the relationships between sulfur metabolism and growth for any microorganisms, a four part approach to the problem was taken as outlined below.

First, a suite of investigations into the mechanism of sulfate transport was conducted. The transport process is the first step in sulfate assimilation, and a thorough knowledge of factors influencing uptake of sulfate into the cell is necessary.

Second, the assimilation of sulfate into cellular components was monitored with an emphasis on the relationship between sulfur incorporation into protein and bulk protein synthesis. The relative rates of bulk protein synthesis, cell division, sulfate uptake and assimilation, and incorporation patterns for glutamic acid were determined during exponential growth in batch culture to serve as a control for experiments involving nutritional and environmental perturbations. Growth and sulfur metabolism were investigated with respect to a number of physiologically and ecologically meaningful parameters, i.e. carbon, nitrogen, and sulfur limited growth, growth on various carbon and energy sources, and protein synthesis inhibition by chloramphenicol. The influence of low molecular weight organic sulfur compounds on sulfate incorporation into protein and the equilibration of precursor pools were studied in order to understand potential sources of error in the interpretation of field measurements.

Two marine isolates were chosen for detailed study in pure culture to avoid possible misinterpretations arising from the use of a single organism. The two organisms chosen are readily and repeatably isolable from marine habitats and hence constitute a very real portion of the bacterial

assemblage, yet are quite distinct from one another both in general nutritional character and in specific aspects of their sulfur metabolism. The comparison of the two serves to remind us of the great diversity of metabolic capability among organisms in mixed populations and further reinforces consistencies in certain central metabolic processes.

Third, a series of enrichments of natural populations in the wild was performed to determine the relationship between protein sulfur and bulk protein in mixed bacterial assemblages. Additionally, pure cultures were isolated from similar enrichments and the relationship between sulfur metabolism and protein synthesis determined. Representatives of other groups of bacteria not commonly isolated by the enrichment techniques were also analyzed.

Finally, the rates of sulfate incorporation into protein by natural populations of bacteria were compared with standing crop parameters and other rate measurements in an effort to learn about relationships among various macromolecular processes in marine bacteria and environmental conditions which influence them.

CHAPTER 2.

GENERAL METHODS

The diverse and experimental nature of the work presented herein renders impractical the consolidation of all methods in a single section. Therefore generally used methods appear in this Chapter. Specific methods relating to the characterization of the sulfate transport systems, intermediary sulfur metabolism and growth, and natural population studies are found in their respective chapters.

Organisms and Culture Conditions

Pseudomonas halodurans

Pseudomonas halodurans was obtained as a pure culture from Dr. Galen E. Jones, University of New Hampshire. It has been described in detail by A. Rosenberg (Ph.D. Thesis, University of New Hampshire, 1977). Although it is a new species, it has not yet been published as such. Pertinent aspects of its physiology include a high degree of tolerance to salt (over 5-fold greater than that found in seawater) and other environmental stresses, broad nutritional capabilities, and extended viability in a variety of induced stationary phases. It grows well in basal salts medium containing sodium glutamate (hereafter referred to as glutamate) as the sole organic supplement, reductively assimilates nitrate, and does not produce poly-β-hydroxybutyrate (PHB). It is an obligate aerobe.

Alteromonas luteo-violaceus

The second organism was originally isolated from a seawater toilet over the Puerto Rico Trench on R/V Oceanus Cruise #40 (February, 1978; Station location 20°48'N, 65°07'W) using natural seawater enriched with glutamate. It is coccobacillary in morphology, with the major axis 1.7-2.0 μm and the minor axis 1.0-1.5 μm. It is obligately aerobic, motile in minimal medium containing glutamate, incapable of nitrate assimilation, capable of PHB production, and of extremely limited nutritional versatility. It grows on gelatin and starch in liquid medium, suggesting extracellular protease and amylase activities. The protease is extremely potent and the cells are apparently autolytic even during exponential growth, resulting in low plating efficiency and short term viability in the stationary phase. It has the unusual characteristic of producing β-glutamate (β-amino glutaric acid) equal to about 10% of the free glutamate pool when grown on glucose (S. Henrichs, personal communication) although it cannot use this compound as a sole carbon and energy source.

The organism produces a distinctive violet pigment when grown on gelatin, glutamate, or acetate but not on the other substrates tested, including peptone (0.5%)-yeast extract (0.25%) medium. Pigment production is pronounced in the early stationary phase. When grown in continuous culture on basal salts medium plus glutamate, pigment production oscillated with ultimate selection for non-pigmented variants. On occassion, viable counts on solid minimal medium containing glutamate give rise to a wide assortment of colonial morphologies with varied pigmentation, how-

ever, the smooth, convex, deeply-pigmented form can be recovered from any of the forms eventually. The pigment has absorption maxima of 577 (primary), 372, and 315nm in ethanolic solution (Figure 2-1), and acidification of the ethanol extract shifts the primary absorbance maximum to a double peak at 692 and 635nm. The pigment fluoresces strongly at 632nm in vivo and 544nm (with a small peak at 632nm) in ethanolic solution (Figure 2-2).

The characteristics of nutrition and pigment production are very similar to the description of Chromobacterium violaceans in Bergey's 8th Edition (Buchanan and Gibbons, 1974) and a marine representative, Chromobacterium marinum (Hamilton and Austin, 1967), but also to the Alteromonas luteo-violaceus of Gauthier (1976). The only apparent distinction of taxonomic value is the moles % (G+C), which is about 42 for Alteromonas and 65-72 for Chromobacterium. DNA from cells grown on peptone-yeast extract was extracted by the method of Marmur (1961) and neutral $CsCl_2$ density gradient centrifugation analysis for the % (G+C) was kindly provided by Dr. M. Mandel. A buoyant density of 1.703 g/cm^3 relative to B. subtilis phage RC DNA (1.742 g/cm^3; 42,040 RPM, 25°C, 23 hours) indicates a % (G+C) of 44, identifying this organism as Alteromonas luteo-violaceus.

To acquaint the reader with the great degree of nutritional disparity between the two organisms described above, the utilization of various carbon and energy sources, reduction of nitrate for assimilation, and use of thiosulfate as the sole source of sulfur for P. halodurans and A. luteo-violaceus is summarized in Table 2-1.

Figure 2-1. Absorption spectrum of the pigment from A. luteo-violaceus. Exponential phase cells were harvested by centrifugation and resuspended in one-half the original volume of 80% EtOH. After vortexing, the suspension was centrifuged and the absorption spectrum of the supernatant fluid recorded. The solution was then made to 10% (v/v) with concentrated H_2SO_4 and the absorption spectrum again recorded.

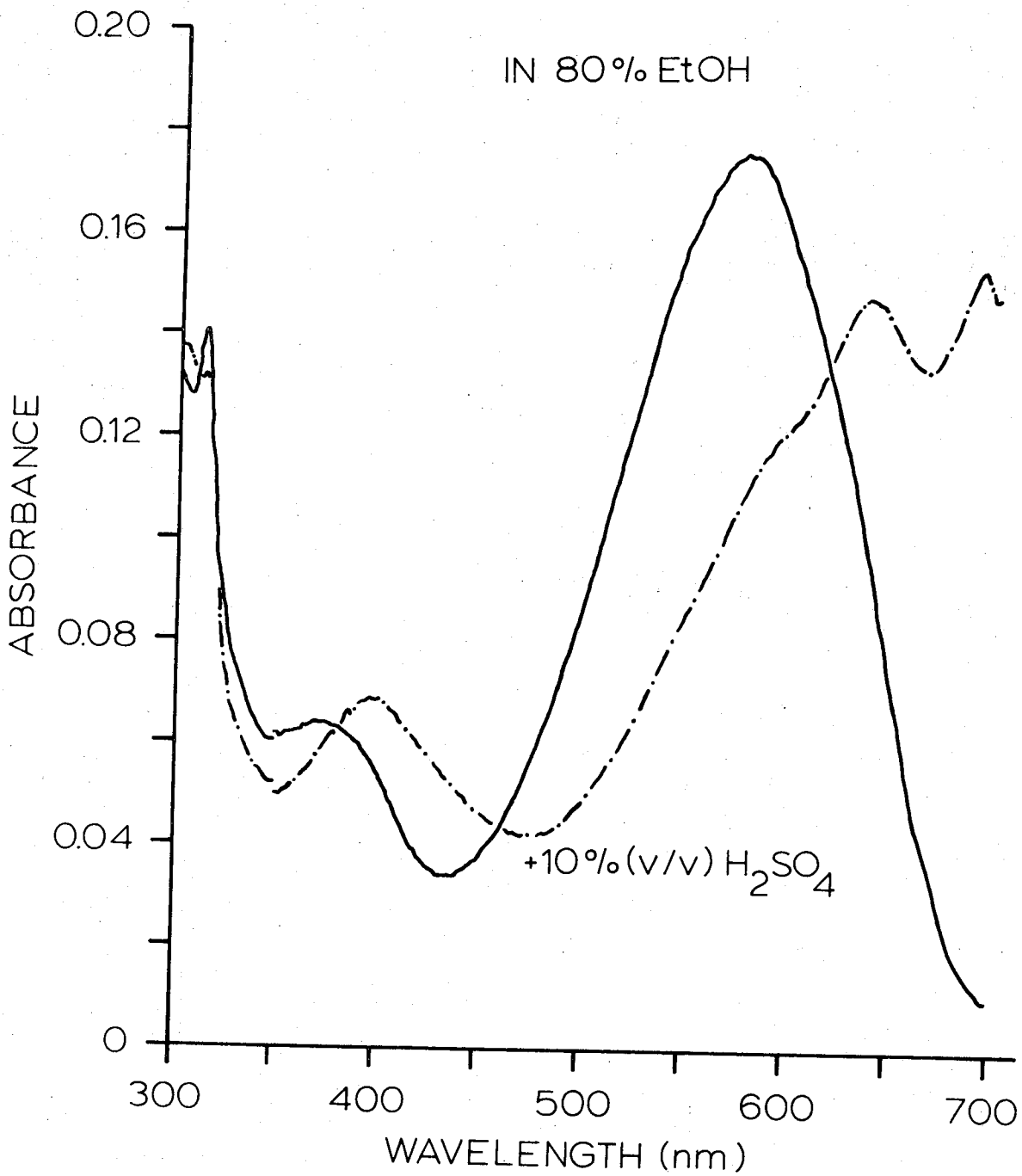

IN 80% EtOH
+10% (v/v) H_2SO_4
ABSORBANCE
0.20
0.16
0.12
0.08
0.04
0
300
400
500
600
700
WAVELENGTH (nm)

Figure 2-2. Fluorescence spectrum of the pigment from A. luteo-violaceus in vivo and in ethanolic solution. A washed suspension of whole cells and an aliquot of the unacidified ethanol extract from Figure 2-1 were excited at 315nm and the fluorescence spectra recorded.

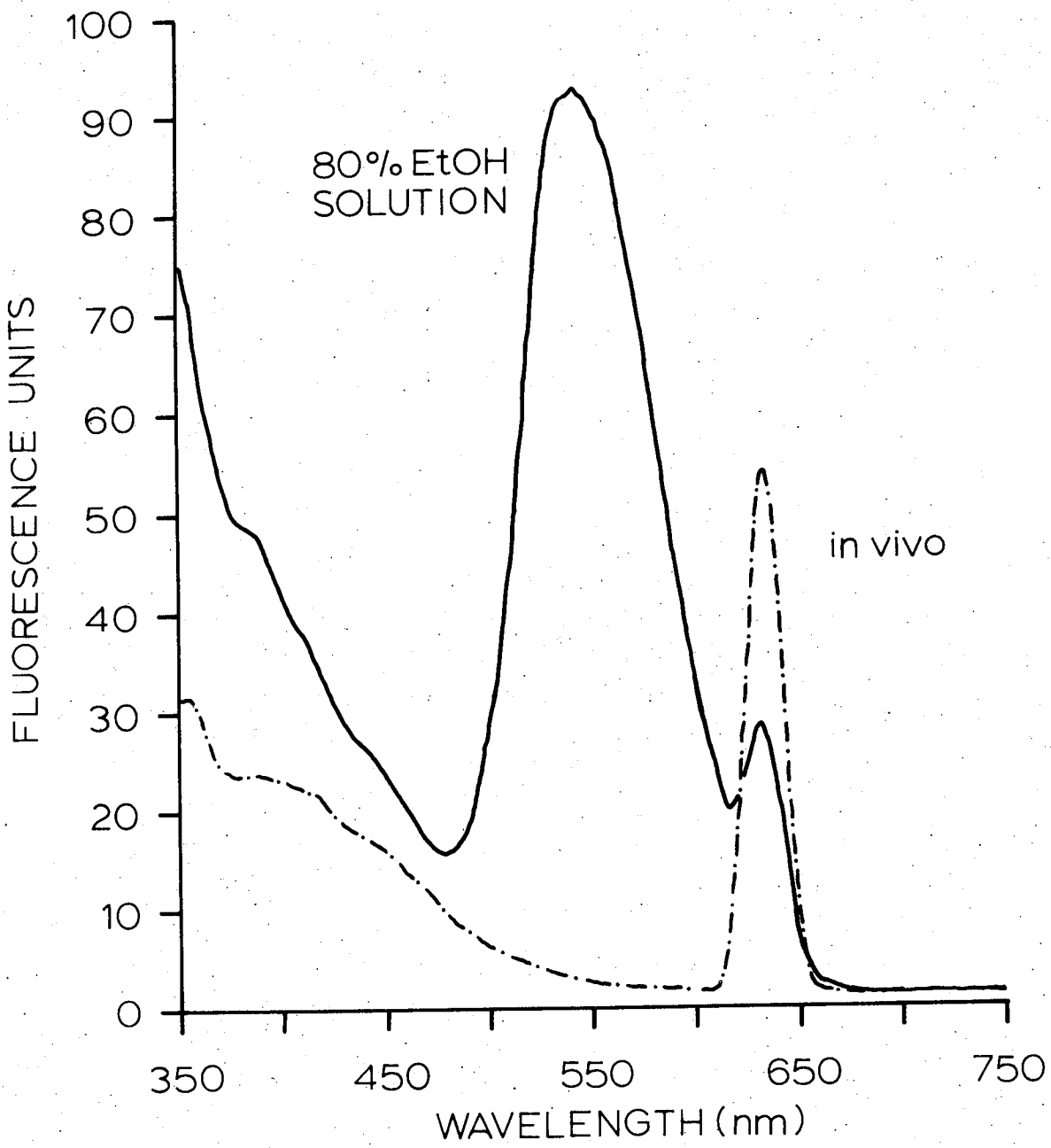

100
90
80
70
60
50
40
30
20
10
0
FLUORESCENCE UNITS
80% EtOH
SOLUTION
in vivo
350
450
550
650
750
WAVELENGTH (nm)

Table 2-1. Substrate Utilization by *Pseudomonas halodurans* and *Alteromonas luteo-violaceus*[a]

Substrate	Concentration	*P. halodurans*		*A. luteo-violaceus*	
		Growth	Time[b]	Growth	Time
Acetate	10mM	++	1	+	2
Adenine	5.5mM	–		–	
DL-α-Alanine	6.3mM	++	1	++	2
β-Alanine	6.3mM	++	1	–	
L-Aspartate	8.8mM	++	1	–	
L-Asparagine	5.0mM	W	3	++	2
Butyrate	5.0mM	++	2	–	
Citrate	10mM	++	1	–	
Creatine	6.0mM	–		–	
Crotonic Acid	8.7mM	+	2	–	
Ethanol	206mM	++	1	–	
Formate	10mM	–		–	
Fructose	10mM	++	1	–	
Fumarate	5.3mM	++	1	–	
Galactose	10mM	++	1	–	
Gelatin	1mg/ml	–		++	1
Glucololactone	10mM	++	1	–	
D-Glucose	10mM	++	2	++	3
L-Glutamate	10mM	++	1	++	1
L-Glutamine	6.3mM	++	1	+	2
Glycine	10mM	–		–	
Glycerol	10mM	++	1	–	
L-Histidine	5.9mM	–		–	
DL-Isoleucine	5.2mM	W	4	W	5
2-Ketoglutarate	5.1mM	++	1	–	
D-Lactate	10mM	++	1	–	
Lactose	10mM	–		–	
DL-Leucine	9.9mM	W	3	–	
L-Lysine	5.2mM	++	3	–	

Table 2-1. (Continued)

Substrate	Concentration	P. halodurans Growth	P. halodurans Time[b]	A. luteo-violaceus Growth	A. luteo-violaceus Time
Malonate	5.0mM	++	2	-	
Maltose	4.7mM	++	1	++	2
Mannitol	5.0mM	++	1	-	
Mannose	10mM	+	2	++	3
Methanol	313mM	-		-	
Propionate	5.1mM	+	2	+	4
Pyruvate	10mM	++	1	+	4
D-(-)-Ribose	10mM	++	2	-	
DL-Serine	5.1mM	W	4	-	
Starch	1.5mg/ml	-		+	2
Succinate	10mM	++	1	-	
Sucrose	10mM	++	1	-	
L-Threonine	5.3mM	-		-	
L-Tryptophane	5.1mM	W	4	-	
DL-Valine	7.3mM	-		-	
Xylose	10mM	-		-	
No carbon source		-		-	
D-Glucose, no N	10mM	-		-	
D-Glucose, no S	10mM	-		-	
D-Glucose, NO_3^-	10mM	++	2	-	
D-Glucose, $S_2O_3^=$	10mM	++	2	-	

[a] RLC-water medium containing 1mM $SO_4^=$, 500 µM NH_4^+, 40 µM $HPO_4^=$, and trace metals was supplemented with sterile, neutralized solutions of substrate to give the final concentrations indicated. Substitution for NH_4^+ and $SO_4^=$ was made for the NO_3^- and $S_2O_3^=$ utilization study. Tubes containing 10ml of medium were inoculated with washed cells to a final concentration of about $2x10^5$ cells/ml and shaken at 250 RPM at 22°C. The appearance of turbidity was used as the growth index: ++, luxuriant; +, good; W, weak; -, no growth.

[b] Days to reach maximum turbidity.

Relatively Low Contamination Artificial Seawater:

The study of assimilatory sulfur metabolism, especially aspects of sulfate transport, requires the ability to regulate or completely eliminate sulfate from the medium. A natural seawater medium containing over 25mM sulfate does not permit the sensitivity necessary to detect rapid changes in intracellular distribution of sulfur metabolites present in small quantities. Initially, a synthetic seawater medium of the same composition as the Lyman and Fleming (1940) formulation was used, simply substituting chloride equivalents for the usual sulfate salts of magnesium and the trace elements. Very shortly after the isolation of A. luteo-violaceus, however, it became clear that a serious contamination of some sulfur compound was present in the medium. In a survey of thiosulfate utilization by cruise isolates, A. luteo-violaceus retained nearly maximum growth rate and yield in the control lacking added sulfur, even after several transfers. Calculation of potential contamination from stock reagent grade chemicals revealed that the bulk consituent, NaCl, was contributing up to 7 μM sulfate. Lower levels of sulfate in NaCl could be obtained through the use of J. T. Baker "Ultrex" NaCl, but at $97/kilo this method of reducing sulfate contamination was not feasable. Many thanks are due to Dr. Ollie Zaffiriou of the W. H. O. I. chemistry department for suggesting that NaCl be made by neutralization of NaOH, since reagent grade NaOH can frequently be obtained with only one tenth the sulfate contamination found in the best commercial grade NaCl. This modification of the "sulfate-free" Lyman and Fleming formulation yielded an artificial seawater with relatively low contamination, designated RLC-water. Its

components and estimated vs. actual sulfate contamination are listed in Table 2-2. Inorganic sulfate contamination of the components of RLC-water is determined on concentrated stock solutions and the final medium using the method of the American Public Health Association (1975). It is indeed gratifying that all reagents contain less than or equal to the amount of sulfate listed on the specification sheet.

Medium Supplements:

Changes in pH during autoclaving of RLC-water are avoided by adding 0.22 μm filter-sterilized $NaHCO_3$ aseptically to the autoclaved basal salts medium. Routine culture and maintenance medium contains the following additional sterile supplements to the autoclaved basal salts mixture, with their respective final concentrations, sodium glutamate (10mM), NH_4Cl (500 μM), KH_2PO_4 (50 μM), and 1ml/l IMR trace elements solution (Eppley et al., 1967). The nitrogen, phosphate, and trace metal supplements are collectively referred to as "inorganic nutrients" in later sections. The complete medium assays at less than 2 μM $SO_4^=$. Growth medium contained 1mM Na_2SO_4 unless otherwise noted. All cultures are grown at pH 7.8-8.0 at 20 ± 2°C on a gyrotary shaker (New Brunswick Scientific, Edison, NJ) at 250 RPM.

Cell Counts:

Direct cell counts are made using the acridine orange epifluorescence method (Daley and Hobbie, 1975). A 0.45 μm cellulose nitrate filter is

Table 2-2. Inorganic Sulfate Contamination of Artificial Seawater Preparations

Compound	Grams per Liter			$SO_4^{=}$ Contamination (μM)	
	Lyman and Fleming, 1940[a]	Both	RLC-Water	Maximum Predicted[b]	Assay[c]
NaCl	23.75		-	9.89	4.78
NaOH	-		16.256	0.85	0.24[d]
HCl	-		14.817	0.09	-
$MgCl_2.6H_2O$		10.63		2.21	0.27
$CaCl_2.2H_2O$		1.45		1.51	0.78
KCl		0.664		0.07	0.06
$NaHCO_3$		0.192		0.06	0.03
KBr		0.096		0.05	ND[e]
H_3BO_3		0.026		0.03	0.01
$SrCl_2.6H_2O$		0.040		0.01	0.01
H_2O		To make 1000 ml			0.09
		Totals:	Lyman and Fleming:	13.83	6.03
			RLC-water:	4.88	1.49

[a]Equimolar chloride salts are substituted for the normal sulfate salts.
[b]From stated maximum limits of impurities for reagent grade chemicals.
[c]Turbidometric assay as described on 10-1000-fold concentrated solutions.
[d]NaOH + HCl at pH 7.0.
[e]Not determined due to Br^- interference.

used beneath a stained (0.2% Irgalan Black in 2% acetic acid) 0.2 μm Nucleopore polycarbonate membrane filter to aid in even distribution of the cells. An aliquot (usually 1.75ml) of the cell suspension or appropriate dilution is pipetted into the funnel, followed by enough Acridine Orange solution (0.1% in 0.02M Tris buffer, pH 7.8) to give 0.01% final concentration. After 5 minutes, the sample is filtered gently, and the filter placed on a glass slide followed by a drop of low fluorescence immersion oil (Cargille Laboratories, Cedar Grove, N. J.), a cover slip, and another drop of immersion oil. The sample is counted on a Zeiss microscope fitted with the epifluorescence attachment. At least 18 fields are counted in a cross pattern from top to bottom and side to side to average out non-uniformity in cell distribution. In some cases dividing pairs are counted as well as the total number of cells.

Viability is determined by plating dilutions of the culture on complete medium containing 15 g/l agar (Difco). Duplicate plates at two or three different dilutions are generally used for the determination of the number of colony forming units (CFU).

Protein Determination:

In the pure culture experiments, protein is determined on trichloroacetic acid (TCA) insoluble material. Cell suspensions (5-25ml) are brought to 10% (w/v; final concentration) TCA with 100% (w/v) TCA, mixed thoroughly, and refrigerated for at least 30 minutes but less than 4 days. The precipitate is centrifuged (20,000xg, 20 minutes), the supernatant fluid aspirated off, and the pellet drained of excess TCA. The

pellet is then dissolved in an appropriate amount of 0.1N NaOH to give about 100-300 µg protein/ml. Aliquots of the sample (0.1ml) are neutralized with an equal amount of 0.15N HCl and the protein concentration assayed by the Coomassie Brilliant Blue dye-binding technique of Bradford (1976). The assay mixture contains, in a final volume of 5.1ml: 0.1ml sample, 0.1ml 0.15N HCl, and 4.9ml diluted dye reagent (1:5, v/v; BioRad Laboratories, Richmond, Ca.). The acidification step enhances the sensitivity of the assay slightly and extends the range of linear increase of the $O.D._{595}$ with protein concentration (0-400 µg/ml; sensitivity limit 40-50 µg/ml). It has been determined in separate experiments that protein remains stable in 10 % TCA for at least 4 days. On occassion the micro modification of the assay is used, especially for samples of the protein residue remaining after the biochemical fractionation procedure described below. This assay contains, in a final volume of 1.25ml: 0.5ml sample, 0.5ml 0.15N HCl, and 0.25ml concentrated dye reagent. The limit of sensitivity of this assay is 5-10 µg protein/ml. The absorption at 595 nm is recorded on a Beckman DU monochrometer with a Gilford Model 252 photometer in 1cm quartz cuvettes against a distilled water blank. Sample values are interpolated from the slope of the least-squares regression line over the linear portion of the standard curve.

Particulate Organic Carbon and Nitrogen:

Aliquots of cell suspensions (10-25ml) or water samples from natural environments (1-10 liters) are filtered through Whatman GF/F filters, folded in eighths, placed in small aluminum foil holders (5x30mm), and

stored desiccated over silica gel in glass tubes (10x75mm) with foil caps. All materials are combusted in a muffle furnace at 450°C for 6 hours prior to use. The samples are analyzed by Phil Clarner using a Perkin-Elmer Model 240 CHN analyzer.

Carbohydrate:

Samples filtered as for particulate organic carbon are assayed for carbohydrate by the modification of the anthrone procedure proposed by Jermyn (1975). Recovery of toadfish glycogen (provided by J. J. Stegeman) as glucose equivalents was 98% by this method.

Respiration of Organic Substrates:

Experiments involving the determination of organic carbon metabolism are carried out in foam-plugged flasks with atmospheric gas exchange. Due to the equilibration of the medium with atmospheric CO_2 the traditional wick method (Hobbie and Crawford, 1969) cannot be used to estimate $^{14}CO_2$ evolution from ^{14}C-labeled organic substrates. Determination of $^{14}CO_2$ evolution in these open systems takes advantage of the volatility of CO_2 in acid medium: an aliquot of the cell suspension (0.25-1.0ml) is acidified with 0.25ml 2N HCl or 2N H_2SO_4 and an air stream is passed vigorously over the surface of the solution in a scintillation vial for 15 minutes. The sample is neutralized with 0.5ml 1M Tris buffer, pH 13, and Aquasol liquid scintillation counting fluid is added. The difference between the sample counts and a like treatment at zero time is

the acid-volatile radioactivity. This procedure is a small-scale version of the method used for the measurement of excreted organic carbon by phytoplankton, and eliminates virtually all $^{14}CO_2$ from the medium (Anderson and Zeutschel, 1970; Berman and Holm-Hansen, 1974; Nalewajko et al., 1976). Zero time values for UL-^{14}C-labeled glucose and glutamate are within 1-2 % of unprocessed controls, but up to 5 % of ^{14}C-labeled acetate may be volatilized under these conditions. Although not as sensitive as the wick method (limit of detection about 3% of the total substrate respired), it can be used effectively in open systems. Respiration determined by this method is identified as acid-volatile radioactivity. A comparison of respiration measured by the two methods is presented in Table 2-3. When the results of the wick method are corrected for recovery based on $^{14}CO_2$ addition, the two methods agree well for transformation of ^{14}C-glutamate by an obligately aerobic bacterium, P. halodurans.

Filtration of Isotopically-Labeled Cells:

The small amount of total sulfur metabolism in bacteria coupled with the high sulfate concentration of seawater necessitates the use of high activities of $^{35}SO_4^=$, as much as 25 µCi/ml in field samples. This poses major isotope adsorption blank problems, encountered even in sulfate uptake studies in freshwater systems (Jordan et al., 1978). The problem has been resolved to a large extent by the use of a specially constructed punch funnel (Figure 2-3), which serves to excise only the cell-retaining, easily rinsed portion of the filter. A comparison of cell-free blanks using the normal flanged funnel and the punch funnel

Table 2-3. Recovery of $^{14}CO_2$ and Respired ^{14}C-Glutamate from Seawater Measured by Two Different Techniques[a]

	Wick Method[b]		Acid Volatile[c]	
Volume (ml)	DPM per ml	% Recovery	DPM per ml	% Recovery
		$^{14}CO_2$		
1	13,103 ± 399	95.1	13,738 ± 29	99.7
2.5	12,383 ± 98	89.9	-	-
5	12,291 ± 177	89.2	-	-
10	12,048 ± 183	87.5	-	-
20	11,585 ± 32	84.1	-	-

	Wick Method (10ml)		Acid Volatile	
Hours of Incubation	DPM per ml	Corrected DPM per ml[d]	DPM per ml	% Recovery[e]
		^{14}C-Glutamate Respired by P. halodurans		
0	8 ± 3	9 ± 3	-46 ± 69	-
7	3878 ± 76	4432 ± 87	4297 ± 17	97.0

[a]Aged natural seawater containing inorganic nutrients and 100 µM glutamic acid was inoculated with (a) $^{14}CO_2$ (final activity 13,774 ± 5 DPM per ml) or (b) UL-^{14}C-glutamic acid (final activity 11,078 ± 90 DPM per ml) and about $1x10^6$ P. halodurans cells per ml. Recovery of $^{14}CO_2$ was determined on triplicate 1ml samples (acid volatile method) or duplicate 1-20ml samples (wick absorption method).

[b]In a 25ml flask fitted with a serum stopper and bucket, samples were acidified with 0.3ml 2N H_2SO_4. Evolved $^{14}CO_2$ was trapped on a paper wick wetted with 0.3ml Protosol during 2 hours of mild agitation.

[c]A 1ml sample was acidified with 0.3ml 2N H_2SO_4, air sparged for 10 minutes, neutralized with TRIS buffer, and 10ml Aquasol added. Results are expressed as DPM volatilized per ml.

[d]Based on recovery of $14CO_2$ for a 10ml sample.

[e]Relative to wick absorption.

is presented in Table 2-4. The original design was made from polycarbonate tubing and could not cut a membrane filter, but the newer version (illustrated) with a stainless steel punch does work with cellulose nitrate and equivalent filters as well as the glass fiber filters used in this study. The punch funnel is used for all isotope filtrations regardless of activity: the bore diameter was chosen such that the excised portion of the filter is amenable for use with the conical centrifuge-type tissue grinders used in the fractionation procedure described below.

Using a vacuum of less than 15" of mercury, samples of 1-50ml are filtered through Reeve Angel 984H Ultrafine or Whatman GF/F glass fiber filters. The filter is rinsed three times with 0.5M NaCl (about 3ml per rinse), and the cell-retaining portion excised. The sample may then be fractionated (see below) or placed in a vial with scintillation cocktail and counted whole.

BIOCHEMICAL FRACTIONATION OF RADIOACTIVELY LABELED SAMPLES

The method used to separate major biochemical fractions for determination of the distribution of radioisotopic tracers is that of Roberts et al. (1963). Their work includes a detailed chromatographic analysis of materials contained in each of the fractions and it is assumed that the observed sequential elution of components is applicable to bacteria in general. For the samples from R/V Oceanus cruise 84 the modification of Neidhardt and Magasanik (1960) was added to allow separation of RNA from the DNA + polysaccharide fraction. In essence, samples are treated with

Figure 2-3. Punch funnel used for filtration of radioisotopically-labelled samples. The funnel (upper portion) is 3/4" polycarbonate tubing, and the lower portion is 316 stainless steel. The scale bar represents 1/2". A small square of Whatman #1 or similar filter paper is placed over a 25mm fritted glass base to preserve the frit surface and prevent clogging by fibers from the glass filters. A 25mm Reeve Angel 984H or Whatman GF/F filter is placed on top, followed by the punch funnel. To reduce loss of vacuum for samples larger than 10ml, a thin piece of rubber punched with a hole slightly larger than 1/2" may be placed on top of the filter. This funnel may also be used with membrane filters.

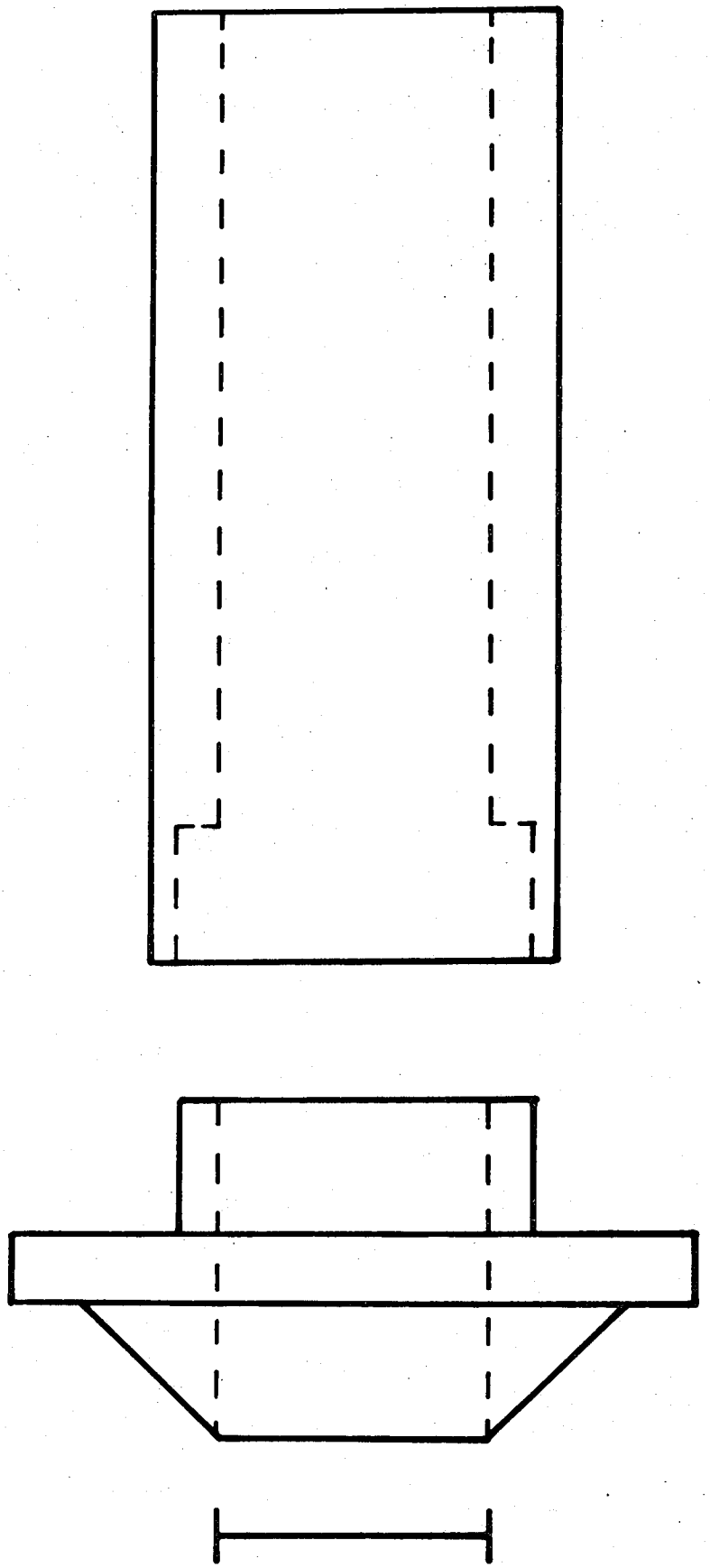


Table 2-4. Retention of $^{35}SO_4^{=}$ by Millipore HAWP (0.45 μm) Membrane Filters and Reeve Angel 984H Ultrafine Glass Fiber Filters Using the Flanged Funnel vs. Retention by Glass Fiber Filters Using the Punch Funnel Shown in Figure 2-3[a]

Volume Filtered (ml)	DPM Retained		
	Flanged Funnel		Punch Funnel
	HAWP	984H	984H
1	333 ± 6	1622 ± 160	128 ± 8
2.5	607 ± 8	3550 ± 123	206 ± 48
5	942 ± 134	5554 ± 5	244 ± 14
10	2167 ± 167	8339 ± 148	393 ± 23
25	3491 ± 671	17,010 ± 2249	506 ± 113

[a] 6 μCi $^{35}SO_4^{=}$/ml in 0.22 μm filtered seawater; values are the average and error of duplicate filtrations.

solvents which sequentially solubilize major classes of biochemical compounds (i.e. low-molecular weight (LMW) soluble compounds, lipids, RNA, hot acid-soluble materials (DNA + polysaccharides), ethanol-soluble protein, and residual protein). Details of the procedure are outlined below.

a) Centrifugation Procedures

Generally, the fractionation procedure is performed on samples which have been filtered onto glass fiber filters as described above. Glass fiber filters were chosen for several reasons: the filters disintegrate easily in the conical centrifuge-type ground glass tissue grinders (Bellco Glass; Vineland, N. J.) used for the extraction procedure, and the very high surface to volume ratio of the fibers provides a large surface for the adsorption of precipitated macromolecules and greatly facilitates resuspension of pellets in subsequent extraction steps. Additionally, the occassional aspiration of small amounts of the fibers along with the supernatant liquid introduces little error in distribution because of the large volume of the pellet (ca. 50 µl). The filters can be combusted to remove organic carbon and nitrogen contaminants and hence particulate analyses can be directly compared with the results of isotope experiments. The filters retain >99.6% of exponential phase P. halodurans and A. luteo-violaceus cells as determined by direct counts, and comparison of GF/F and 0.2 µm Nucleopore filter retention of isotopically labeled natural populations indicates that less than 10 % of the labeled material passes through the glass filters in field experiments (see Chapter 5). One drawback to the use of these filters is that the large pellet

volume necessitates a rinsing step after each extraction to remove solubilized counts trapped in the pellet. In view of the aforetomentioned advantages, this is a small price to pay.

The use of glass conical centrifuge-type tissue grinders eliminates cross-contamination among samples and transfer losses because extraction and centrifugation are accomplished in the same vessel. Initially, centrifugation was carried out in the SS-34 rotor (Sorvall; Newton, Ct.) at 8000 RPM (7710xg) for 20 minutes in an RC2-B centrifuge. A minor problem associated with the use of the conical tube and a fixed-angle rotor is that the pellet tends to hang up at the junction of the cone, resulting in some pellet resuspension during aspiration of the supernatant fluid. This was eliminated by the acquisition of an HS-4 swinging bucket rotor (Sorvall); samples spun at 5000 RPM (4500xg) for 20 minutes give a clean pellet at the bottom of the conical tube. In addition to the larger sample capacity of this rotor (20 tubes vs. 8 in the SS-34), the reproducibility of isotope distribution in the various fractions is increased significantly. Centrifugation at 20°C is followed by aspiration of the supernatant fluid with Pasteur pipets into 10ml graduated cylinders, in the case of LMW soluble, RNA, and hot acid soluble fractions, or into 16x125mm screw-cap tubes in the case of alcohol and alcohol-ether soluble fractions. The rinses for each fraction are combined with the appropriate original supernatant fluid, vortexed thoroughly, the volume recorded, and an aliquot taken for liquid scintillation counting as described below.

b) Fractionation Procedure

Samples are ground with the ground glass pestle in a small volume of 10% TCA (ca. 50 µl) and the pestle rinsed with about 1-1.5ml 10% TCA. After 30 minutes, the sample is centrifuged, the supernatant fluid withdrawn, and the pellet vortexed in an additional 1-1.5ml 10% TCA. After centrifugation, the superantant fluid is combined with the first, yielding the LMW soluble fraction, containing inorganic ions, free amino acids and peptides, vitamins, and metabolic intermediates.

The pellet is resuspended in 80% EtOH (pH adjusted to 6.5-7 with dilute NaOH or HCl as necessary) and incubated in a water bath at 60°C for 20 minutes. The suspension is centrifuged and the supernatant fluid aspirated into a screw-cap tube. The pellet is then extracted with about 4ml 80% EtOH:diethyl ether (1:1; v/v) at 60°C for 20 minutes, centrifuged, and the supernatant fluid combined with the EtOH-soluble material. The pellet is further extracted with 2ml of 80% EtOH:diethyl ether for 10 minutes at 60°C and this supernatant combined with the previous two. The resulting fraction is the alcohol-soluble, alcohol-ether-soluble material containing lipids and alcohol-soluble protein.

The optional base-catalyzed hydrolysis of RNA (not done in laboratory pure culture experiments) is accomplished by resuspending the pellet in 1ml 1N KOH at 37°C for 60 minutes. The suspension is then neutralized with 0.5ml 2N HCl, and made to 10% TCA by the addition of 0.167ml 100% TCA with vigorous mixing. The DNA, polysaccharides, and protein are allowed to precipitate for 30 minutes in an ice bath, and the suspension centrifuged. The supernatant fluid and a rinse of about 1ml 10% TCA are

combined in a graduated cylinder, resulting in a fraction containing monomeric RNA residues.

Polysaccharides plus total nucleic acids (in laboratory experiments) or DNA (in field experiments) are solubilized by incubating the residue from the above extractions in 1.5ml 10% TCA at 95°C for 20 minutes, followed by centrifugation and a further 10 minute incubation at 95°C in about 1ml 10 % TCA. The supernatant fluids are combined, yielding the hot acid-soluble fraction.

The pellet, containing residual protein, is dissolved in 0.1N NaOH.

c) Further Separations:

For samples labeled with $^{35}SO_4^{=}$, inorganic sulfate contamination of the LMW soluble materials obscurs the contribution of LMW organic components. The inorganic sulfate is therefore removed by preciptation with barium as follows: to 1.75ml of the LMW soluble fraction are added, with vortexing, 50 µl 50mM Na_2SO_4 followed by 0.25ml 1M $BaCl_2$ (final concentrations: $SO_4^{=}$, 1.22mM; Ba^{++}, 122mM). The precipitate forms during a 2 hour period at 4°C, after which it is removed by centrifugation in a clinical centrifuge at full speed for 10 minutes. A 1ml aliquot is taken for counting to give the LMW organic fraction. If the amount of label present as sulfate is desired as well, the pellet is rinsed twice with 1M $BaCl_2$ and resuspended in water for counting. The sum of soluble plus precipitated materials rarely differs from counts of unprocessed acid-soluble material by more than 5%, but a small loss of ^{14}C-labeled LMW soluble compounds is observed (see Chapter 5).

The supernatant fluid from the combined alcohol-soluble and alcohol-ether soluble fractions may be separated into two components, lipids and alcohol-soluble proteins. To the tube containing these fractions is added, with vortexing, 5ml diethyl ether followed by 3.5ml distilled water. The fluid will separate into two phases; an upper ether phase containing lipids, and a lower aqueous phase containing alcohol-soluble proteins. The amount of water required to effect phase separation may vary slightly depending on the total volume of the mixture. After a 3-5 minute spin at high speed in a clinical centrifuge to effect complete separation of the two phases, the upper layer is pipetted into a scintillation vial. The aqueous phase is re-extracted with 5ml of diethyl ether, centrifuged, and the upper organic layer combined with the first. The aqueous phase is pipetted into another vial, the tube rinsed with 95 % EtOH, and the rinse combined with the aqueous material. The volume of the samples is reduced to approximately 0.5ml by passing an air stream over the vials in a sand bath at about 45°C and 10ml Aquasol added for counting. Both samples are usually counted in their entirety. The alcohol-soluble, ether-insoluble component (mostly protein) is rarely over 5% of the total cellular isotope with any label. Cells labeled with ^{35}S contain virtually no ether-soluble (lipid) radioactivity (less than 2 % of the total ^{35}S) and often in pure culture experiments the alcohol-soluble plus alcohol-ether soluble fraction is not separated prior to counting.

d) Liquid Scintillation Counting

All samples are counted in Aquasol liquid scintillation fluid (New England Nuclear, Boston, Ma.) in a Beckman LS100-C liquid scintillation counter. Most samples are counted for 50 minutes or to an error of 1%, whichever comes first. Correction for chemical quenching is made by the use of the channels ratio technique, with a quench curve constructed with the solvents normally used (seawater, 10% TCA, EtOH, etc.). Highly acidic or basic samples are neutralized with 1M Tris buffer of appropriate pH before addition of Aquasol.

Samples labeled with ^{32}P (half-life 14.7d) or ^{35}S (half-life 87.9d) are counted with vials of the original medium placed among samples at appropriate intervals so that the specific activity can be calculated. Since the activity of the medium decays at the same rate as the samples, recalculation of the specific activity at intervals short relative to the half-life of the isotope eliminates the need for decay-correction programs.

The atomic weight of radioisotopes used for tracers of biological activity is usually higher than that of the naturally-occurring element, and account must be taken for the discrimination of isotopes by microorganisms to provide truly quantitative results. Until recently, all information on sulfur isotope discrimination by bacteria was confined to dissimilatory sulfate reduction, but the applicability of the discrimination factor (up to 3.5 % in favor of ^{32}S over the stable isotope ^{34}S) was questionable for studies of sulfur assimilation. Specific analysis of

$^{34}S/^{32}S$ ratios of incorporated sulfur derived from sulfate in Clostridium pasteurianum grown under conditions preventing dissimilatory sulfate reduction (Laishley et al., 1976; McCready et al., 1975) revealed a small (1.2 ± 0.6 o/oo) positive enrichment of the heavy isotope ^{34}S. In most cases, organisms discriminate against the higher atomic weight compound, and the addition of one atomic weight unit in ^{35}S, the radioisotope of sulfur used in this work, may bring the small enrichment to a near zero value. Therefore no correction has been applied to the calculations for sulfate incorporation. No data are available for discrimination resulting from the use of ^{14}C-labeled organic compounds, so no correction is possible.

Radiochemicals:

Carrier-free $^{35}SO_4^=$ (sodium salt), $^{35}SSO_3^=$ (sodium salt; 10-30mCi/mMole), and UL-^{14}C-glutamic acid (285mCi/mMole) were obtained from Amersham (Chicago, Ill.). Ethanolic solutions of isotopes were evaporated and reconstituted with distilled water prior to use.

Other Chemicals:

Dicyclohexylcarbodiimide (DCCD) and carbonylcyanide m-chlorophenylhydrazone (CCCP) were obtained from U.S. Biochemicals (Cleveland, Ohio); para-hydroxymercuribenzoate (pHMB) and 2,4-dinitrophenol (2,4-DNP) were from Sigma (St. Louis, Mo.). Na_2SeO_4 was the gift of I.K. Smith.

Acridine Orange was from Matheson, Coleman, and Bell (Norwood, Ohio). All other chemicals are reagent grade. Special precautions are taken to obtain components of the artificial seawater with the lowest possible sulfate contamination.

CHAPTER 3

SULFATE TRANSPORT IN MARINE BACTERIA

INTRODUCTION

The sulfate transport systems of bacteria isolated from marine habitats have not been characterized. Seawater contains about 25 mM sulfate, a concentration three orders of magnitude higher than most freshwater environments, and this saturating concentration has existed for geological time. Study of the sulfate uptake characteristics of marine bacteria may therefore provide information relevant to the understanding of adaptation by microorganisms to the much more dilute freshwater and terrestrial environment. In addition, knowledge of the factors regulating sulfate uptake and conditions which may affect the transport of this nutrient will aid in the interpretation of sulfate metabolism studies, both in pure culture and in natural populations.

Sulfate uptake systems have been characterized from a wide variety of organisms including fungi (Marzluf, 1970a; Yamamoto and Segel, 1966), cyanobacteria (Utkilen et al., 1976), algae (Deane and O'Brien, 1975; Ramus, 1974; Vallee and Jeanjean, 1968a), enteric bacteria (Dreyfuss, 1964), and higher plants (Hart and Filner, 1969; Leggett and Epstein, 1956; Smith, 1975, 1976; Vange et al., 1974). Among these studies are found several common characteristics which suggest a strong evolutionary cohesiveness in the development of this transport system among microorganisms and higher plants. The Michaelis constant (K_m) for sulfate uptake spans a range of 1–50 μM, the only higher value being that of 200 μM reported for the conidial stage of Neurospora crassa (Marzluf, 1970a). Active or energy-requiring transport has been demonstrated

through the use of the ATP-ase inhibitor DCCD* (Jeanjean and Broda, 1977; Smith, 1976), proton or electrical gradient uncouplers CCCP (Deane and O'Brien, 1975; Jeanjean and Broda, 1977; Smith, 1976) and 2,4-DNP (Holmern et al., 1974; Renosto and Ferrari, 1975; Smith, 1976; Yamamoto and Segel, 1966), and the respiratory poisons cyanide and azide (Roberts and Marluf, 1971; Yamamoto and Segel, 1966). Additional evidence for active transport is found in the accumulation of sulfate against an external concentration gradient (Utkilen et al., 1976; Vallee and Jeanjean, 1968a; Yamamoto and Segel, 1966). Sulfhydryl reagents (e.g. pHMB, NEM) are effective in abolishing sulfate uptake (Holmern et al., 1974; Marzluf, 1974; Smith, 1976; Vallee and Jeanjean, 1968b), implicating an essential sulfhydryl group proximal to the active site.

Anions of the class $XO_4^{=}$, where X=Cr, Se, Mo, and W, are structural analogs of sulfate, and are found to be effective inhibitors of sulfate transport (Deane and O'Brien, 1975; Pardee et al., 1966). Competitive inhibition of uptake by these anions (Smith, 1976; Vange et al., 1974) reveals an inverse relationship between the inhibitory power of the analog and the atomic weight of X, demonstrating effective size selectivity (Vange et al., 1974). Sulfate and its analogs are all effective in liberating inorganic pyrophosphate from ATP in the intracellular activation of sulfate to adenosine-5'-phosphosulfate (Bandurski et al., 1956).

*Abbreviations used: ATP, adenosine triphosphate; CCCP, carbonylcyanide m-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; 2,4-DNP, 2,4-dinitrophenol; pHMB, para-hydroxymercuribenzoate; NEM, N-ethyl maleimide; DTT, dithiothreitol.

It is likely that these structurally similar compounds are transported by the same permease, as shown for chromate in Neurospora crassa (Roberts and Marluf, 1971).

The application of competitive inhibition by sulfate analogs to natural populations may be of practical value in the case of thiosulfate ($S_2O_3^=$). The use of sulfate uptake by bacteria as a measure of bacterial production has been attempted in freshwater habitats (Jassby, 1975; Monheimer, 1974a) but met with little success due to low rates of sulfate uptake combined with high isotope adsorption blanks (Jordan et al., 1978). Although no constant relationship was found between microbial carbon assimilation and sulfate uptake (Monheimer, 1978b), evidence derived from the data of Datko et al. (1978b) suggests that a predictable relationship exists between sulfate incorporation into protein and de novo protein synthesis in microorganisms. This was found to be true for marine bacteria in the present work (Chapters 4 & 5). However, the high sulfate concentration of seawater, 25mM, imposes a very great isotope dilution barrier for sulfate uptake studies.

A possible means for circumventing the problem of isotope dilution is to outcompete sulfate for uptake and metabolism using a competitive inhibitor which can replace sulfate in assimilatory sulfur metabolism. Thiosulfate seems to be the ideal candidate for a number of reasons. The substitution of a reduced sulfur atom for one of the four equivalent oxygen atoms of sulfate yields an analog which is not only an effective inhibitor of sulfate uptake (Marzluf, 1970a; Roberts et al., 1963; Smith, 1976; Utkilen et al., 1976; Vange et al., 1974; Yamamoto and Segel, 1966)

but also supports normal growth as the sole sulfur source in a wide variety of organisms (Hart and Filner, 1969; Hodson et al., 1971; Leinweber and Monty, 1963; Marzluf, 1970b; Ramus, 1974; Roberts et al., 1963).

The sulfate and thiosulfate transport systems are regulated by the same or very closely linked genetic loci in both Salmonella typhimurium (Leinweber and Monty, 1963) and Chlorella pyrenoidosa (Hodson et al., 1971). Nutritional studies with mutants of sulfate transport and reduction in these organisms leave little doubt that the two systems are identical. Mutants deficient in sulfate transport do not grow on thiosulfate. Furthermore, mutants specifically deficient in sulfate reduction but retaining transport capacity are able to grow with thiosulfate as the sole sulfur source, but to only half the yield attainable by the wild type, expected if only one sulfur atom of thiosulfate is available for biosynthetic reactions. In support of this hypothesis, biochemical studies demonstrated that only the reduced (sulfane) moiety could be utilized, indicating that the oxidized moiety of thiosulfate undergoes reduction by the same system as sulfate.

Thiosulfate is not only an effective inhibitor of sulfate uptake: the kinetics of inhibition often indicate a significantly higher affinity of the transport system for thiosulfate (Dreyfuss, 1964; Roberts et al., 1963). Moreover, the reduced (sulfane) moiety of thiosulfate is preferentially incorporated into the sulfur-containing amino acids relative to the sulfite moiety or sulfate (Dreyfuss and Monty, 1963; Hodson et al., 1968a). The apparent absence of reduced sulfur compounds in the aerobic marine environment (Schell, 1974; Lee, Mague, Tuttle, personal communications) would permit the quantitative addition of ^{35}S-labeled thiosul-

fate at concentrations large enough to outcompete sulfate for uptake and metabolism yet significantly lower than 25mM. Alteration of metabolic activity would be minimal because sulfate is never a growth-limiting nutrient in marine environments.

The similarity of sulfate and thiosulfate with respect to structure, nutritional response of mutants, and genetics suggests that the two are transported by the same permease, but this question has never been studied kinetically. If a common transport system is in operation, uptake patterns for both compounds should respond similarly to derepression during sulfur starvation and growth on organic sulfur sources, competition by $XO_4^{=}$ anions, and uncoupling by inhibitors of energy metabolism. Also the two compounds should be mutual competitive inhibitors. This chapter presents uptake data demonstrating these characteristics for the sulfate transport system of a marine bacterium, Pseudomonas halodurans, to provide support for a possible means of lowering the isotope dilution factor for studies of sulfur assimilation by marine microorganisms.

In addition to kinetic analysis of the effects of thiosulfate on sulfate transport, it is necessary to demonstrate that marine bacteria universally utilize thiosulfate as a sulfur source for growth. During a survey of thiosulfate utilization by a number of marine isolates, however, several responses of one of them, Alteromonas luteo-violaceus, prompted me to examine its sulfate uptake system in further detail. Although similar to the transport system of the pseudomonad and previously reported systems in some respects, key differences in its response to thiosulfate, the sulfhydryl reagent pHMB, and regulation of transport capacity suggest that this permease is unique among sulfate transport enzymes. Side by

side comparison of characteristics of the sulfate transport systems of these two marine bacteria emphasizes the differences between them.

MATERIALS AND METHODS

Organisms and Culture Conditions: Pseudomonas halodurans, Alteromonas luteo-violaceus, and the RLC-water medium have been described in Chapter 2. Growth medium for sulfate uptake studies contains 10mM glutamate, inorganic nutrients, and 1mM sulfate unless otherwise noted.

Sulfate Uptake Assay: Cells in the late exponential phase of growth in complete medium ($1-3x10^8$/ml) are harvested by centrifugation at 5000xg for 10 minutes, rinsed once with basal salts medium, and resuspended to the same density in complete medium minus sulfur. For kinetics studies, the washed culture is starved for sulfur for 2 hours, then assayed for transport as follows: 9.8 ml of the cell suspension are added to tubes containing 0.1ml of the inhibitor or appropriate solvent and 0.1ml of ^{35}S-labelled sulfate or thiosulfate. Carrier-free sulfate is added to a final activity of 1-2 μCi/ml with the addition of sterile Na_2SO_4 to achieve the desired concentrations (11.5-201.5 μM final concentration including background sulfate). Thiosulfate is used without the addition of carrier because of the low specific activity available (10-30DPM per pMole) at final concentrations of 2.5-25 μM. The mixture is vortexed thoroughly, and 1ml samples are withdrawn at 60 second intervals for 5 minutes into tubes containing 0.1ml 1M Na_2SO_4 or $Na_2S_2O_3$ as ap-

propriate to dilute the label (final concentration over 100mM). Subsamples of 1ml are then filtered through Reeve Angel 984H Ultrafine or Whatman GF/F glass fiber filters and rinsed with 0.5M NaCl. Termination of the reaction by isotope dilution does not result in the loss of label transported into the cells nor does further detectable uptake of the label occur for at least 3 hours after sampling, as determined in separate experiments (data not shown). The uptake rate is determined from the slope of the least-squares regression of pMoles taken up vs. time.

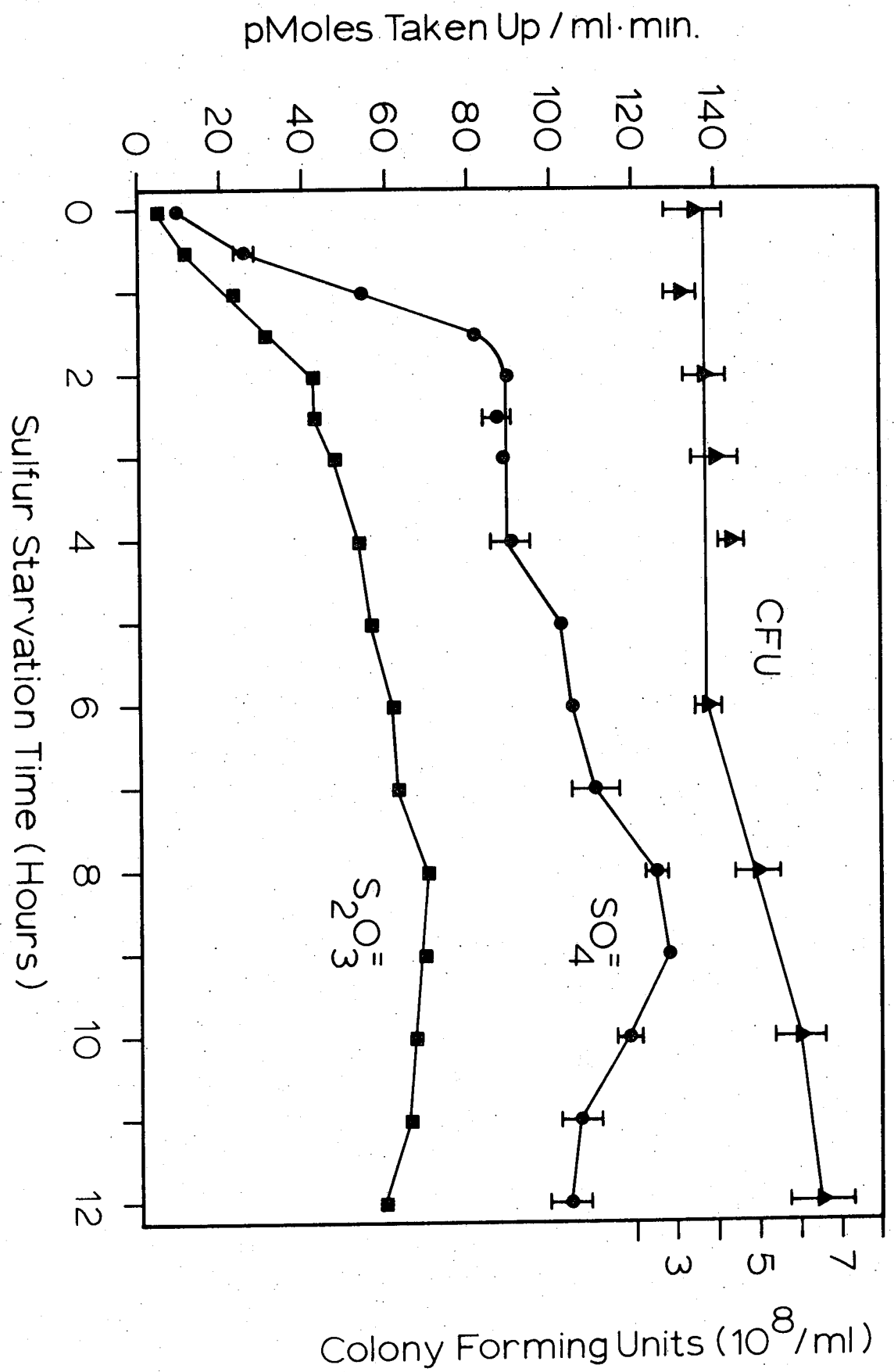
Other Methods: Methods used for direct and viable cell counts, protein, and liquid scintillation counting are found in Chapter 2, as are the sources of chemicals and radioisotopes.

RESULTS

Effect of Sulfur Starvation on Sulfate and Thiosulfate Uptake by P. halodurans

The sensitivity of sulfate uptake assays often can be increased by measurement after a period of sulfur starvation if the transport system is subject to derepression (Deane and O'Brien, 1975; Jeanjean and Broda, 1977; Utkilen et al., 1976; Yamamoto and Segel, 1966). In order to determine the optimum starvation time for P. halodurans, the derepression of sulfate and thiosulfate uptake capacity was followed during the course of sulfur starvation. The results are shown in Figure 3-1. Both uptake rate profiles are similar, characterized by a rapid increase during the first

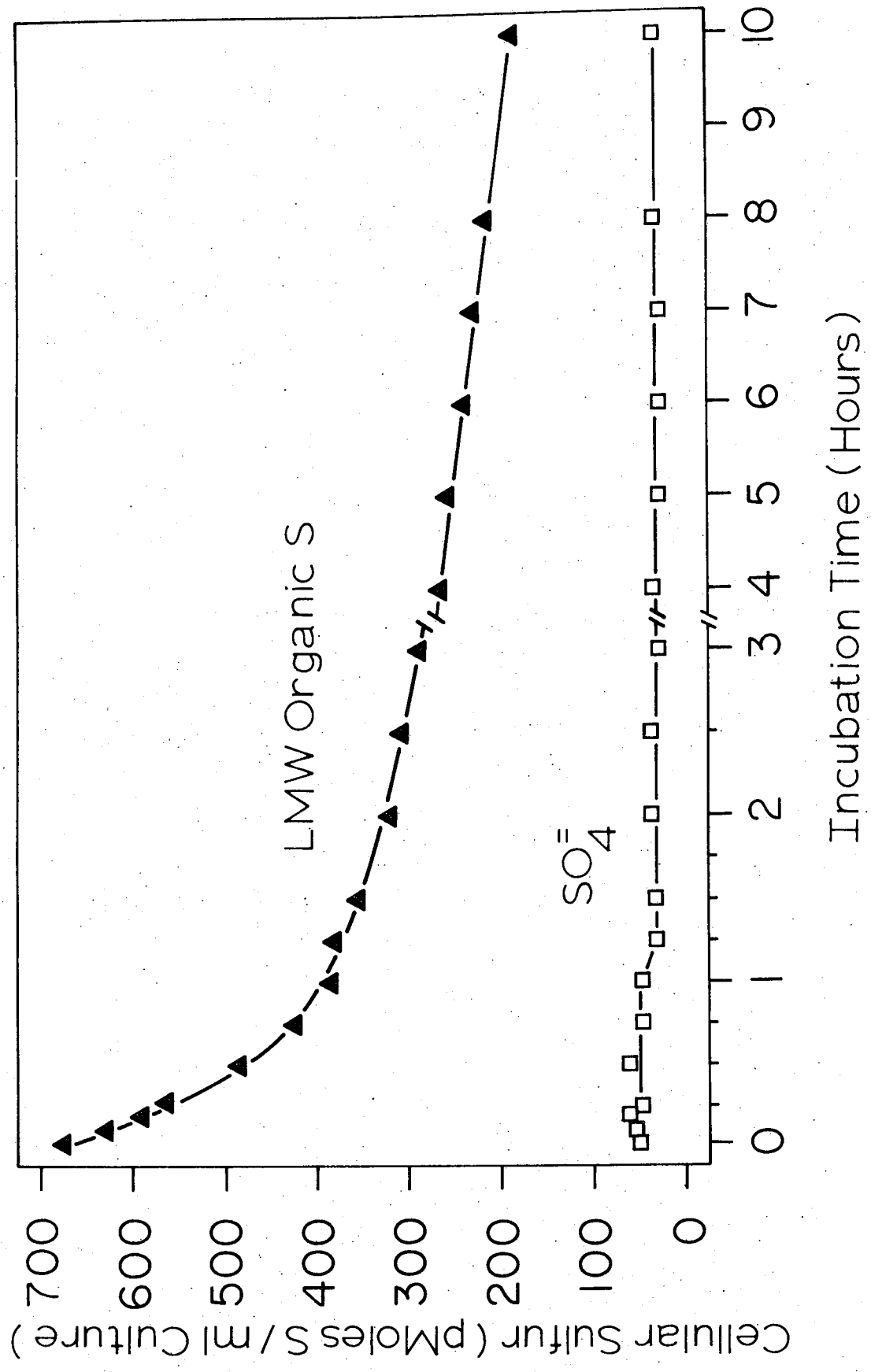
Figure 3-1. Viable cell counts, sulfate and thiosulfate uptake rates during sulfur starvation of P. halodurans. Final assay concentrations and specific activities were 200 µM $SO_4^=$ (17 DPM/pMole) and 20 µM $S_2O_3^=$ (17 DPM/pMole). Uptake rate data are the averages of duplicate determinations; error bars are shown when larger than the symbol. Viable counts are the mean ± one standard error of 2 plates at each of 3 dilutions.


pMoles Taken Up / ml·min.
0
20
40
60
80
100
120
140
Sulfur Starvation Time (Hours)
0
2
4
6
8
10
12
$S_2O_3^=$
$SO_4^=$
CFU
3
5
7
Colony Forming Units (10^8/ml)

two hours followed by a slow rise coincident with an increase in both direct and viable cell counts. The uptake rate per cell rises only slightly during the period 2-8 hours, demonstrating that two hours of sulfur starvation are sufficient for the complete derepression of transport capacity under these conditions. The increase in cell numbers is apparently due to the metabolism of endogenous sulfur reserves rather than the uptake of the 1.5 μM sulfate contamination, as no such increase is observed when the cells are initially grown on 250 μM sulfate, a concentration nearing sulfur-limited growth for this organism (see Chapter 4).

In the case of sulfate transport and metabolism in marine bacteria, sulfate transport rates appear to be tightly coupled to growth requirements for sulfur, and it is difficult to distinguish between repression and feedback inhibition. If protein synthesis is blocked by an inhibitor such as chloramphenicol, not only is de novo protein synthesis prevented, but also the drainage of low-molecular weight (L.M.W.) organic sulfur pools which may be responsible for feedback inhibition (see Chapter 4). In fact, the rate of disappearance of L.M.W. compounds from the soluble pools of P. halodurans is virtually the mirror-image of the uptake rate profile (Figure 3-2) when followed during the course of sulfur starvation. A rapid decline in L.M.W. organic sulfur during the first two hours is followed by a prolonged, slow, linear drainage of this pool during which time a similarly prolonged, slow increase in total sulfate uptake capacity is observed. The stability of the inorganic sulfate pool in this experiment indicates that sulfate itself has no regulatory activity in sulfate transport in this bacterium. The concentration of the L.M.W. or-

Figure 3-2. Disappearance of the low-molecular weight organic sulfur pool during sulfur starvation of P. halodurans. Experimental details in Figure 4-11.

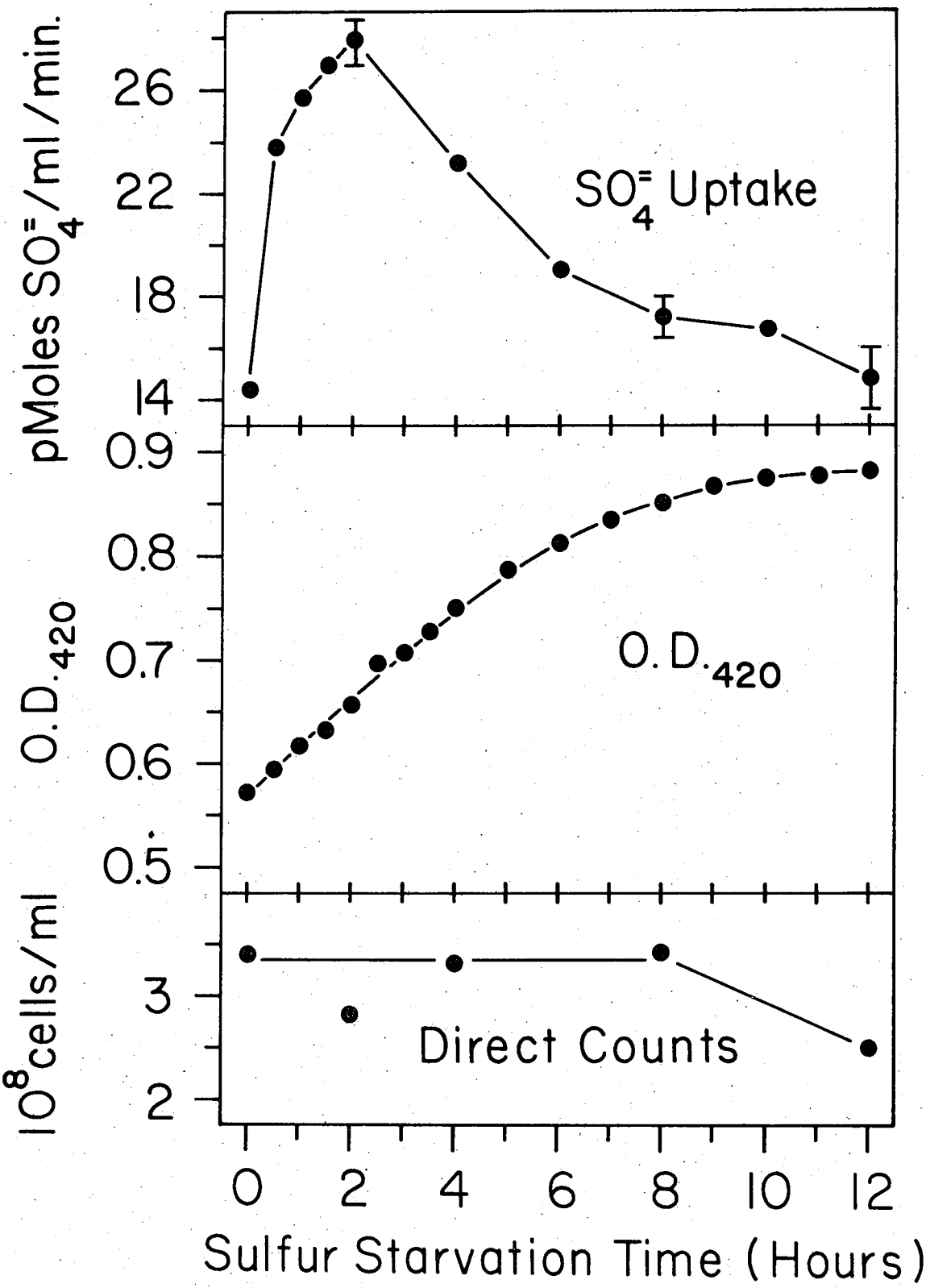

Cellular Sulfur (pMoles S / ml Culture)
700
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
Incubation Time (Hours)
LMW Organic S
$SO_4^=$

ganic sulfur compounds may exert allosteric influence on the enzymes responsible for sulfate reduction, since a sharp drop in the free sulfate pool occurs at about 1 hour, when most of the L.M.W. organic sulfur pool has disappeared. Although a small change in relative terms, a decrease of 38% in the free sulfate pool is observed at this time.

Effect of Sulfur Starvation on Sulfate Uptake by A. luteo-violaceus

A similar initial response of sulfate transport capacity to sulfur starvation is observed with A. luteo-violaceus (Figure 3-3). Unlike some transport systems subject to derepression (Smith, 1975; Utkilen et al., 1976; Yamamoto and Segel, 1966), however, there is no plateau in the uptake rate. The direct counts remain constant for several hours after this point, but plating efficiency drops rapidly after two hours of sulfur starvation. The lower plating efficiency as well as the increases in $O.D._{420}$ shown in Figure 3-3 coincide with a dramatic change in cell size as the normally coccobacillary cells (2 x 1 μm) grow into long, slender filaments (up to 10 μm). Clumping of cells is undoubtedly a factor in the reduced viability estimate, but death and ultimately autolysis, as indicated by the observed decrease in direct counts, are also significant factors. In this experiment no transport assays were performed in close succession around the point of maximum uptake rate, but during the inhibition experiments below it was found that the uptake rate begins a slow and steady decline immediately after the maximum is observed. This requires that the measurement of kinetic parameters be undertaken just before two hours of sulfur starvation, when the sensitiv-

Figure 3-3. Derepression of sulfate uptake capacity by sulfur starvation in Alteromonas luteo-violaceus. Washed cells were resuspended in complete medium minus sulfur at an initial density of 3.5×10^8 cells/ml. Aliquots were withdrawn at intervals for measurement of $O.D._{420}$, direct cell counts, and uptake of $^{35}SO_4^=$ (final concentration, 26.5 μM; specific activity, 45 DPM/pMole) as described in Materials and Methods.


pMoles $SO_4^=$/ml/min.
26
22
18
14
$SO_4^=$ Uptake
O.D.$_{420}$
0.9
0.8
0.7
0.6
0.5
O.D.$_{420}$
10^8cells/ml
3
2
Direct Counts
0
2
4
6
8
10
12
Sulfur Starvation Time (Hours)

ity gained by the derepression of the transport system was not jeopardized by a loss of viability.

As with P. halodurans, the disappearance of L.M.W. organic sulfur pools during sulfur starvation of A. luteo-violaceus mirrors the increase in sulfate uptake rate (Figure 3-4). However, after the initial rapid drainage, no further decrease in this pool occurs, and metabolism of cellular inorganic sulfate occurs only during the first few minutes of sulfur starvation. The cessation of L.M.W. organic sulfur metabolism coincides with the onset of a rapid decrease in viability, and after a short time the action of the organism's powerful protease results in loss of protein from the cells (see Chapter 4). Thus sulfur starvation is lethal to this organism.

Mutual Competition of Sulfate and Thiosulfate for Uptake in P. halodurans

Prerequisite to the identity of sulfate and thiosulfate transport systems is their mutual competition for uptake. Thiosulfate is a potent competitor for sulfate uptake in P. halodurans, as demonstrated in Figure 3-5. The thiosulfate uptake system is likewise competitively inhibited by sulfate (Figure 3-6), although a substantially greater concentration of sulfate is required to achieve inhibition.

Graphical determination of the K_i' for a competitive inhibitor requires several concentrations of inhibitor (I) in addition to the uninhibited control. A Dixon plot of 1/v vs. I for thiosulfate inhibition of sulfate uptake is shown in Figure 3-7. The K_i' resulting from the 10 intersections is 12.3 $\pm$ 2.9 μM $S_2O_3^=$ (mean ± one standard error).

Figure 3-4. Disappearance of the low-molecular weight organic sulfur pool during sulfur starvation of A. luteo-violaceus. Experimental details in Figure 4-10.

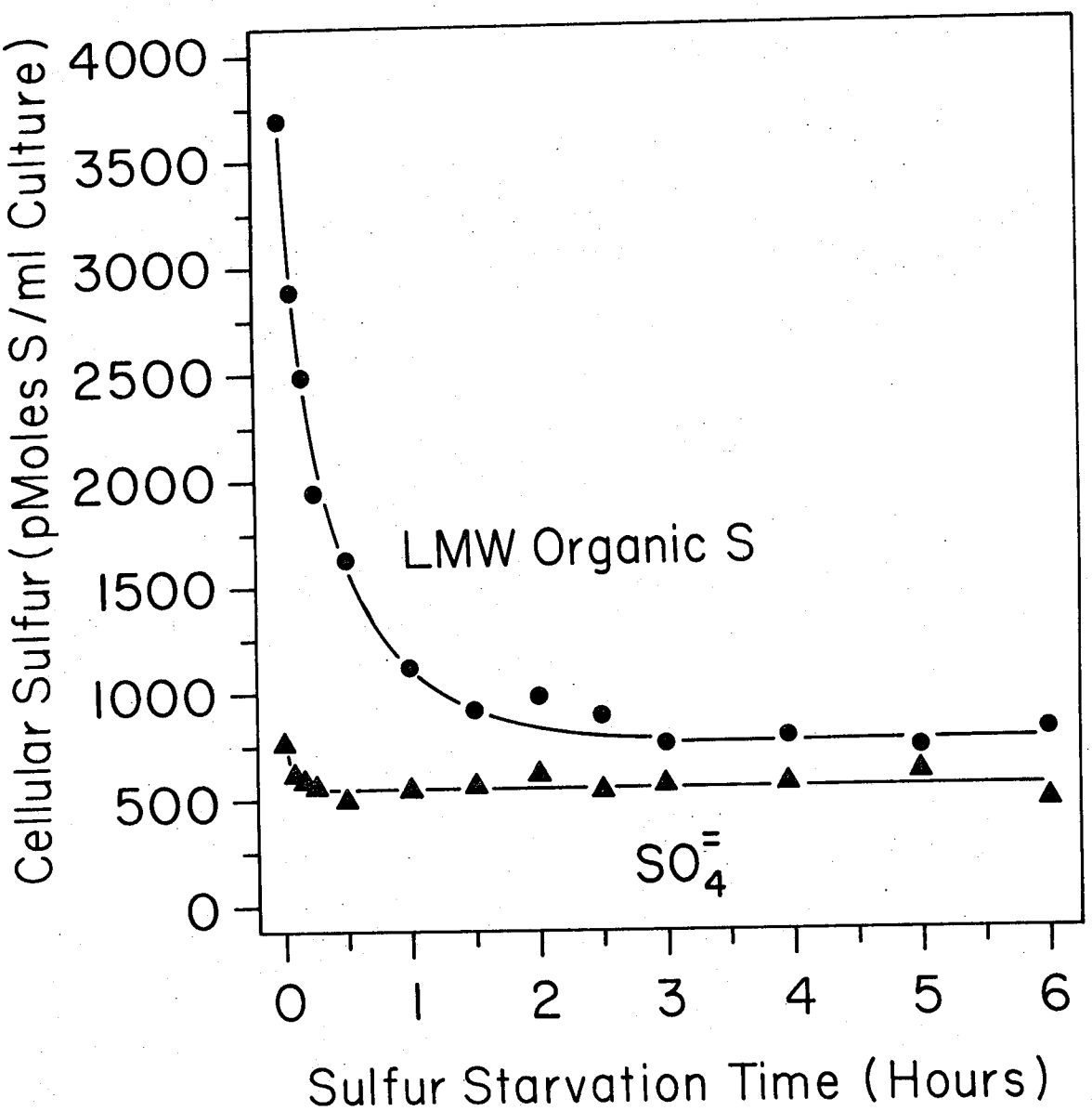

Cellular Sulfur (pMoles S/ml Culture)
4000
3500
3000
2500
2000
1500
1000
500
0
LMW Organic S
$SO_4^=$
0
1
2
3
4
5
6
Sulfur Starvation Time (Hours)

Figure 3-5. Lineweaver-Burk plot of competitive inhibition of sulfate uptake by thiosulfate in P. halodurans. Sulfate uptake was assayed at 6.5, 11.5, 21.5, 51.5, 101.5, and 251.5 μM final concentration with 1 μCi/ml $^{35}SO_4^{=}$ final activity. The cell density was 3.0×10^8/ml.

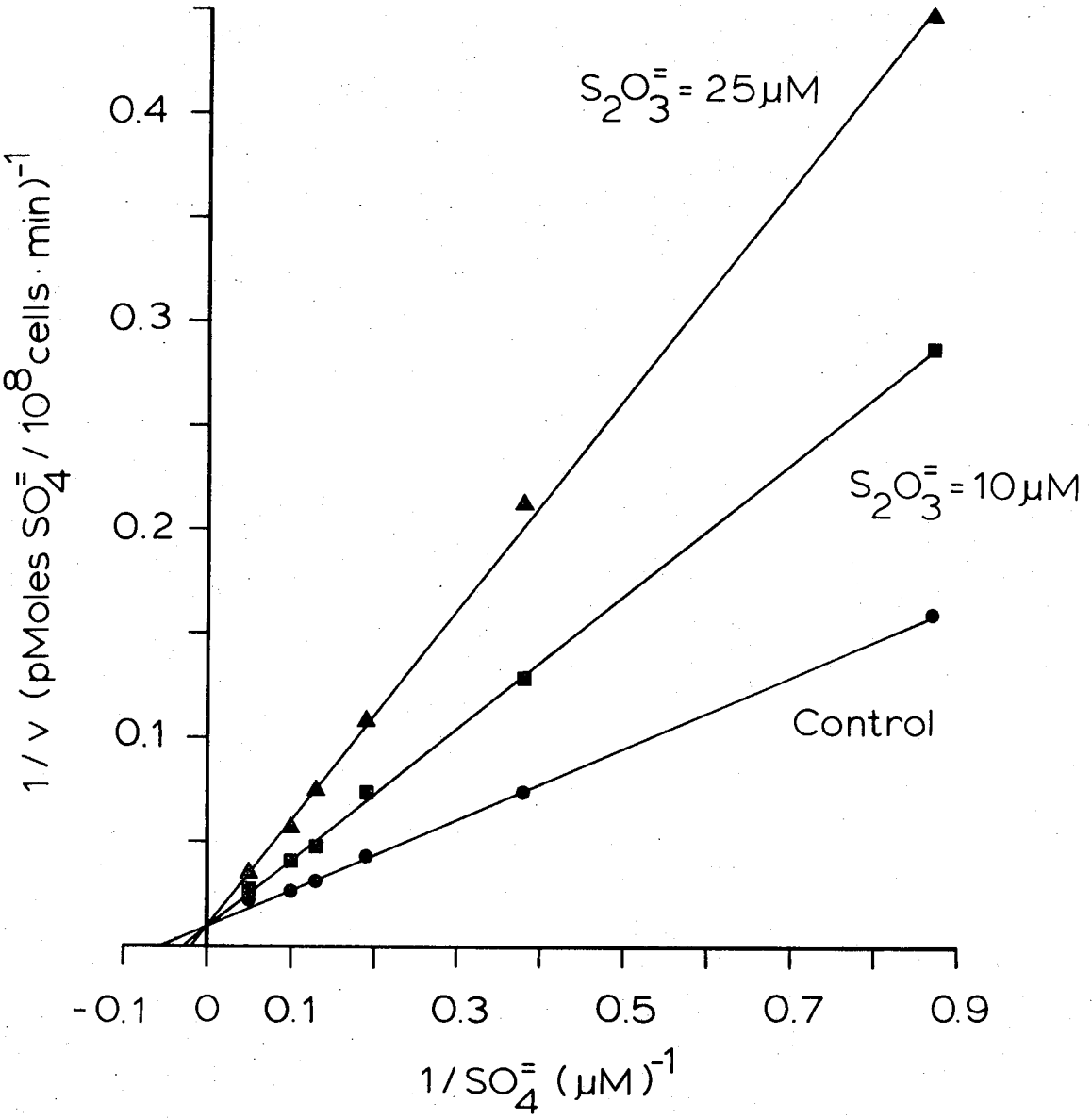

$S_2O_3^= = 25\mu M$
$S_2O_3^= = 10\mu M$
Control
0.4
0.3
0.2
0.1
-0.1
0
0.1
0.3
0.5
0.7
0.9
$1/v$ (pMoles $SO_4^=$ / 10^8 cells · min)$^{-1}$
$1/SO_4^=$ (μM)$^{-1}$

Figure 3-6. Lineweaver-Burk plot of competitive inhibition of thiosulfate uptake by sulfate in P. halodurans. Thiosulfate uptake was assayed at 2.5, 5, 10, 15, 25, and 50 μM final concentration at a specific activity of 11 DPM/pMole. The cell density was 3.2×10^8/ml.

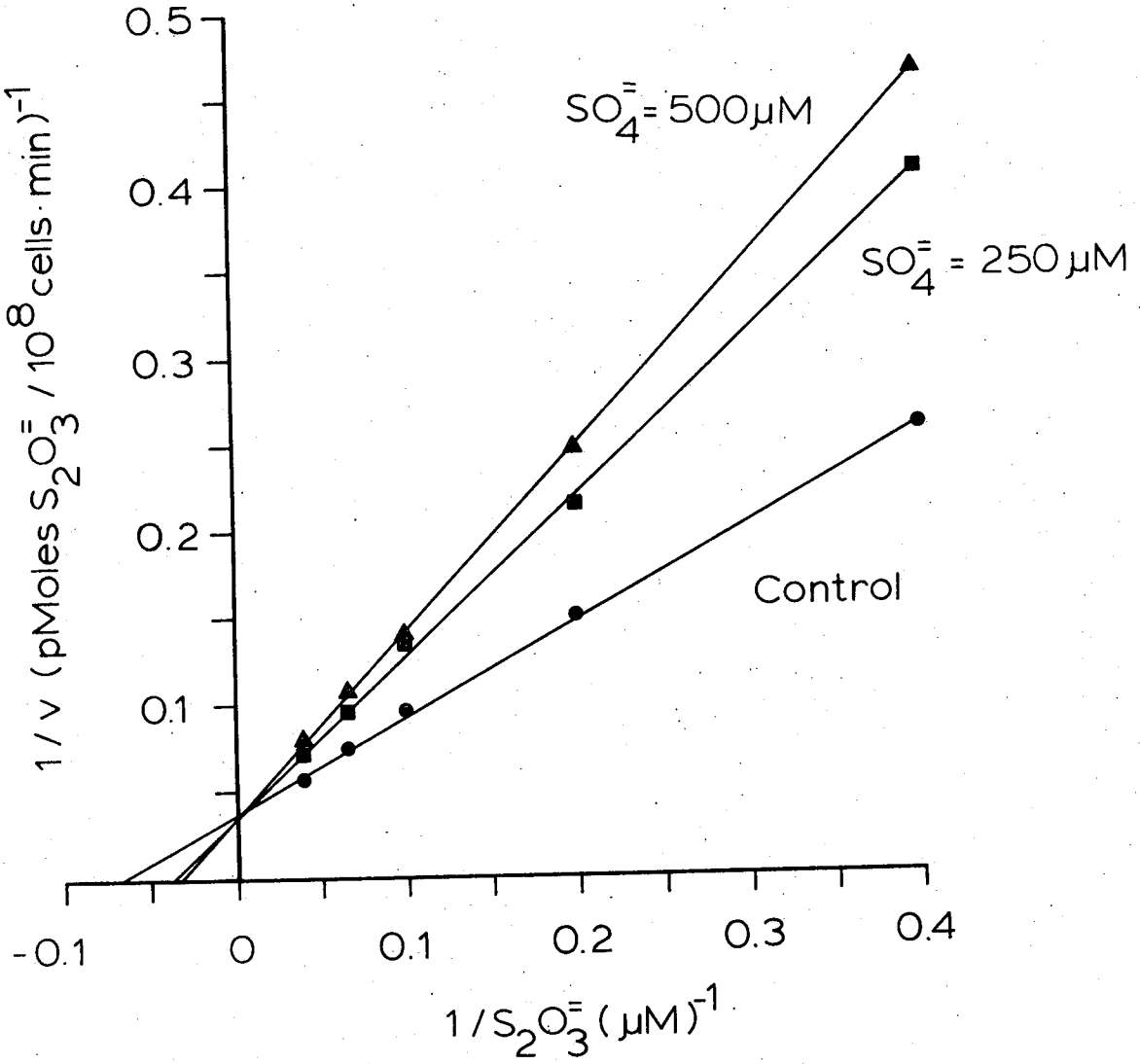
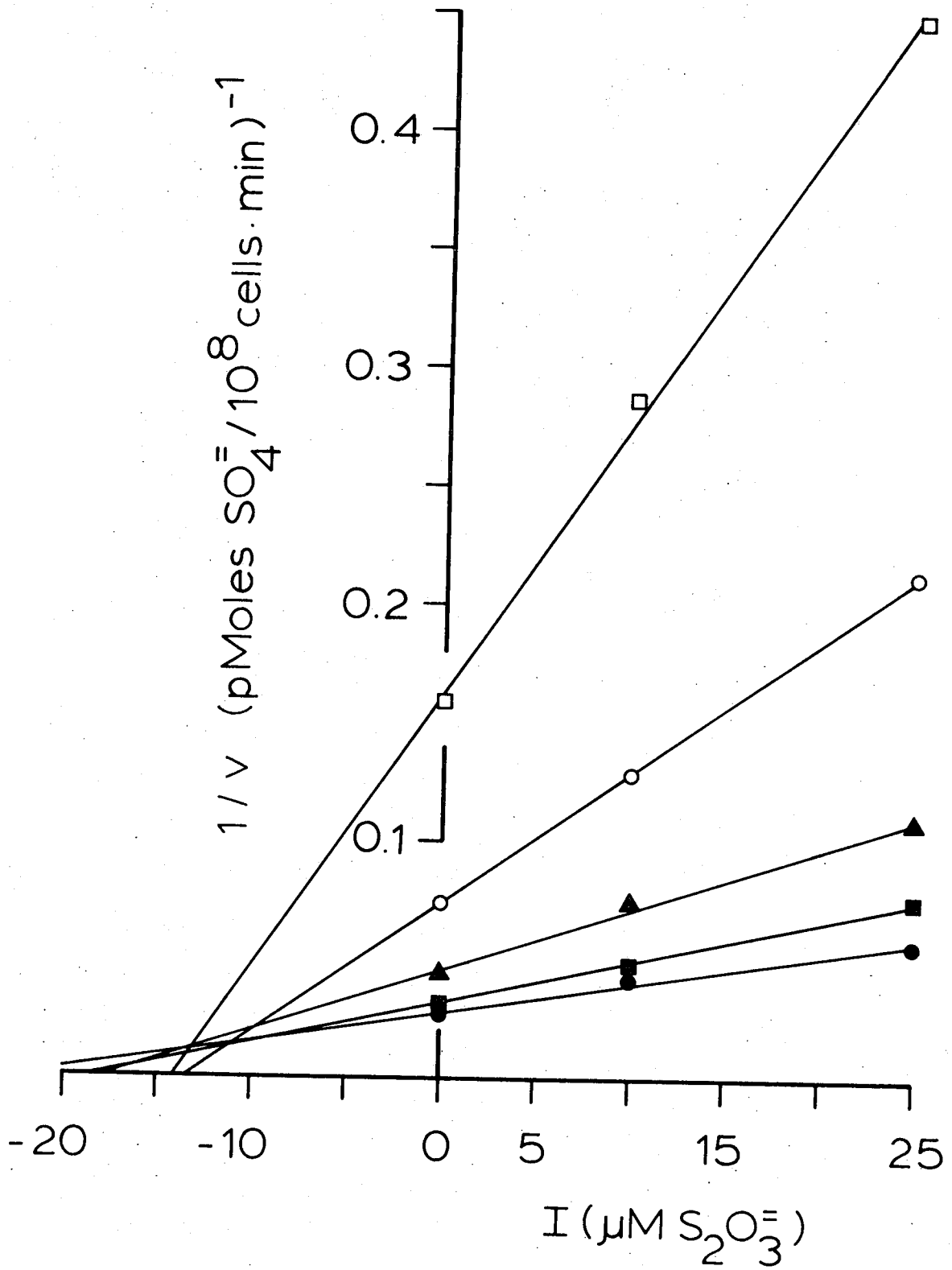

0.5
0.4
0.3
0.2
0.1
-0.1
0
0.1
0.2
0.3
0.4
$SO_4^= = 500\mu M$
$SO_4^= = 250\mu M$
Control
$1/v$ (pMoles $S_2O_3^=$ / 10^8 cells · min)$^{-1}$
$1/S_2O_3^=$ (μM)$^{-1}$

Figure 3-7. Dixon plot of the data from Figure 3-5.


1 / v (pMoles $SO_4^=$ / 10^8 cells · min)$^{-1}$
0.4
0.3
0.2
0.1
-20
-10
0
5
15
25
I (μM $S_2O_3^=$)

In surveys of the type attempted in this chapter, however, such data are not practical to obtain. An alternative method of determining the K_i' is to solve Equation 3-1 for K_i':

$$\frac{-1}{K_m'} = \frac{-1}{K_m \left(1 + \frac{I}{K_i'} \right)} \qquad \text{[Equation 3-1]}$$

where K_m=the half-saturation constant for uptake in the absence of inhibitor, I=the inhibitor concentration, and K_m'=the apparent half-saturation constant in the presence of the inhibitor (Webb, 1963; p.151). K_m' is determined from the X-intercept of the Lineweaver-Burk plot for the inhibited reaction. For the data in Figure 3-5, with a K_m of 187 μM the calculated K_i' is 12.6 ± 1.9 μM thiosulfate, in agreement with the value obtained with the Dixon plot. This method has a lower sensitivity but is less affected by minor deviations from linearity of Lineweaver-Burk plots often associated with whole cell uptake studies. The agreement of the two methods within an experiment and the difficulty of graphically interpreting data such as that shown for chromate inhibition below led to the use of Equation 3-1 as the method for determining K_i' in this study. The K_m used for the calculations is the mean of all determinations for both organisms.

The Lineweaver-Burk estimate of kinetic constants is sensitive to error arising from variations in the measured rates at low substrate concentrations. Two other transformations of the Monod equation can be used; v vs. v/S and S/v vs. S plots, each of which are subject to different errors. All three plots were constructed for each experiment: in the majority of cases agreement of constants among plots was better than 10 %

for both K_m and V_{max}. A larger difference among plots was used as a criterion for the elimination of an erroneous point from the calculations (c.f. Table 3-1). The reported values are derived from the Lineweaver-Burk plot. All kinetic constants will be summarized below.

Sulfate and Thiosulfate Uptake over a Wide Range of Concentrations by P. halodurans

In many instances inorganic nutrient transport systems display a phenomenon known as luxury uptake, in which rapid accumulation of the nutrient in excess of growth requirements occurs, especially following starvation for the nutrient (McCarthy and Goldman, 1979; Segel and Johnson, 1961; Vallee and Jeanjean, 1968a). This is manifested by a high initial uptake rate followed by an often abrupt rate decrease as internal pools are saturated. Unsuccessful attempts to demonstrate this for sulfate uptake by P. halodurans revealed an unusual regulatory feature: when the uptake of sulfate by sulfur-starved cells is determined over an extended range of sulfate concentrations, there is little response of the uptake rate to sulfate additions beyond 200-250 μM (Table 3-2). The observed maximum uptake rate is only half that predicted from the intercept of the Lineweaver-Burk plot. Uptake is linear with time at all concentrations until increases due to cell growth become manifest (Figure 3-8).

Under the conditions of growth used in these experiments, a freshly inoculated culture of P. halodurans requires about 4500pMoles S to double $1x10^8$ cells with a generation time of about 70 minutes (Chapter 4). The observed maximum sulfate uptake rate for sulfur-starved cells, 53 pMoles

Table 3-1. Comparison of Kinetic Constants Derived from 1/v vs. 1/S, v vs. v/S, and S/v vs. S Plots of Control Sulfate Uptake Data from Figure 3-5[a]

	11.5 - 201.5 μM $SO_4^=$			11.5 - 101.5 μM $SO_4^=$		
Plot	r	K_m	V_{max}	r	K_m	V_{max}
1/v vs. 1/S	0.9993	159	94	1.0000	185	108
v vs. v/S	-0.9233	116	75	-0.9967	186	108
S/v vs. S	0.9791	102	69	0.9978	187	109
$\bar{X}$		126	79		186	108
S. D.		30	13		1	1
C. V. (%)		23.6	16.5		0.5	0.5

[a]The correlation coefficient for the least-squares linear regression is given for each plot. Standard units for the K_m (μM) and V_{max} (pMoles sulfate/10^8cells/minute) are used.

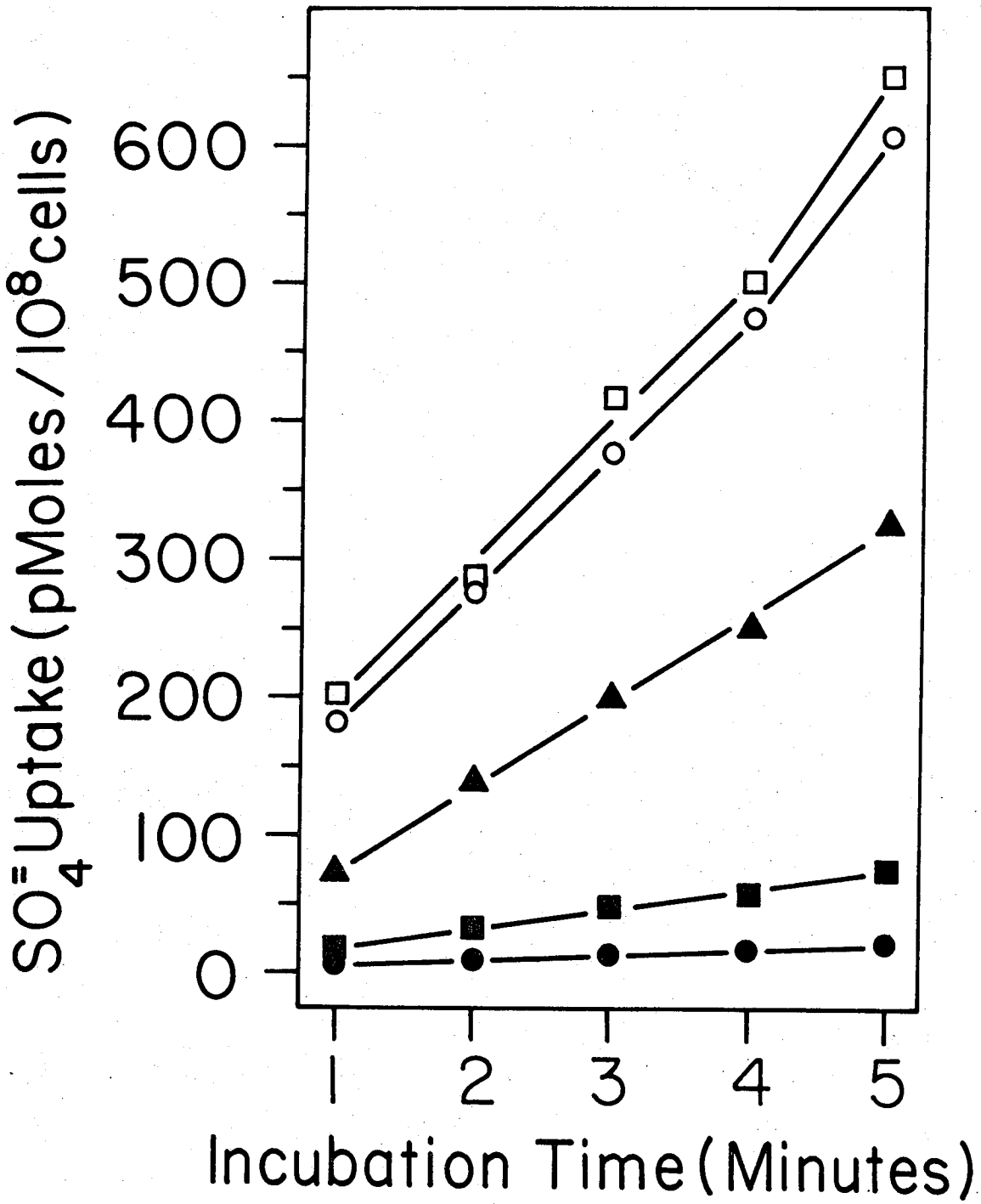
Table 3-2. Sulfate Uptake Rates Over a Wide Range of Sulfate Concentrations by Pseudomonas halodurans[a]

Sulfate Concentration (μM)	Uptake Rate pMoles $SO_4^=/10^8$cells/minute	
	Observed	Predicted[b]
2.5	1.1	1.2
6.5	2.8	3.1
16.5	7.0	7.5
26.5	10.7	11.6
51.5	17.4	20.4
101.5	35.0	33.8
251.5	48.0	56.6
501.5	47.8	73.2
1001.5	52.9	85.9

[a]Washed cells in sulfur-free medium were starved for sulfur for two hours, then assayed for sulfate uptake as described in materials and methods. The cell density was 2.7×10^8/ml, and the final activity of $^{35}SO_4^=$ was 1 μCi/ml.

[b]Calculated from the Monod equation using the kinetic constants in Table 3-4.

Figure 3-8. Sulfate uptake by Pseudomonas halodurans at concentrations of 6.5-1002 μM. Experimental details in Table 3-2. Symbols: ● , 6.5 μM; ■ , 26.5 μM; ▲ , 101.5 μM; □ , 501.5 μM; ○ , 1002 μM.


$SO_4^{=}$ Uptake (pMoles / 10^8 cells)
600
500
400
300
200
100
0
1
2
3
4
5
Incubation Time (Minutes)

$SO_4^=/10^8$cells/minute, provides this much sulfur in 85 minutes. This indicates that the transport system is regulated to provide only the amount of sulfur necessary for biosynthesis. Accumulation of excess sulfate in intracellular pools has not been observed for this organism, even following sulfur starvation.

The V_{max} calculated for thiosulfate uptake by sulfur-starved P. halodurans, 29.5 pMoles $S_2O_3^=/10^8$cells/minute (equal to 59 pMoles $S/10^8$cells/minute), is in good agreement with the V_{max} observed for sulfate uptake. However, the data in Figure 3-9 reveal a completely different reaction to higher concentrations of thiosulfate. Michaelis-Menten kinetics are obeyed at thiosulfate concentrations up to 40 μM, above which the uptake rate increases more rapidly than predicted by extrapolation of the Lineweaver-Burk plot. A low affinity transport process is suggested by Figure 3-10, a double reciprocal plot of the difference between predicted and observed rates (Δv) versus thiosulfate concentration. An approximate K_m of 9.5 mM $S_2O_3^=$ and V_{max} of 770 pMoles $S_2O_3^=/10^8$cells/minute is indicated. The preponderance of transport systems which are sensitive to sulfhydryl reagents provides many sites for reaction with the reduced sulfur atom of thiosulfate. Such nonspecific uptake mediated by an unrelated transport system would not be regulated in the fashion described for sulfate; hence uptake rates in excess of growth requirements could be obtained.

Sulfate Uptake over a Wide Range of Concentrations by A. luteo-violaceus

The kinetics of sulfate uptake over a wide range of sulfate concen-

Figure 3-9. Lineweaver-Burk plot of thiosulfate uptake by P. halodurans in the range 2.5-1000 μM. Specific activity 12 DPM/pMole, cell density, 2.7×10^8/ml. Closed symbols show the mean and error of two separate experiments; open symbols are single determinations.

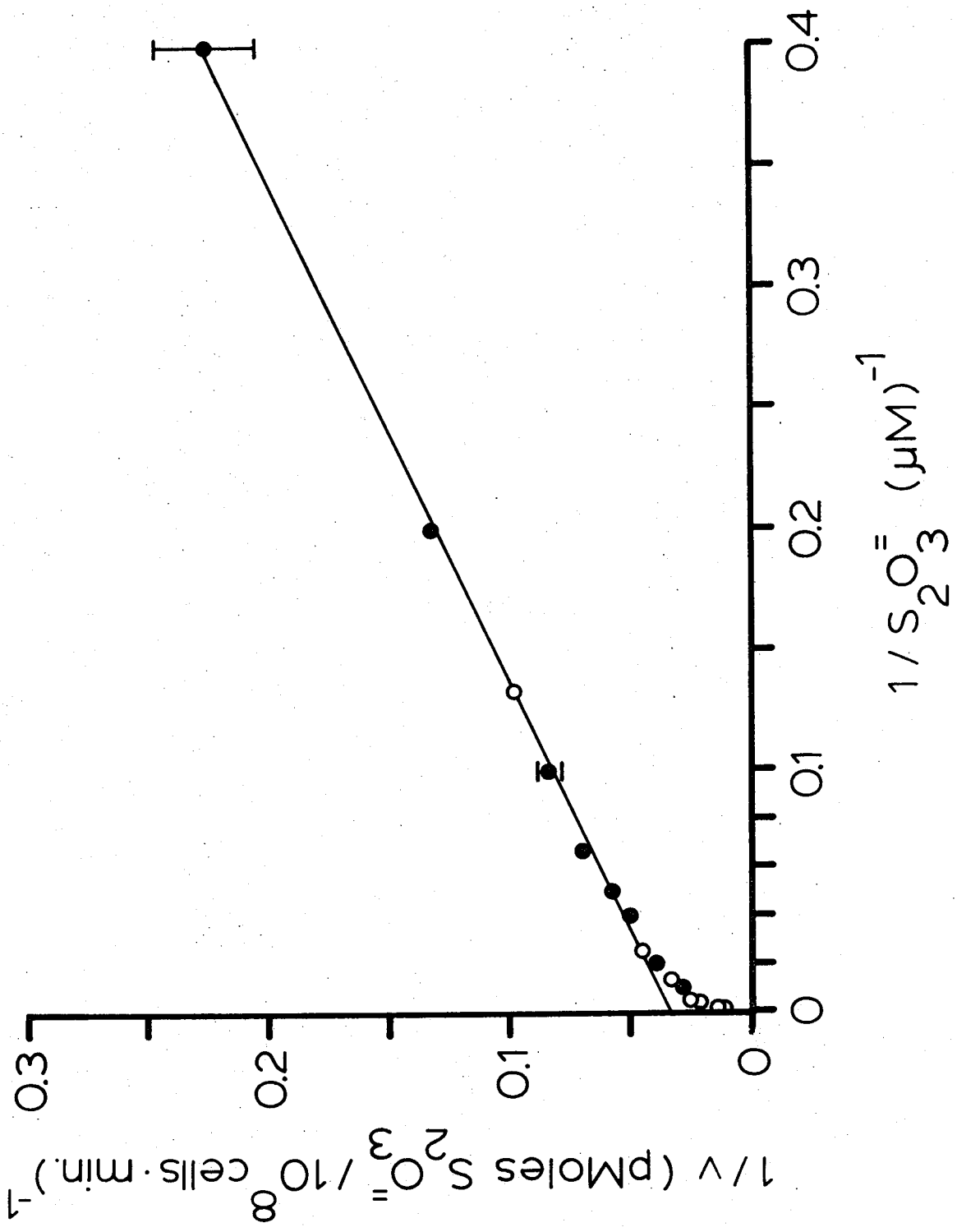
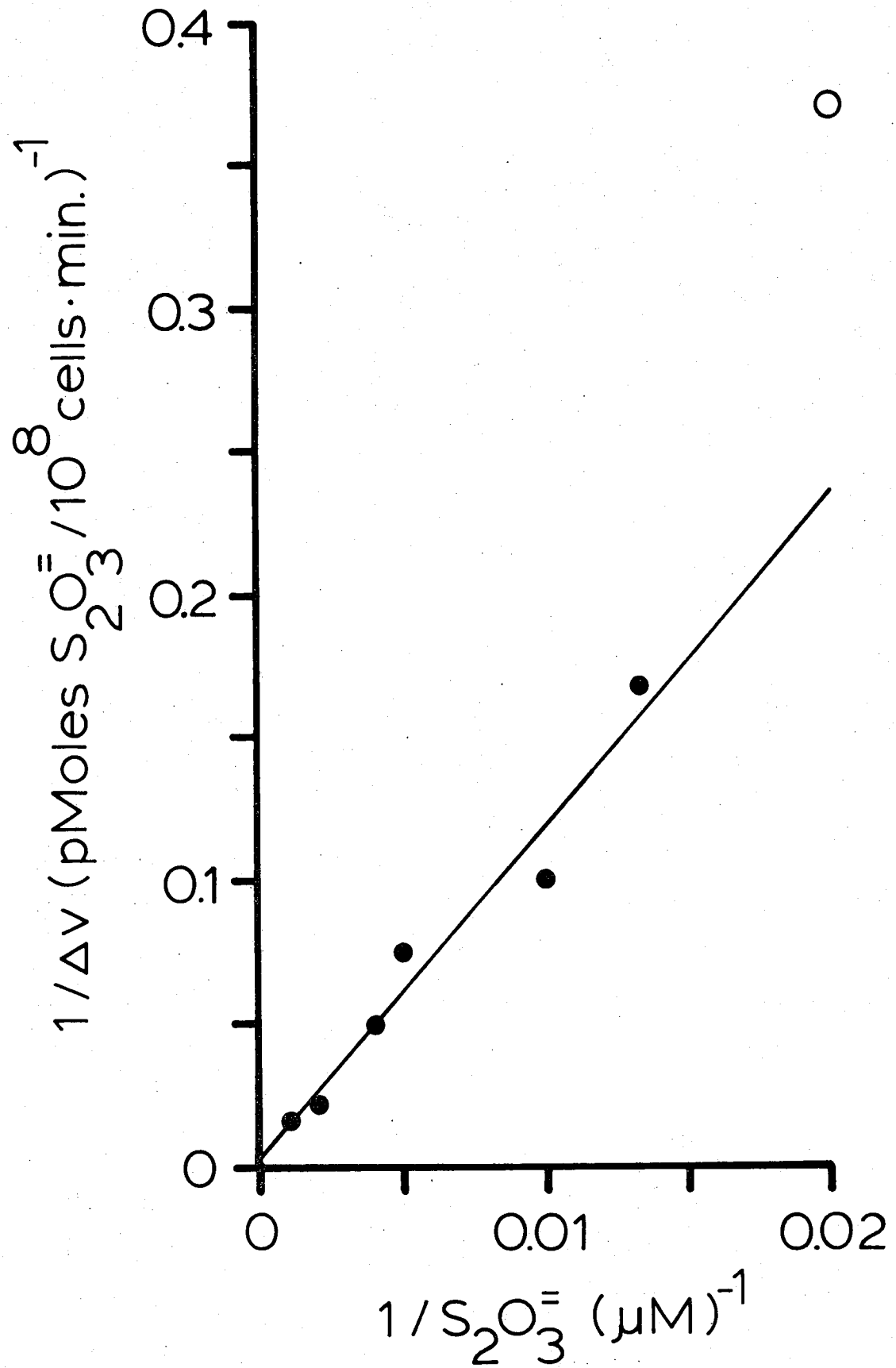

0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
1 / v (pMoles $S_2O_3^=$ / 10^8 cells · min.)$^{-1}$
1 / $S_2O_3^=$ (μM)$^{-1}$

Figure 3-10. Lineweaver-Burk plot of the difference between observed and extrapolated rates of thiosulfate uptake at concentrations greater than 40 μM, derived from data in Figure 3-9. The open symbol was not included in the regression because the error of the difference was greater than the value itself.


0.4
0.3
0.2
0.1
0
0
0.01
0.02
$1/\Delta v$ (pMoles $S_2O_3^{=}/10^8$ cells·min.)$^{-1}$
$1/S_2O_3^{=}$ (μM)$^{-1}$

trations were investigated at the time of maximum transport capacity in A. luteo-violaceus to obtain the V_{max} and determine the range over which Michaelis-Menten kinetics are observed. The uptake rate increases over the entire range of 6.5 - 1001.5 µM $SO_4^=$ and obeys Michaelis-Menten kinetics within experimental error. The apparent K_m is 178 µM and a V_{max} is 146 pMoles $SO_4^=/10^8$cells/minute. Uptake is not linear with time at higher substrate concentrations (Figure 3-11): feedback inhibition may be indicated by the decline in uptake rate after 500-600 pMoles $SO_4^=/10^8$cells have been taken up.

Effect of Thiosulfate on Sulfate Uptake by A. luteo-violaceus

A remarkable feature of the sulfate transport system of A. luteo-violaceus is its weak response to thiosulfate, normally a very effective inhibitor of sulfate uptake (Bradfield et al., 1970; Dreyfuss, 1964; Marzluf, 1970a; Pardee et al., 1966; Roberts and Marluf, 1971; Smith, 1976; Utkilen et al., 1976; Vange et al., 1974; Yamamoto and Segel, 1966). No concentration-dependent inhibition is observed with thiosulfate at concentrations up to 10 times that of sulfate (Table 3-3), and only 59 % inhibition is obtained when thiosulfate is present at 50 times the sulfate concentration. Furthermore, attempts to measure thiosulfate uptake using $Na_2^{35}SSO_3$ (3.19×10^8cells/ml; 14 DPM/pMole) after two hours of sulfur starvation revealed barely detectable uptake (4 pMoles per 10^8cells per minute) at 500 µM, the highest concentration tested. At this concentration, the thiosulfate uptake rate for P. halodurans is about 77 pMoles/10^8cells/minute. When inoculated into complete medium

Figure 3-11. Sulfate uptake by A. luteo-violaceus at concentrations of 6.5-1002 μM. A washed cell suspension (3.2×10^8/ml) was starved for sulfur for two hours, then assayed for sulfate uptake as described in Materials and Methods. $^{35}SO_4^=$ was present at a final activity of 1-2 μCi/ml depending on the sulfate concentration, indicated in μM.

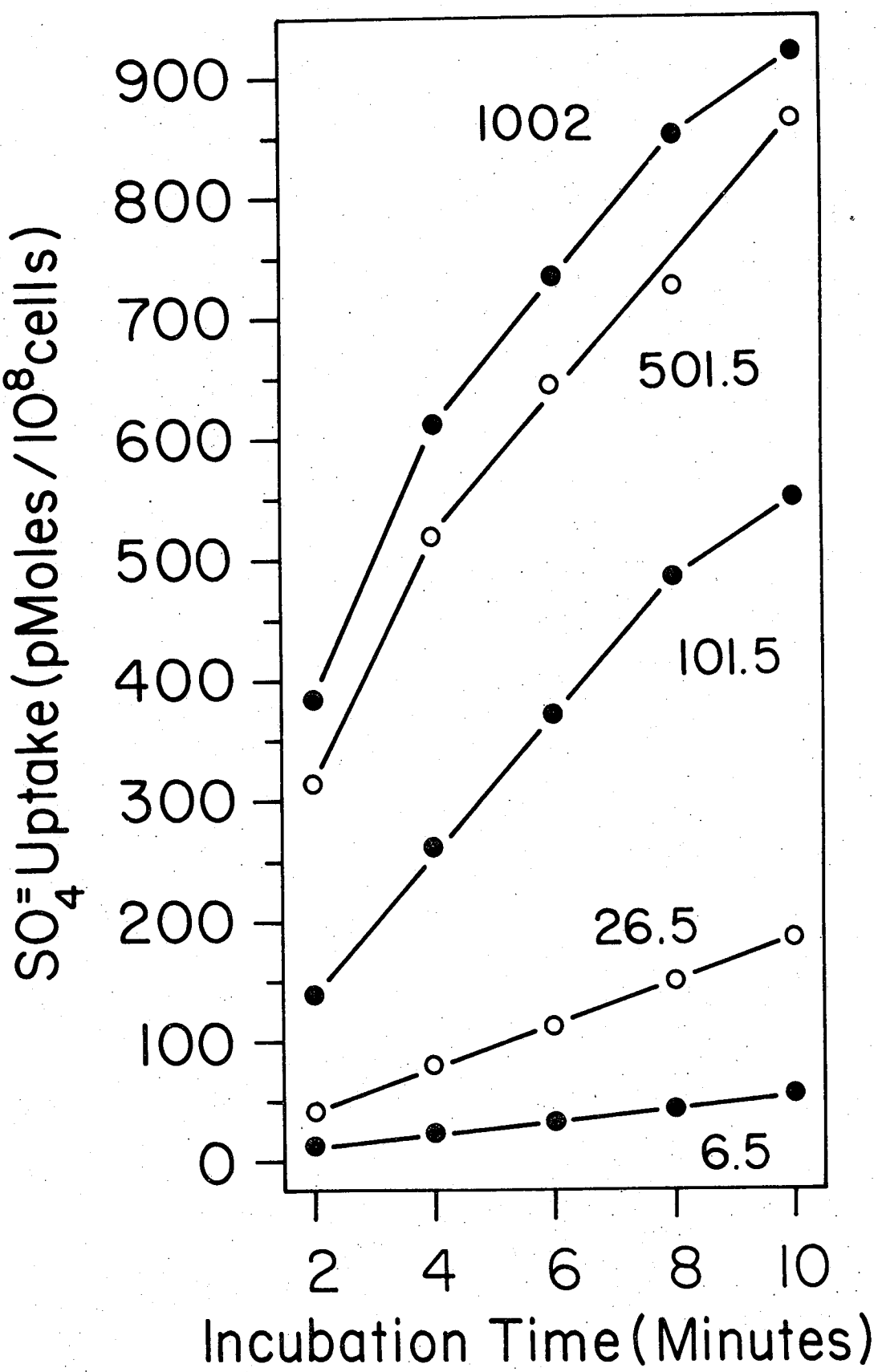

900
800
700
600
500
400
300
200
100
0
$SO_4^=$ Uptake (pMoles / 10^8 cells)
1002
501.5
101.5
26.5
6.5
2
4
6
8
10
Incubation Time (Minutes)

Table 3-3. Thiosulfate Inhibition of Sulfate Transport by *Alteromonas luteo-violaceus*[a]

Thiosulfate Concentration (μM)	Sulfate Uptake Rate (pMoles $SO_4^=/10^8$cells/minute)	% Inhibition
0	57.6	0
10	54.9	5
100	57.6	0
500	49.8	14
1000	50.8	12
5000	23.7	59

[a]A washed cell suspension was starved for sulfur for two hours, then assayed for sulfate transport activity as described in Materials and Methods. The reaction mixture contained, in a final volume of 10ml: 9.8ml cell suspension (2.36×10^8cells/ml), 0.1ml $^{35}SO_4^=$(final concentration, 101.5 μM; 19DPM/pMole), and 0.1ml thiosulfate solution to give the final concentrations indicated.

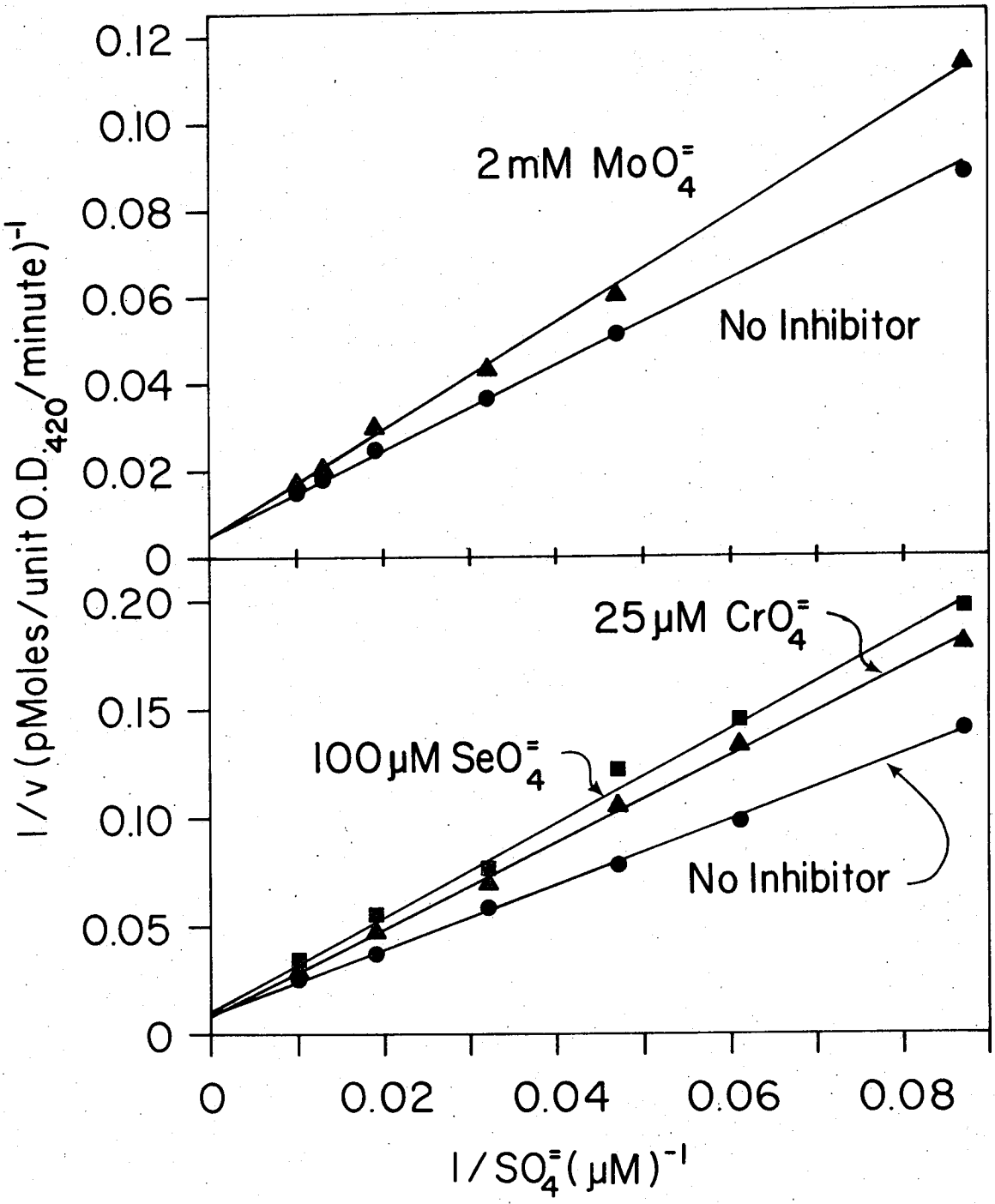
containing sulfate or thiosulfate at 1mM final concentration, the thiosulfate culture displays a lag time of 6-7 days relative to sulfate, but then grows at the same rate as the sulfate-grown cells. It is likely that the onset of growth in cultures containing thiosulfate as the sole source of sulfur is due to spontaneous oxidation of thiosulfate, yielding sulfate as a final product, since the organism requires only 20 μM sulfate to acheive its maximum growth rate under the conditions of these experiments.

For the remainder of this chapter, the parallel analysis of sulfate transport systems for the two marine bacteria will be supplemented with data relevant to the transport of thiosulfate in P. halodurans in order to demonstrate the remarkable similarity of the sulfate and thiosulfate transport characteristics of this organism. Although inhibition of sulfate uptake by thiosulfate has been demonstrated at high concentrations with A. luteo-violaceus, its lack of growth on thiosulfate as a sole source of sulfur suggests that attempts to measure uptake characteristics of this compound would be of little value.

Competition for Sulfate and Thiosulfate Uptake by Sulfate Analogs

The competitive inhibition of sulfate uptake by $XO_4^=$ sulfate analogs (X=Cr, Se, and Mo) common to other transport systems (Jeanjean and Broda, 1977; Pardee et al., 1966; Ramus, 1974; Smith, 1976; Vange et al., 1974; Yamamoto and Segel, 1966) is observed with A. luteo-violaceus. Figure 3-12 demonstrates competition by chromate, selenate, and molybdate. Since several sequential uptake assays are required to demonstrate com-

Figure 3-12. Lineweaver-Burk plots of competitive inhibition of sulfate uptake by $XO_4^=$ analogs in A. luteo-violaceus. Washed cell suspensions ($1-3x10^8$/ml) were starved for sulfur for 90 minutes, then assayed for sulfate uptake at 16.5, 21.5, 31.5, 51.5, 76.5, and 101.5 μM $^{35}SO_4^=$ (final activity: upper panel, 1 μCi/ml; lower panel, 2 μCi/ml).


0.12
0.10
0.08
0.06
0.04
0.02
0
2mM $MoO_4^=$
No Inhibitor
0.20
0.15
0.10
0.05
0
25μM $CrO_4^=$
100μM $SeO_4^=$
No Inhibitor
0
0.02
0.04
0.06
0.08
$1/v$ (pMoles/unit O.D.$_{420}$/minute)$^{-1}$
$1/SO_4^=(\mu M)^{-1}$

petitive inhibition, the absence of a stable uptake rate in A. luteo-violaceus poses a minor problem. The uptake rate per unit $O.D._{420}$ is constant between 90-120 minutes of sulfur starvation due to parallel increases in both parameters (Figure 3-3), however, so the uptake rates have been normalized to the $O.D._{420}$. The V_{max} in these units varies due to pigmentation and other factors, but is constant in individual cultures.

Structural analogs of sulfate also competitively inhibit sulfate uptake in P. halodurans (Figure 3-13). The data for chromate inhibition represent only the first minute of uptake, since this compound rapidly inactivates the transport system and rates are not linear at low sulfate concentrations. From the concentrations of inhibitor required to achieve the degree of inhibition shown, it can be seen readily that the inhibitor effectiveness is inversely proportional to the molecular weight of the analog.

If thiosulfate and sulfate are transported by the same permease in P. halodurans, a similar response of thiosulfate uptake to $XO_4^{=}$ inhibitors would be expected to occur. Competitive inhibition of thiosulfate transport is observed (Figure 3-14) with the same pattern of specificity as for sulfate transport inhibition, although considerably greater amounts of inhibitor are required. Data for chromate are again for the first minute of uptake only.

Kinetic constants for P. halodurans and A. luteo-violaceus are summarized in Table 3-4. Pseudomonas halodurans has over ten-fold greater affinity for thiosulfate than for sulfate. This is also reflected in the relative inhibitory power of the $XO_4^{=}$ analogs: although inhibition of

Figure 3-13. Lineweaver-Burk plot of sulfate uptake inhibition by $XO_4^=$ analogs in P. halodurans. Sulfate uptake was assayed at 11.5, 26.5, 51.5, 76.5, 101.5, and 201.5 μM final concentration with 1 μCi $^{35}SO_4^=$/ml final activity. Cell density in individual experiments ranged from 3.0-3.8x10^8/ml.

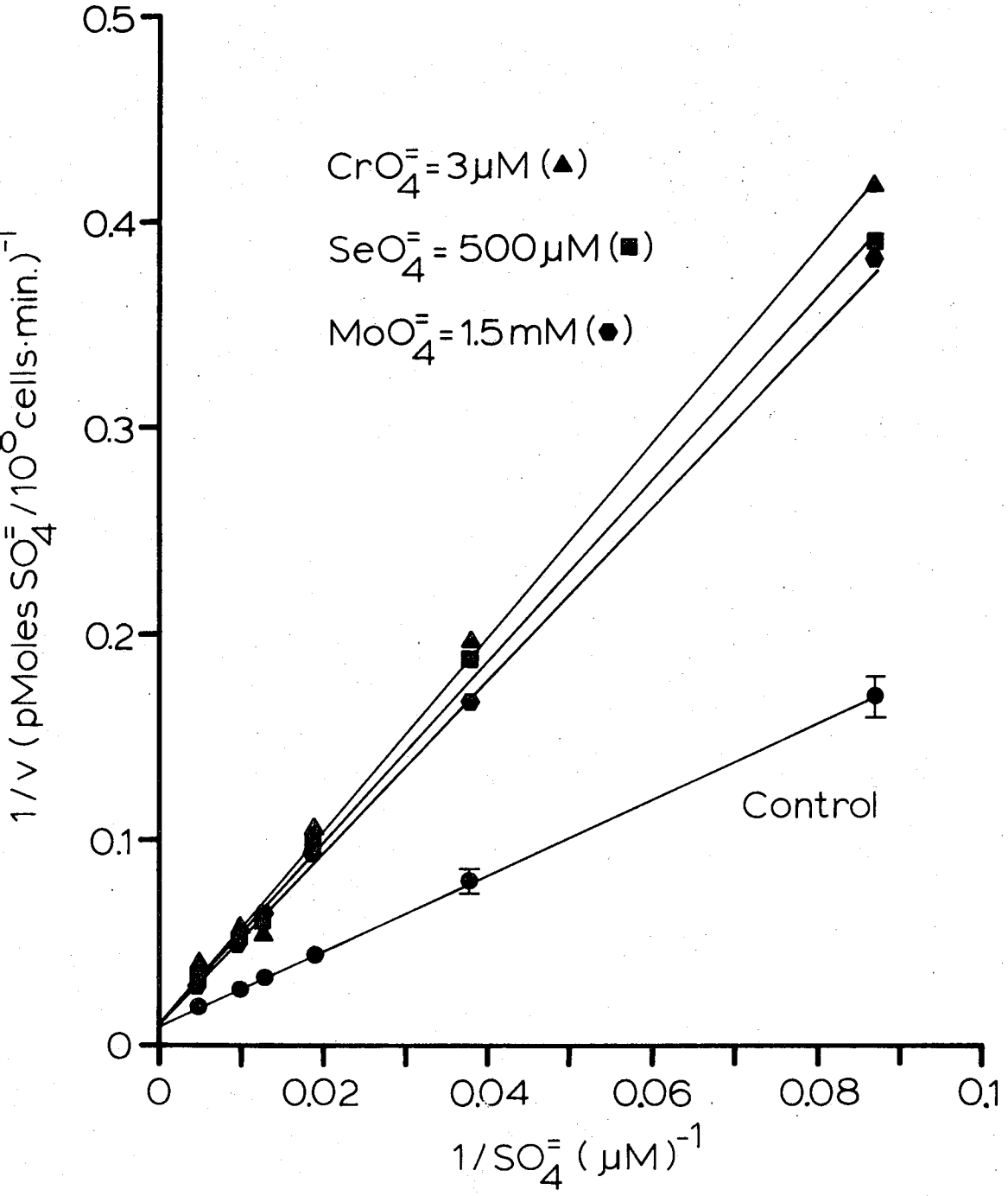

0.5
0.4
0.3
0.2
0.1
0
0
0.02
0.04
0.06
0.08
0.1
$CrO_4^{=}$ = 3μM (▲)
$SeO_4^{=}$ = 500μM (■)
$MoO_4^{=}$ = 1.5mM (●)
Control
1/v (pMoles $SO_4^{=}$ / 10^8 cells·min.)$^{-1}$
1/$SO_4^{=}$ (μM)$^{-1}$

Figure 3-14. Lineweaver-Burk plot of thiosulfate uptake inhibition by $XO_4^=$ analogs in P. halodurans. Thiosulfate was used without additional carrier (specific activity 10-13 DPM/pMole) at final concentrations of 2.5, 5, 7.5, 10, 15, 20, 25, and 40 μM. Cell density in individual experiments ranged from $2.9\text{-}3.1 \times 10^8$/ml.

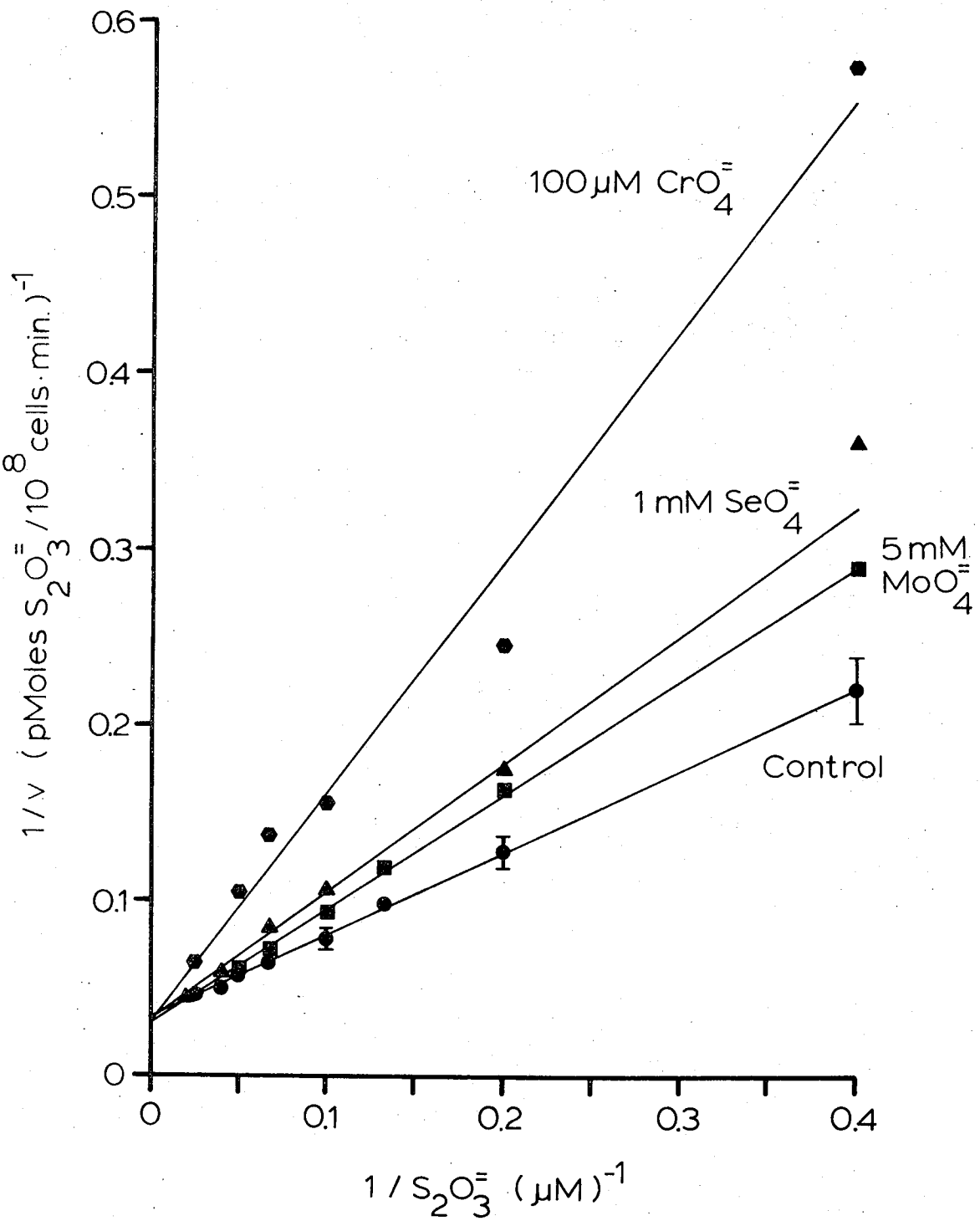

0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
100 µM $CrO_4^=$
1 mM $SeO_4^=$
5 mM $MoO_4^=$
Control
$1/v$ (pMoles $S_2O_3^=/10^8$ cells·min.)$^{-1}$
$1/S_2O_3^=$ (µM)$^{-1}$

Table 3-4. Kinetic Constants for Sulfate and Thiosulfate Uptake by *P. halodurans* and for Sulfate Uptake by *A. luteo-violaceus* with Inhibition Constants for Competition by Sulfate Analogs[a]

	P. halodurans		*A. luteo-violaceus*
	Thiosulfate	Sulfate	Sulfate
K_m (μM)	14.7 ± 1.5(8)	214 ± 34(9)	186 ± 18(5)
V_{max} (pMoles/10^8cells/minute)	30.5 ± 3.0(7)	108 ± 22(9)	146
Competitor	K_i' (μM)		
$CrO_4^=$	52	3	73
$SeO_4^=$	1989 ± 174(2)	569	238
$MoO_4^=$	9109 ± 562(2)	1327 ± 120(3)	12,200
$S_2O_3^=$	-	16.6 ± 1.8(2)	ND[b]
$SO_4^=$	397 ± 75(3)	-	-

[a]Data summarized from Figures 3-5, 6, 12, 13, and 14. The K_i' is calculated with Equation 3-1 using the mean K_m for each compound. Numbers in parentheses indicate the number of experiments if greater than one.

[b]Not determined.

uptake of both sulfur compounds follows the same molecular weight specificity pattern, much higher concentrations of the analogs are required to effect half-maximal inhibition of thiosulfate transport than are required for sulfate. The K_i' for thiosulfate inhibition of sulfate uptake is equal to the K_m determined for thiosulfate uptake, whereas the K_i' for the reverse competition is about twice the K_m. The size selectivity demonstrated for the P. halodurans transport systems is also found for sulfate transport by A. luteo-violaceus.

Effect of Active Transport Inhibitors on Sulfate and Thiosulfate Uptake

Inhibitors of active transport found effective for other sulfate uptake systems are inhibitory to both sulfate and thiosulfate uptake by P. halodurans. The response of both systems to DCCD, 2,4-DNP, CCCP, and azide is remarkably similar (Table 3-5). A small degree of inhibition due to DCCD was observed in this particular experiment, but in an earlier experiment no effect was noted, even with up to 15 minutes of preincubation with the inhibitor. The lack of inhibition of active transport by DCCD has been reported for amino acid transport in another marine bacterium as well (Pearce et al., 1977). Concentrations of DCCD greater than 100 μM cannot be used in seawater due to the formation of an insoluble precipitate. Dual starvation for sulfur and glutamate, the carbon and energy source, reduced both sulfate and thiosulfate uptake considerably, in further support of energy-dependent transport of the two anions.

Sulfate uptake by A. luteo-violaceus also shows a pronounced dependence on energy coupling (Table 3-6). In contrast to active transport

Table 3-5. Effects of Active Transport Inhibitors on Sulfate and Thiosulfate Uptake by Pseudomonas halodurans[a]

Addition	$SO_4^{=}$ Uptake		$S_2O_3^{=}$ Uptake	
	Rate[b]	Inhibition	Rate	% Inhibition
Distilled Water	60.7	0	15.0	0
NaN_3 (100 μM)	53.4	12[c]	12.0	20[c]
NaN_3 (1 mM)	29.0	52	6.8	55
-Glutamate	6.9	89	4.2	72
Ethanol (1)	62.0	0	15.7	0
DCCD (100 μM)	48.0	23[d]	13.5	14[d]
2,4-DNP (100 μM)	52.2	16	13.1	17
2,4-DNP (1 mM)	0	100	1.0	94
CCCP (5 μM)	57.0	8	12.9	18
CCCP (50 μM)	26.5	57	7.4	53

[a]Washed cell suspensions were starved for sulfur for two hours, then assayed for sulfate and thiosulfate uptake as described in materials and methods. The reaction mixture contained, in a final volume of 10 ml, 9.8 ml cell suspension, 0.1 ml $^{35}SO_4^{=}$ (final concentration 200 μM; 18 DPM per pMole) or $^{35}S_2O_3^{=}$ (final concentration 15 μM; 8 DPM per pMole), and 0.1 ml 100-fold concentrated inhibitor to give the final concentrations indicated. The cell density was 4.1×10^8 per ml.

[b]Rates are expressed in terms of pMoles $SO_4^{=}$ or $S_2O_3^{=}$ /10^8cells/minute.

[c]Inhibition relative to distilled water control for NaN_3 and -Glutamate.

[d]Inhibition relative to 1 EtOH control for 2,4-DNP, CCCP, and DCCD.

Table 3-6. Effects of Active Transport Inhibitors on Sulfate Uptake by Alteromonas luteo-violaceus[a]

Inhibitor	Uptake Rate ($pMoles\ SO_4^{=}/10^8 cells/min.$)	% Inhibition
Distilled Water	53.4	0
NaN_3 (100 µM)	46.2	14[b]
NaN_3 (1 mM)	10.8	80
-Glutamate	20.8	61
EtOH (1%)	52.8	0
2,4-DNP (100 µM)	42.6	19[c]
2,4-DNP (1 mM)	0	100
CCCP (5 µM)	29.2	45
CCCP (50 µM)	0	100
DCCD[d] (100 µM; t_0)	23.7	55
DCCD (100 µM: t_{-5})	15.3	71

[a]Washed cells were starved for sulfur for two hours, then assayed for sulfate uptake as described in Materials and Methods. The reaction mixture contained, in 10ml: 9.8ml cell suspension ($1.9x10^8$/ml), 0.1ml $^{35}SO_4^{=}$ (final concentration 101.5 µM; 35 DPM/pMole), and 0.1ml 100-fold concentrated inhibitor to give the final concentrations indicated.

[b]Inhibition relative to distilled water control for NaN_3 and -glutamate.

[c]Inhibition relative to 1% EtOH control for 2,4-DNP, CCCP, and DCCD.

[d]DCCD was added either simultaneously with sulfate (t_0) or 5 minutes prior to assay (t_{-5}).

systems of P. halodurans and another marine bacterium (Pearce et al., 1977), the ATPase inhibitor DCCD is effective in abolishing transport, and the effect is enhanced by a 5 minute preincubation with the inhibitor.

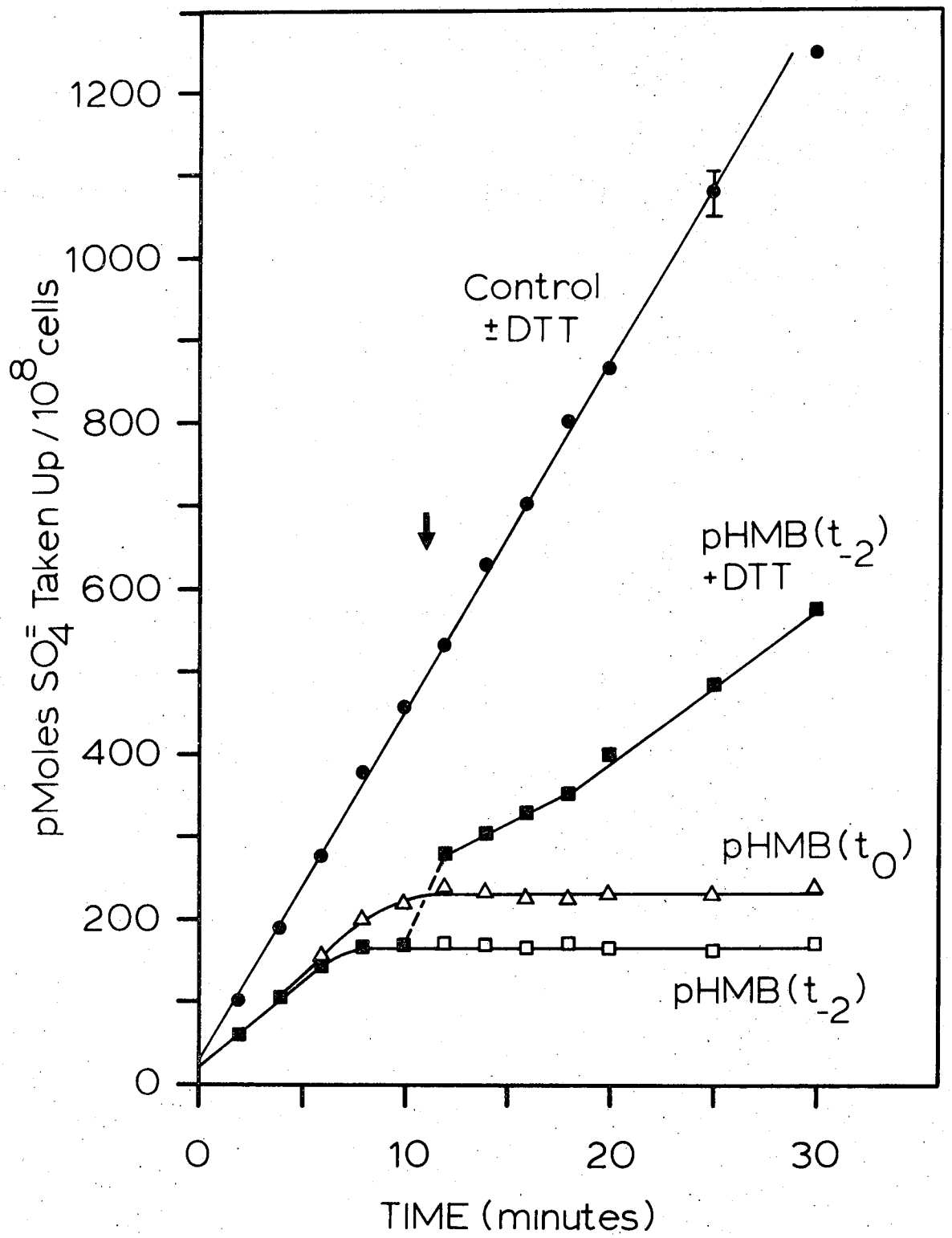
Inhibition of Sulfate Transport by pHMB

As previously noted, other sulfate transport systems are sensitive to inhibition by sulfhydryl reagents, indicating a free sulfhydryl group at or proximal to the active site of the permease. Low concentrations of pHMB effect a rapid and complete blockage of sulfate transport by P. halodurans (Figure 3-15) which is reversible by DTT. Preincubation of the cells with pHMB did not increase its effectiveness. The addition of DTT restored uptake to 44 % of control rates in inhibited cells; higher concentrations inhibited uptake by control suspensions and were not used.

Thiosulfate uptake inhibition cannot be demonstrated in this manner because it reacts chemically with the mercurial. A cell suspension of P. halodurans incubated with pHMB and then rinsed with fresh medium to remove unbound inhibitor did not take up either sulfate or thiosulfate; however, no activity could be recovered with DTT. The 15 minutes required to rinse the cells was probably sufficient for other effects of pHMB to become manifest.

The lack of response to thiosulfate in A. luteo-violaceus is supported by the unusual pattern of inhibition achieved with the sulfhydryl reagent pHMB. Transport enzymes which are sensitive to sulfhydryl reagents are generally characterized by a rapid and complete blockage of transport which is partially reversible by DTT (Jeanjean et al., 1975;

Figure 3-15. Effects of 10 μM pHMB on sulfate uptake by P. halodurans. Sulfate (100 μM; 40 DPM/pMole) was added either simultaneously (t_0) or after a 2 minute preincubation with pHMB (t_{-2}). Dithiothreitol (100 μM) was added at the arrow to one control and the sample preincu- bated with pHMB. The cell density was 3.2×10^8/ml.


1200
1000
800
600
400
200
0
pMoles $SO_4^=$ Taken Up / 10^8 cells
Control
± DTT
pHMB(t_{-2})
+DTT
pHMB(t_0)
pHMB(t_{-2})
0
10
20
30
TIME (minutes)

Kaback and Barnes, 1971; Marzluf, 1974). However, only mild inhibition of sulfate uptake by pHMB is found for A. luteo-violaceus, and the extent of inhibition does not increase with exposure time or preincubation (Figure 3-16). The sulfate uptake rate is increased slightly by the addition of DTT, but the characteristics of inhibition suggest that reduced sulfate uptake is not directly due to the action of pHMB but rather to a side effect, perhaps reduction of transport of glutamate, the energy source in these experiments.

Regulation of Sulfate and Thiosulfate Transport Capacity by Growth on Organic Sulfur Sources

The time course of appearance of sulfate and thiosulfate uptake during sulfur starvation of P. halodurans was similar, suggesting a common regulatory mechanism. Additionally, however, repression of sulfate and thiosulfate transport systems resulting from growth on various sulfur sources and subsequent derepression by sulfur starvation must be coordinate for the two systems to be considered identical. The instantaneous rates of sulfate and thiosulfate uptake were assayed with cells grown on sulfate, thiosulfate, cyst(e)ine, methionine, and glutathione harvested in the late exponential phase. Assays were again made after 2 and 8 hours of sulfur starvation, corresponding to the primary and secondary maxima of Figure 3-1. The results are shown in Table 3-7.

Organic sulfur compounds are poor sulfur sources for the growth of P. halodurans. The growth rates with cyst(e)ine and methionine as the sole source of sulfur are about half that acheived with either sulfate or thio-

Figure 3-16. Inhibition of sulfate uptake by pHMB in A. luteo-violaceus. A washed cell suspension (3.8×10^8 cells/ml) was starved for sulfur for two hours, then assayed for sulfate transport at 101.5 µM $^{35}SO_4^=$ (specific activity 25 DPM/pMole) in the presence or absence of 10 µM pHMB (final concentration). The arrow indicates the time of DTT addition (final concentration, 100 µM) or distilled water. Symbols: no addition or +DTT, ● ; pHMB added at 0 time, ▲ ; pHMB added at 0 time +DTT, △ ; pHMB added 2 minutes before sulfate, ■ . Control data are the mean of three samples (one +DTT) and filled triangles are the average of duplicate incubations before DTT addition; error bars fit within the symbol.

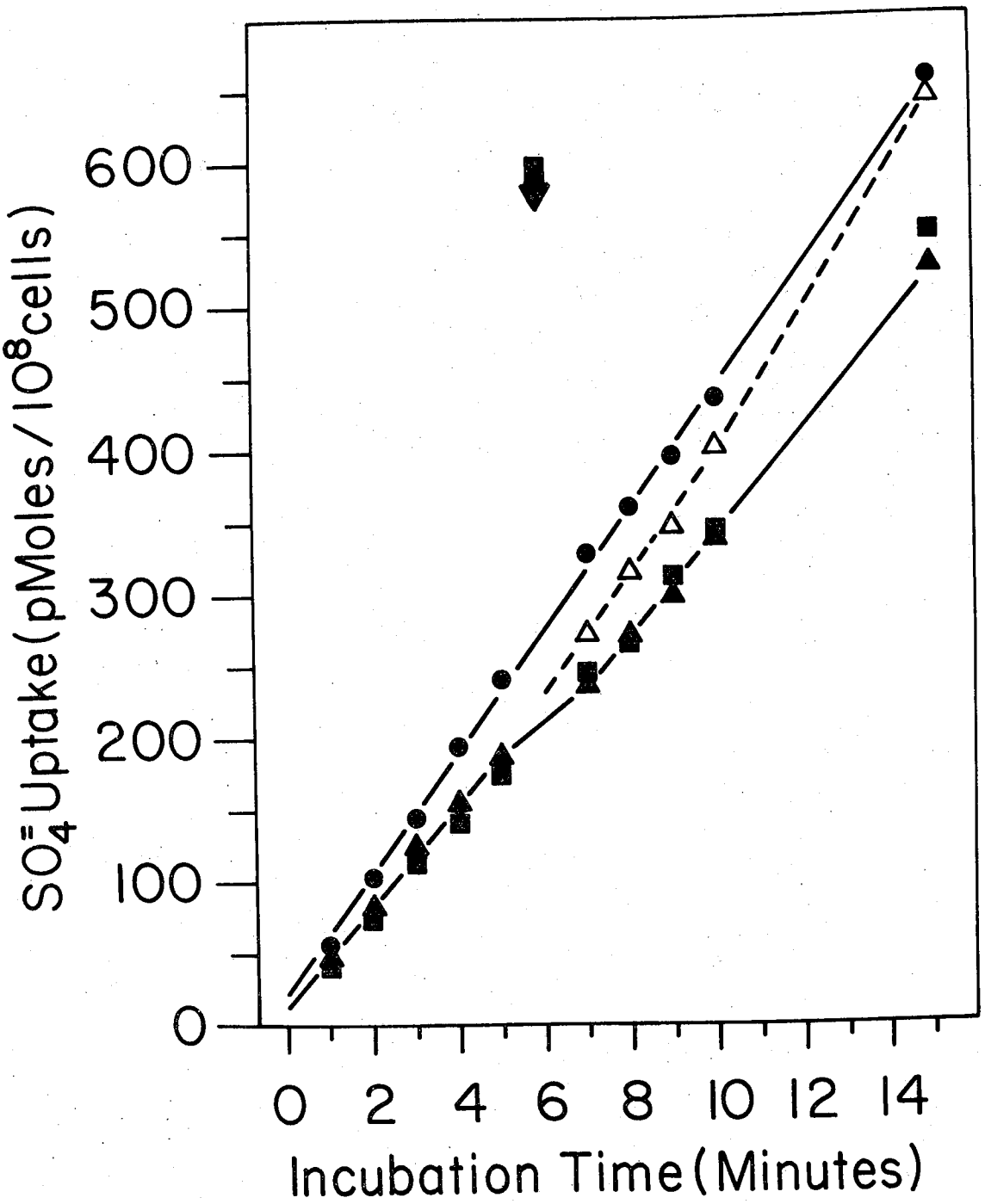

600
500
400
300
200
100
0
$SO_4^=$ Uptake (pMoles/10^8cells)
0
2
4
6
8
10
12
14
Incubation Time (Minutes)

Table 3-7. Derepression of Sulfate and Thiosulfate Transport Capacity by Growth on Organic Sulfur Sources and Sulfur Starvation in P. halodurans[a]

Sulfur Source[b]	Time[c]	10^8cells per ml	mg Protein per 10^8cells	Uptake Rate[d] Sulfate	Uptake Rate[d] Thiosulfate
Sulfate	0	1.45	18.4 ± 0.3	6.1 ± 1.1	5.9 ± 0
$k=0.87hr^{-1}$	2	1.49	21.2 ± 0.8	32.6 ± 0.5	16.7 ± 0.1
	8	2.08	20.7 ± 0.3	49.9 ± 0.1	14.9 ± 2.1
Thiosulfate	0	1.40	17.8 ± 1.2	4.8 ± 0.2	5.8 ± 0.3
$k=0.89hr^{-1}$	2	1.55	19.7 ± 0.5	32.0 ± 0.6	16.5 ± 0.1
	8	2.35	17.9 ± 0.3	47.2 ± 3.3	18.9 ± 0.1
Cyst(e)ine	0	2.01	16.3 ± 0.6	44.1 ± 4.8	23.4 ± 0.2
$k=0.45hr^{-1}$	2	2.46	19.5 ± 0.1	91.5 ± 0	43.4 ± 1.2
	8	4.97	13.9 ± 0.1	45.2 ± 1.2	21.0 ± 1.1
Glutathione	0	1.67	18.6 ± 0.2	61.7 ± 0.3	31.5 ± 0.5
$k=0.32hr^{-1}$	2	2.07	20.2 ± 0	61.7 ± 0.4	41.3 ± 0
	8	4.43	13.1 ± 0.1	24.9 ± 0.7	14.9 ± 0.2
Methionine	0	1.30	23.8 ± 0.4	63.8 ± 2.1	34.8 ± 0.2
$k=0.46hr^{-1}$	2	2.00	21.1 ± 0.5	89.5 ± 0.6	37.3 ± 0.6
	8	3.31	18.6 ± 0.2	62.2 ± 1.3	22.4 ± 1.0

[a]1500ml complete medium minus sulfur were inoculated with washed cells to a final density of about $2x10^4$/ml and shaken for 10 minutes to evenly distribute the cells. 250ml aliquots were aseptically transferred into sterile 300ml flasks and 0.2 µm filter-sterilized sulfur sources were added to a final concentration of 500 µM sulfur (i.e. 500 µM each L-cysteine, DL-methionine, reduced glutathione, and sulfate; 250 µM thiosulfate). The cultures were shaken at 250 RPM at 20°C. Late exponential phase cells were harvested by centrifugation, rinsed with basal salts medium, and resuspended in complete medium minus sulfur. At 0, 2, and 8 hours of sulfur starvation, aliquots were removed for direct counts, total protein, and assay of sulfate and thiosulfate transport as described in materials and methods.

[b]The growth rate, k, was determined from direct counts taken at intervals during exponential growth.

[c]Hours of sulfur starvation.

[d]The reaction mixture contained, in 10ml: 9.9ml cell suspension and 0.1ml $^{35}SO_4^{=}$ (final concentration 100 µM, 32 DPM/pMole) or $^{35}S_2O_3^{=}$ (final concentration 20 µM, 8 DPM/pMole). The uptake rate is expressed as pMoles $SO_4^{=}$ or $S_2O_3^{=}/10^8$cells/minute.

sulfate. The methionine-grown cells reached $1x10^7$ cells/ml with a lag time of 18 hours relative to those grown on sulfate; this lengthy delay cannot be attributed to the slower growth rate alone and suggests that synthesis of enzymes for the conversion of methionine to cysteine must be induced for growth on methionine to occur. These cells also exhibit a markedly higher protein content. Cultures provided with reduced glutathione grow at the slowest rate; however, extrapolation of the direct count regression line to the time of inoculation indicates a more rapid growth rate prior to the appearance of turbidity. It is not known whether this is due to the dimerization of glutathione by autooxidation and concommittant difficulty of transport or to other effects.

In contrast to previously studied sulfate transport systems (Dreyfuss, 1964; Marzluf, 1970a; Yamamoto and Segel, 1966), all three organic sulfur sources derepress both sulfate and thiosulfate uptake capacity effectively. Uptake rates prior to sulfur starvation are highest in cultures grown on methionine and glutathione, being about 50% higher than cyst(e)ine-grown cells and ten-fold higher than those grown on sulfate or thiosulfate. However, the characteristically low initial uptake rates for both sulfate and thiosulfate by sulfate-grown cells (see also Figure 3-1) are also observed for the culture using thiosulfate as the sole source of sulfur.

Further derepression of sulfate transport capacity occurs as a result of sulfur starvation for all cultures except those grown on glutathione as the sole sulfur source. Sulfate and thiosulfate-grown cells show a primary increase of about 6-fold during the first two hours of sulfur starvation, followed by a small increase during the next 6 hours. The

cysteine-supplemented culture exhibits a doubling of transport capability, whereas an increase of slightly less than 50 accompanies sulfur starvation of the methionine-grown culture: both return to the initial value as a result of cell division and dilution of the transport proteins among daughter cells. Glutathione-grown cells demonstrate no increase during the first two hours, but decline by over 50 during the subsequent six hours of sulfur starvation because of cell division. It should be noted that under no circumstances does the total transport capacity (per ml of culture) decline; all decreases in activity reported in Table 3-7 are due to increases in cell numbers during sulfur starvation. This is also true for total protein.

Thiosulfate uptake rates follow virtually the same pattern as those for sulfate. All three organic sulfur sources derepress thiosulfate transport capacity. Using the kinetic constants in Table 3-4 for sulfate and thiosulfate uptake, the $\% V_{max}$ at the assay concentrations are 32% and 57 % respectively, or 33.4 and 16.9 pMoles/10^8cells/minute, yielding an uptake ratio of 1.98 pMoles $SO_4^=$ transported for each pMole of $S_2O_3^=$. This predicted ratio is observed for derepression by cyst(e)ine (1.88), methionine (1.83), and glutathione (1.96) prior to sulfur starvation. The trends for sulfate and thiosulfate derepression both by growth on various sulfur sources and by subsequent sulfur starvation of those cultures are very similar in 12 of the 15 cases, with a mean $SO_4^=:S_2O_3^=$ uptake ratio of 1.99 ± 0.63 for all data, in good agreement with the ratio predicted from data in Table 3-4.

Feedback inhibition of sulfate uptake by products of sulfate metabolism in A. luteo-violaceus is suggested by the data in Figure 3-11: the

reduction in transport rate at high sulfate concentrations is too rapid to be attributable to repression of permease synthesis. This is supported by the inhibition of sulfate uptake by organic sulfur compounds: when assayed at 101.5 μM sulfate, 1mM methionine has little or no detectable effect, whereas cyst(e)ine and glutathione strongly inhibit sulfate uptake (100 and 85 , respectively). Since A. luteo-violaceus is insensitive to pHMB, it is not likely that this is an effect of the sulfhydryl group of cysteine or glutathione but rather a fast response to intracellular organic sulfur pool filling. Additionally, the sulfate uptake rate decreases rapidly with time in the presence of glutathione, suggesting metabolic conversion to cysteine. Growth of A. luteo-violaceus in media containing organic sulfur compounds as the sole source of sulfur and assay of the sulfate uptake rate during subsequent sulfur starvation of the cultures provides further evidence for regulation by cysteine or glutathione. The growth rate is similar (0.26-0.29 hr^{-1}) for cultures grown on cyst(e)ine, methionine, glutathione, and sulfate, in contrast to P. halodurans. Table 3-8 shows that cysteine and glutathione repress the sulfate transport capacity of A. luteo-violaceus to a greater extent than sulfate itself, whereas methionine is effective in derepressing the system. Sulfur starvation led to increased sulfate uptake rates in all cultures similarly, therefore it is not possible to distinguish between feedback inhibition and repression. Notably only the methionine-grown culture demonstrated non-linear uptake patterns with an abrupt rate break at 4 minutes in 1 and 2-hour starved assays, when the instantaneous rate was much greater than the biosynthetic requirement for sulfur. The fact that P. halodurans sulfate transport is derepressed by all three organic

Table 3-8. Derepression of Sulfate Uptake Capacity of *Alteromonas luteo-violaceus* Grown on Various Sulfur Sources[a]

Hours of Sulfur Starvation	pMoles $SO_4^=/10^8$cells/minute[b]			
	Sulfur Source for Growth			
	Sulfate	Methionine	Cyst(e)ine	Glutathione
0	4.8 ± 0.4	20.8 ± 4.2	0.9 ± 0.1	0
1	23.3 ± 0.4	68.3 ± 1.9 [c]	16.2 ± 0.2	9.4 ± 0.5
2	33.7 ± 0.4	109.4 ± 0.2 [c]	29.6 ± 0	45.4 ± 6.8

[a]Washed cells were inoculated to about $1x10^4$cells/ml into flasks containing 100ml complete medium with the indicated 0.2 µm filter-sterilized sulfur source at 1mM final concentration. Growth was followed by optical density at 420nm; at an optical density of about 0.5 the cells were harvested, rinsed with basal salts medium, and resuspended in complete medium minus sulfur. Sulfate uptake rates were measured as described in Materials and Methods during sulfur starvation at 101.5 µM $^{35}SO_4^=$ final concentration (20DPM/pMole). Initial cell densities were: sulfate, $1.52x10^8$/ml; methionine, $1.42x10^8$/ml; cyst(e)ine, $1.73x10^8$/ml; glutathione, $1.06x10^8$/ml.

[b]Based on initial cell density.

[c]First 4 minutes of uptake only.

sulfur sources and shows no indication of inhibition by products of sulfate metabolism argues that feedback inhibition is at least partly responsible for the observations with A. luteo-violaceus.

DISCUSSION

The properties of the sulfate transport system of Pseudomonas halodurans are qualitatively similar to those of other microorganisms. Common characteristics include (a) dependence of transport on energy coupling which is susceptible to gradient uncouplers and respiratory poisons (Deane and O'Brien, 1975; Holmern et al., 1974; Jeanjean and Broda, 1977; Roberts and Marluf, 1971; Smith, 1976; Yamamoto and Segel, 1966), (b) size-specific competition of uptake by $XO_4^{=}$ analogs (X=Cr, Se, and Mo in decreasing order of effectiveness)(Deane and O'Brien, 1975; Pardee et al., 1966; Smith, 1976; Vange et al., 1974), (c) effective inhibition by thiosulfate (Marzluf, 1970a; Roberts et al., 1963; Smith, 1976; Utkilen et al., 1976; Vange et al., 1974; Yamamoto and Segel, 1966), (d) reversible inhibition of uptake by sulfhydryl reagents (Holmern et al., 1974; Marzluf, 1974; Smith, 1976; Vallee and Jeanjean, 1968b), and (e) derepression of sulfate transport capacity during sulfur starvation (Deane and O'Brien, 1975; Jeanjean and Broda, 1977; Utkilen et al., 1976; Yamamoto and Segel, 1966).

Several differences stand out, however. The K_m for sulfate uptake by P. halodurans is over 200 μM, about ten times the average for previously reported systems, probably reflecting the high sulfate concentration (greater than 25 mM) of seawater. The sulfur-containing amino acids

cyst(e)ine and methionine and the peptide glutathione strongly derepress sulfate uptake, in strict contrast to the other sulfate transport systems (Dreyfuss, 1964; Marzluf, 1970a; Yamamoto and Segel, 1966). Cells grown on these compounds as sole sources of sulfur have initial uptake rates up to ten times higher than sulfate or thiosulfate-grown cells, and further derepression is acheived by subsequent sulfur starvation. Since thiosulfate contains an oxidized sulfur atom which apparently undergoes reduction in the same manner as sulfate (Hodson et al., 1971; Leinweber and Monty, 1963), it can be concluded that repression is due to some organic sulfur compound which is either a precursor to cysteine or which is not directly related to sulfate reduction. This is supported by the observation that low molecular weight organic sulfur compounds (soluble in 10% trichloroacteic acid but not precipitable with barium) rapidly disappear during the first two hours of sulfur starvation, whereas inorganic sulfate remains at a constant, low level. The compound may be needed for biosynthetic reactions other than those utilizing cysteine or methionine, perhaps the synthesis of an essential sulfate ester requiring an oxidized form of sulfur. A rate-limiting reversal of the sulfate reduction pathway to yield an oxidized product is suggested by the fact that the growth rate on the organic sulfur compounds is only half that achieved with inorganic sulfur sources. An analogous situation is the synthesis of the plant sulfolipid by Chlorella (Sinensky, 1977), which cannot be carried out when the organism is grown on cysteine.

The genetic analysis of the sulfate and thiosulfate transport system of Salmonella typhimurium (Leinweber and Monty, 1963) and an increasing amount of circumstantial evidence obtained from various studies of sul-

fate transport systems (Marzluf, 1970a; Roberts et al., 1963; Smith, 1976; Utkilen et al., 1976; Vange et al., 1974; Yamamoto and Segel, 1966) strongly support the concept of a common carrier for the transport of the two structurally similar sulfur compounds, and probably for the class of $XO_4^=$ analogs as well. The fact that sulfate reduction mutants retaining transport capacity will grow on thiosulfate as the sole source of sulfur in both Salmonella (Leinweber and Monty, 1963) and Chlorella (Hodson et al., 1971), whereas sulfate transport mutants possessing reduction capability will not is very convincing evidence.

The data presented in this chapter are in further support of the concept of a dual-substrate permease for sulfate and thiosulfate. The uptake of both compounds by P. halodurans is affected by energy-dissipating agents identically. Transport of the two structurally related anions is competitively inhibited by the sulfate analogs chromate, selenate, and molybdate in the same order of decreasing effectiveness and increasing molecular weight. Above all, sulfate and thiosulfate are mutual competitive inhibitors of uptake, with the half-saturation constant for thiosulfate uptake equal to its K_i' for inhibition of sulfate uptake. The uptake of both compounds is coordinately regulated by low molecular weight sulfur compounds, and the derepression patterns for sulfur starvation and growth on organic sulfur sources are strikingly similar.

Apparent anomalies in the relationship between inhibition constants for the sulfate analogs, the K_m for sulfate transport versus the K_i' for sulfate inhibition of thiosulfate transport, and the much lower K_m for thiosulfate transport are consistent with the model of active transport originally proposed by Kaback and Barnes (1971). The model invokes a

pore-type carrier in which the pore opening and transport process is controlled by the reversible reduction of one or more disulfide bridges. This model is attractive with regard to the sulfate and thiosulfate transport data for P. halodurans for a number of reasons. The very low chemical reactivity of sulfate, emphasized by the requirement for sulfate activation with ATP before further metabolism, argues against a covalent intermediate during transport. The rapid inhibition of uptake by proton gradient uncouplers and respiratory poisons favors an energy source other than ATP itself, as in the Kaback and Barnes (1971) model, although proton motive force generated by ATP hydrolysis would result in similar behavior. Reversible sensitivity to pHMB indicates the involvement of a sulfhydryl function proximal to the active center. In the absence of covalent binding of sulfate, the steric hindrance of the large organo-mercurial compound could abolish uptake. Alternatively, the binding of the compound to an essential disulfide bridge could disrupt the ability to make the conformational changes necessary to transport the substrate.

A free sulfhydryl group near the active site or an accessible disulfide linkage involved in transport could explain the higher affinity for thiosulfate and the greater resistance of thiosulfate uptake to inhibition by sulfate and its analogs. The sulfane moiety of thiosulfate is a potent nucleophile and reacts readily with free sulfhydryl groups and disulfides; thiosulfate can therefore bind covalently to such a site, rendering the effective concentration higher for transport. Covalently bound thiosulfate could reduce accessibility to the site for competing anions. This is not an equilibrium process regulated solely by diffusion and explains the greater amount of inhibitor required to reduce thiosulfate uptake rates relative to that required to inhibit sulfate transport.

A strong case for involvement of the reduced sulfur atom of thiosulfate is found in the characterization of the sulfate binding protein of Salmonella typhimurium, which contains no sulfur amino acids (Pardee, 1966). The sulfate analogs chromate and selenate inhibit both binding and transport (Dreyfuss and Pardee, 1965). Even though thiosulfate is structurally related to sulfate, however, it is ineffective in inhibiting sulfate binding, whereas it is a good inhibitor of transport (Pardee et al., 1966).

The sulfate transport system of A. luteo-violaceus is strikingly different from that of another obligately aerobic marine bacterium, P. halodurans, yet the two possess several common characteristics which are in contrast to previously reported sulfate permeases. Features common to all systems include competitive inhibition by the sulfate analogs $CrO_4^{=}$, $SeO_4^{=}$, and $MoO_4^{=}$, a strict dependence on energy coupling, and apparent derepression of sulfate transport capacity by sulfate starvation.

The K_m for sulfate uptake is about ten times higher for the two marine microorganisms than for their terrestrial counterparts. This is not unexpected as the 25mM sulfate concentration of seawater should not apply selection pressure for a higher affinity transport system. An additional influence of the high sulfate concentration may be reflected in the close agreement between maximum transport capacity and growth requirements. Although increasing sulfate concentration leads to increasing initial uptake rates by A. luteo-violaceus throughout a wide range of concentration, the uptake rate declines after a short period of time at high concentrations. The same result is achieved in a different manner by P. halodurans, in which the uptake rate is linear with time at all sul-

fate concentrations, but ceases to increase in response to increasing sulfate concentration after its quota for sulfur has been filled . Neither organism can be made to accumulate inorganic sulfate to more than the amount characteristic of exponential growth, unlike the uptake in excess of growth requirements demonstrated by Penicillium chrysogenum (Yamamoto and Segel, 1966), Salmonella typhimurium (Dreyfuss, 1964), or Anacystis nidulans (Utkilen et al., 1976). This is an especially valuable feature if sulfate metabolism is to be considered for use as a tool for the measurement of marine microbial growth.

Regulatory processes for sulfate uptake by the marine bacteria are unusual in that growth on methionine derepresses sulfate transport capacity effectively, contrary to results from other systems (Marzluf, 1970a; Yamamoto and Segel, 1966). Repression by cyst(e)ine (Pardee et al., 1966; Yamamoto and Segel, 1966) and glutathione (Yamamoto and Segel, 1966) is found for A. luteo-violaceus, but not for P. halodurans . Since the free sulfate pool is very small in both marine bacteria, it seems likely that a precursor to cysteine (e.g. APS) is acting as the repressor in P. halodurans whereas cysteine itself may be responsible for repression and/or feedback inhibition in A. luteo-violaceus. A. luteo-violaceus-like organisms have been isolated frequently from surfaces such as Sargassum weed, fish slime and squid tentacles, where its potent protease may release sufficient quantities of cysteine for growth, whereas P. halodurans is found free-living in estuaries (A. Rosenberg, Ph. D. Thesis, University of New Hampshire, 1977) where sulfate is most likely its only sulfur source.

The most unusual feature of the A. luteo-violaceus sulfate transport system is the limited effect of thiosulfate on sulfate transport and lack of growth on this compound. Although structurally similar to sulfate, thiosulfate possesses a reduced sulfur atom in place of one of the four equivalent oxygen atoms of sulfate. This substitution imparts a considerably greater nucleophilic character to thiosulfate, which readily reacts with free sulfhydryl groups and disulfide bridges. When thiosulfate and sulfate are present in equimolar amounts, sulfate uptake is inhibited at least 50 % (Bradfield et al., 1970; Marzluf, 1970a; Pardee et al., 1966; Utkilen et al., 1976; Vange et al., 1974) and often much more (Dreyfuss, 1964; Roberts and Marluf, 1971). When tested, these sulfate transport systems are also sensitive to pHMB, with rapid and nearly complete blockage of uptake (Marzluf, 1974; Vallee and Jeanjean, 1968b). The action of the sulfhydryl reagent and the frequent preference for thiosulfate in transport suggest that the reduced sulfur moiety of thiosulfate is an important component in the mechanism of its transport. In A. luteo-violaceus, however, a 50-fold excess of thiosulfate relative to sulfate is required to achieve slightly more than 50 % inhibition of sulfate uptake. In addition, the sulfhydryl reagent pHMB exerts only slight influence on sulfate transport.

Certain differences in the sulfate transport systems of the two marine bacteria relative to their terrestrial counterparts may reflect the high sulfate concentration characteristic of their habitat and are of interest from an ecological point of view. The small endogenous inorganic sulfate pool of the two organisms and the low rate of its metabolism during sulfur starvation indicate that this pool is either inaccessible or,

more likely, maintained at a threshold level in marine bacteria. In contrast, endogenous sulfate pools are rapidly metabolized during sulfur starvation of fungi (Yamamoto and Segel, 1966) and plants (Leggett and Epstein, 1956; Smith, 1975). The constant availability of sulfate in the marine environment would not be expected to select for sulfate pooling abilities. The resultant absence of detectable luxury uptake of sulfate indicates that sulfate uptake may be a useful tool for the measurement of marine microbial growth. Such work has already been attempted in freshwater ecosystems (Jordan and Peterson, 1978; Monheimer, 1974). The similarity of the sulfate and thiosulfate uptake systems in P. halodurans and other organisms suggests caution, however, in the interpretation of sulfate uptake studies near anaerobic water masses or other sources of reduced sulfur compounds such as coastal wetlands and lakes where the hypolimnion becomes anaerobic (Jassby, 1975). In such habitats thiosulfate, a relatively stable compound in aqueous media and a major product of abiotic sulfide oxidation, could compete for sulfate uptake and metabolism, leading to a serious underestimate of rates of sulfur metabolism. The concept of actually using thiosulfate at high concentration to outcompete sulfate for metabolism and hence reduce the large isotope dilution barrier in seawater was appealing until the sulfate transport system of A. luteo-violaceus was investigated.

The sharp contrast of the sulfate transport system of A. luteo-violaceus to that of P. halodurans and other microorganisms with respect to thiosulfate uptake and inhibition of sulfate transport, response to pHMB, and repression of sulfate transport by growth on organic sulfur sources indicates a substantially different mechanism of sulfate transport in

this bacterium. The peculiar nature of the system is emphasized by the results of a thiosulfate utilization survey of marine bacterial isolates obtained in the vicinity of the Puerto Rico Trench on R/V Oceanus Cruise #40: of 12 isolates with distinctly different colony morphology on solid media, only A. luteo-violaceus did not grow as well on thiosulfate as on sulfate as the sole source of sulfur. The failure of this organism to utilize thiosulfate is not sufficient in itself to discount the possibility of using thiosulfate as a means of measuring marine bacterial protein synthesis as suggested in the introduction. However, other possible artifacts, such as the incorporation of the reduced atom of thiosulfate into cysteine by animals (Schneider and Westley, 1963) have led me to investigate high sensitivity methods for the direct measurement of sulfate incorporation into microbial protein.

CHAPTER 4.

SULFUR METABOLISM, PROTEIN SYNTHESIS, AND GROWTH IN MARINE BACTERIA

INTRODUCTION

Studies of microbial physiology in the laboratory have demonstrated that bacterial metabolism and growth can be uncoupled from one another under a variety of culture conditions. The synthesis of major biochemical components of bacteria, e.g. protein, ribonucleic acid, deoxyribonucleic acid, lipid, polymeric carbohydrate, and low molecular weight compounds may proceed at very different rates depending on the availability of specific nutrients or in response to physical and chemical stresses. Pertinent examples include the differential rates of synthesis of protein, RNA*, and DNA during the transition phase of diauxic growth (Neidhardt and Magasanik, 1960) and in synchronized growth (Asato, 1979), and the preferential synthesis of carbohydrate reserves during nutrient limited growth of bacteria (Antoine and Tepper, 1969; Dawes and Senior, 1973; Herbert, 1961; Sirevag and Ormerod, 1977; Slepecky and Law, 1961) and algae (Hobson and Pariser, 1971; Lehman and Wober, 1976).

The attainment of balanced growth, when all components of biomass and rates of energy-yielding processes increase identically (Herbert, 1961) is often difficult to achieve without rigorously controlled culture conditions such as those obtained with a chemostat. Bacteria in natural habitats are unlikely to be exposed to conditions constant enough to maintain (or even enter) balanced growth (Jannasch, 1974). Therefore, a

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* Abbreviations used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; TCA, trichloroacetic acid; L.M.W., low molecular weight.

thorough understanding of the manifestations of unbalanced growth is necessary for the interpretation of microbial metabolism studies in nature.

The measurement of a single rate process gives only a limited amount of information about the state of growth with either pure cultures or natural populations. It is through the comparison of several rates differing in their sensitivity to uncoupling of growth and metabolism that imbalances may be detected. Preferably a central metabolic rate such as protein or nucleic acid synthesis should be used in conjunction with an indicator of uncoupling, e.g. total carbon metabolism and the distribution of carbon in major macromolecular components. Changes in the ratios of such rates over time indicate the preferential synthesis of compounds suggestive of specific types of imbalance.

No quantitative measurement of short-term microbial growth in natural aquatic habitats currently exists. This is largely due to the complex and undefined nature of dissolved organic compounds in aquatic ecosystems and the extreme breadth of metabolic capabilities of the bacteria found in any water sample. It is generally understood that the measurement of mineralization of individual organic carbon compounds cannot accurately represent the cumulative metabolism of diverse assemblages of microorganisms. For this reason, some ecologists have turned to the measurement of sulfate uptake by natural plankton communities in freshwater ecosystems. It was believed that sulfate uptake by whole cells might provide a measure of total bacterial heterotrophy if (1) sulfate is the only sulfur source, and (2) a predictable relationship exists between sulfate uptake and carbon metabolism. The first assumption is, in principle, correct, but there is no a priori reason why carbon and sulfur metabolism should

be closely related. The wide distribution of carbon in biological molecules and its importance in storage products often formed in quantity during unbalanced growth argue against an invariant relationship between carbon and sulfur metabolism.

Sulfur is an essential element in protein. The sulfur-containing amino acid cysteine is largely responsible for the tertiary structure and stabilty of proteins through involvement as disulfide linkages. Additionally, cysteine residues at or near the active site of many enzymes are important in catalysis (Anfinsen, 1973; Jeanjean et al., 1975; Kaback and Barnes, 1971; Nelson et al., 1971). The other sulfur-containing amino acid, methionine, is important in hydrophobic interactions with other amino acids and membranes. Of the major elements of biomass in bacteria (i.e. C, H, O, N, P, and S), sulfur has the most restricted distribution in the biochemical sense. Except for traces of the sulfur-containing RNA base 4-thiouracil (Carbon et al., 1965; Lipsett, 1965), organic sulfur is found only in low molecular weight soluble materials (amino acids, vitamins, and coenzymes) and protein (Datko et al., 1978a; Roberts et al., 1963). Sulfur metabolism and protein synthesis must therefore be closely related.

In turn, protein synthesis is expected to be highly coupled to growth. Proteins provide the machinery for all biosynthetic processes of cells, and protein storage is evident only in some resting stages such as plant seeds (Elmore and King, 1978). Protein is of considerable trophodynamic interest as well: the ultimate goal of fisheries modelling is to accurately predict the production of higher organism protein for human consumption. A quantitative measurement of protein synthesis would be

useful to the microbial physiologist, who must currently rely on relative rates obtained from amino acid incorporation or insensitive chemical assays. The ecosystem modeller, on the other hand, could use protein synthesis rates from natural habitats to predict potential biomass increases at higher trophic levels.

The enzymology of sulfate reduction by microorganisms has been well characterized. The work of Schiff and co-workers described the intermediates and enzyme reaction conditions necessary for the formation of adenosine-5'-phosphosulfate (APS)(Goldschmidt et al., 1975) and its phosphorylated derivative adenosine-3'-phospho-5'-phosphosulfate (PAPS)(Hodson et al., 1968) from inorganic sulfate, the reduction of carrier-bound sulfite to the level of sulfide (Abrams and Schiff, 1973), and demonstrated the formation of cysteine (Schmidt et al., 1974). The differential reduction of the sulfur-containing intermediates APS by plants and PAPS by bacteria (Goldschmidt et al., 1975) is the major feature discriminating photosynthetic and non-photosynthetic sulfate reduction pathways (Tsang and Schiff, 1975). Others have analysed the mechanism of the cysteine synthetase complex in detail (Becker et al., 1969; Hulanicka et al., 1979; Kredich, 1971; Kredich and Tomkins, 1966), as well as the three step sequence in the conversion of cysteine to methionine (Delaney et al., 1973; Giovanelli et al., 1973; Rowbury and Woods, 1964a,b).

Surprisingly little work has been done on the relationship between sulfur assimilation and microbial growth. However, careful interpretation of data on the flow of sulfur through various intermediates support the hypothesis that sulfur assimilation into protein may be a quantitative indicator of de novo protein synthesis. For example, Datko et al. (1978b)

found that the sulfur amino acid content of Lemna remained in constant proportion to the indicator amino acids of the protein hydrolysate during growth over a range of sulfate concentrations, in spite of a highly variable content of LMW sulfur-containing intermediates.

Bearing in mind the importance of sulfur-containing amino acids in the maintenance of protein structure and function, a relatively constant proportion of sulfur-containing amino acids may be found in bulk protein. Variations in amino acid composition among individual proteins will be averaged out in most cases, since individual proteins rarely account for a significant amount of the total protein.

Numerous proteins have been purified and completely sequenced. Independent analyses of the average composition and variability in individual amino acid composition of proteins have been computed by Reeck and Fisher (1973) and Jukes et al. (1975), further supporting the theory that the sulfur-containing amino acids constitute a relatively constant proportion of the total amino acids in the total cellular protein. In spite of the high variance of the total sulfur amino acids (cysteine plus methionine) as a proportion of the total amino acids among individual proteins (Holmquist, 1978), both analyses (involving completely different data sets) calculate the same mean value of 3.1 moles % of the total amino acids. Summing all of the amino acid molecular weights in the appropriate proportions (discarding 60 water molecules for peptide bond formation), the 61 amino acid "average protein" of Jukes et al. has a molecular weight of 6698 Daltons. The weight % contribution of the elements is: C, 52.8; O, 21.9; N, 17.1; H, 7.0; and S, 1.1, which provides a basis for comparison with the results of growth studies.

The lack of detection of the sulfur-containing amino acids in seawater (Schell, 1974; C. Lee and T. Mague, personal communications) and the limited variety of other sulfur-containing compounds in microorganisms (Busby and Benson, 1973; Datko et al., 1978a; Giovanelli et al., 1973; Roberts et al., 1963) suggests that sulfate may be the only source of sulfur for open ocean microorganisms. A predictable relationship between sulfur incorporation into protein and bulk protein synthesis could lead to a quantitative assay for microbial activity in natural assemblages.

The work presented in this chapter is based on the detailed analysis of sulfur metabolism in Escherichia coli published by Roberts et al. (1963), stimulated by the application of a similar method to studies of phytoplankton growth (Morris et al., 1974). Roberts and co-workers found that sulfur is confined to a very restricted group of compounds, predominantly the sulfur-containing amino acids, and pointed out that "sulfur metabolism therefore provides a relatively simple measure of protein synthesis" (p. 318). Their exhaustive chromatographic separations emphasize the following pertinent characteristics of sulfur metabolism in E. coli: (1) the amount of sulfur per unit dry weight is variable over a 2-fold range, (2) at least 95% of the total cellular sulfur is contained in the amino acids cysteine and methionine, (3) the L.M.W. organic sulfur pool contains primarily glutathione, with traces of cysteine and virtually no methionine, (4) hydrolysis of alcohol soluble, hot TCA soluble, and residual protein fractions yield only cysteine and methionine, (5) the residue protein contains twice as much methionine as cysteine, and (6) cysteine and to a lesser extent methionine compete with sulfate for uptake and metabolism.

The thorough analysis of sulfur-containing compounds in the biochemical fractions published by Roberts et al. (1963) permitted me to place an emphasis on the relationships among cell numbers, major biomass parameters, protein synthesis, and sulfur metabolism during normal and perturbed bacterial growth. The fractionation procedure used in this work (Chapter 2) has been modified from the method of Roberts et al. only in the use of glass fiber filters as an added pellet and site for the adsorption of precipitated macromolecules; the results are therefore strictly comparable to those described above. The use of glass fiber filters enables the fractionation of as few as 10^6 cells and is thereby applicable to the study of natural populations as well as pure cultures using any radioisotopic label.

The potential of using the measurement of sulfate incorporation into bacterial protein as an assay for natural marine bacterial protein synthesis will be greater if the following hypotheses are proven correct:

(1) the protein content of bacteria on a per cell basis is a relatively conservative measure of cell growth (i.e. has a low variability), and

(2) the sulfur content of the bulk protein lies within a narrow range of variation such that nutritional and environmental perturbations do not significantly affect the calculation of protein synthesis rates from sulfate incorporation into protein.

This chapter provides information on the flow of sulfur through major biochemical components during growth of two marine bacteria under a variety of conditions with specific emphasis on protein synthesis and cell division. Additional analyses for particulate organic carbon and nitrogen

and ^{14}C-glutamate metabolism are made to illustrate the potential variability of cellular composition as a function of environmental perturbations of both physiological and ecological relevance.

METHODS

Culture Medium and Inoculation: The RLC-water medium described in Chapter 2 is used for all experiments. Medium is usually inoculated with late exponential or early stationary phase cells at a final density of $1\text{-}5x10^4$cells/ml. Washed cell suspensions in RLC-water are used as inocula when necessary to avoid nutrient carry-over. Immediately after the addition of radioisotope, zero time samples are removed for isotope blanks, and unlabeled samples for particulate organic carbon and nitrogen are filtered as described in Chapter 2. The cultures are incubated at room temperature (20-23°C) on a gyrotary shaker (New Brunswick Scientific, Edison, NJ) at 250 RPM.

Multiple Isotope Labeling: ^{14}C and ^{35}S emit β^- at nearly identical energies and are tedious to separate by dual labeling. Studies involving both isotopes are carried out in several flasks in the following manner. A large amount of culture medium is prepared, inoculated with cells at low density, and shaken for 10 minutes to evenly distribute the cells. Three portions are transferred into sterile flasks, one each receiving ^{14}C, ^{35}S, or distilled water. Additions are kept to less than 1% of the culture volume. The unlabeled flask provides material for CHN analysis, direct counts, and bulk protein.

Sampling: Sampling for all parameters usually begins at the first sign of tubidity (5-6x10^6cells/ml). Whole cell uptake of radioisotope is determined at intervals on 1 ml samples filtered through Whatman GF/F filters and usually accompanies fractionation samples (5ml). All isotope filtrations are washed three times with 0.5M NaCl and make use of the punch funnel described in Chapter 2. Whole cell uptake filters are placed directly in Aquasol; fractionation samples are placed in conical grinder tubes, wetted with 10 % TCA, and refrigerated until processing. Aliquots or appropriate dilutions in 0.2 μm filtered RLC-water are prepared for protein assay, direct and viable cell counts, acid volatile radioactivity, and CHN analysis as described in Chapter 2.

Direct count and bulk protein samples are routinely taken from the ^{14}C and ^{35}S flasks to ensure that equivalent growth occurs in all cultures. In a typical experiment (P. halodurans batch growth; Figure 4-1), paired samples from unlabeled and alternate labeled flasks agree with an average error of 4.5% for direct counts (n=10), 4.5% for viable counts (n=10), and 2.8% for bulk protein (n=5).

Details of specific activity, medium composition, inoculation density, etc. are included in the text for each experiment.

Calculations: The growth rate k (doublings/hour) for exponential increase in direct and viable cell counts, protein, or radioisotope metabolism is calculated by least-squares linear regression of (ln x vs. t) and division of the slope by ln 2. No correction factor for isotope discrimination is necessary for ^{35}S assimilation (McCready et al., 1975), and no

such data are available for ^{14}C-labeled organic compounds so no correction has been applied. Results for protein are usually either the avarage of two samples assayed in duplicate (single isotope experiments) or one sample each from the unlabeled and alternating labeled flasks (dual isotope experiments). Direct counts are either the result of single determinations (single isotope experiments) or single samples from two flasks as for protein (dual label experiments). Ratios among parameters are determined using the actual values rather than regression calculations.

Tables showing the distribution of radioisotope among the major biochemical fractions often include only representative sampling points to illustrate trends. The complete data set for each experiment os found in the Appendix.

Radiochemicals: Carrier-free $^{35}SO_4^=$ and UL-^{14}C-glutamic acid (>230mCi/mMole) are obtained from Amersham (Chicago, IL). Working solutions in distilled water are sterilized by autoclaving.

Other Chemicals: Chloramphenicol and reduced glutathione are purchased from Sigma (St. Louis, MO). Aquasol is produced by New England Nuclear (Boston, MA). All other chemicals are reagent grade.

RESULTS

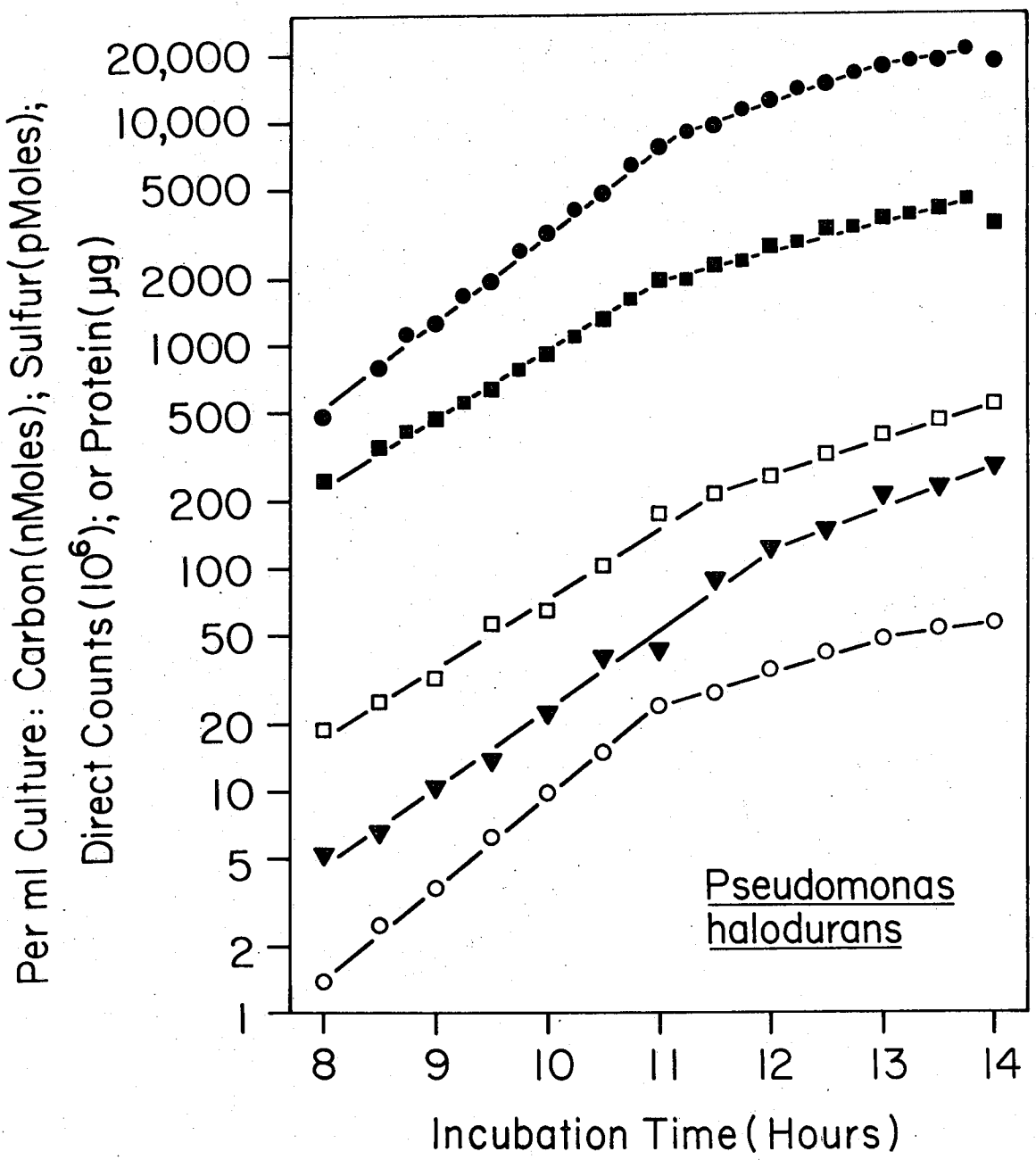
Batch Growth Physiology of P. halodurans

The rates of biosynthetic processes during mid-exponential phase growth of microorganisms in batch culture provide a basis for comparison

with measurements made on perturbed systems. In addition, ratios of the rates and absolute values of cell density, protein synthesis, respiration, and carbon and sulfur metabolism are important indices of growth physiology. Therefore a comprehensive investigation of unperturbed batch growth was made, with measurement of direct and viable cell counts, total protein, metabolism of glutamate and sulfate, and particulate organic carbon and nitrogen. Although the work presented in this thesis is directed towards an understanding of the relationship between sulfur metabolism and protein synthesis, the inclusion of carbon metabolism studies in the experiments described below provides data for comparison with whole cell microbial C:S uptake ratios previously reported (Monheimer, 1974; Jordan and Peterson, 1978). Similar experiments were undertaken with P. halodurans and A. luteo-violaceus.

Major biomass parameters for P. halodurans are shown in Figure 4-1; acid volatile ^{14}C values are very similar to total carbon incorporation and have been drawn one log scale lower for visual clarity. It is immediately apparent that balanced growth, defined as an identical rate of increase in all biomass and energetics parameters, is not obtained with this organism in batch culture. Bulk protein and total sulfur assimilation increase at rates substantially greater than the cell count during the early exponential phase, whereas total carbon assimilation and respiration are somewhat slower. These differences lead to a continuous increase in the total protein and sulfur per cell during exponential growth, with a decrease in the total carbon per cell of smaller magnitude. As a result, the cumulative carbon to sulfur ratio decreases from an initial maximum value of 175 (weight:weight) at 8 hours to 90 at 11 hours of growth.

Figure 4-1. Growth, protein synthesis, carbon and sulfur assimilation by Pseudomonas halodurans during batch growth. An overnight culture in complete medium containing 5mM glutamate and 1mM sulfate was inoculated into 1500ml of fresh medium at a final density of about $1x10^4$cells/ml. After shaking for 10 minutes, the culture was aseptically divided into three portions: individual flasks received either $^{35}SO_4^=$ (final specific activity 3 DPM/pMole), UL-^{14}C-glutamic acid (final specific activity 41 DPM/nMole), or distilled water. Aliquots were withdrawn at intervals for total protein (○), direct cell counts (▼), acid volatile ^{14}C (□ , shown x0.1 for clarity), total ^{14}C assimilated (■), and total ^{35}S assimilated (●). Larger symbols for assimilated ^{14}C and ^{35}S represent the sum of the biochemical fractions (Figure 4-2); smaller symbols are filtered cells counted unprocessed.

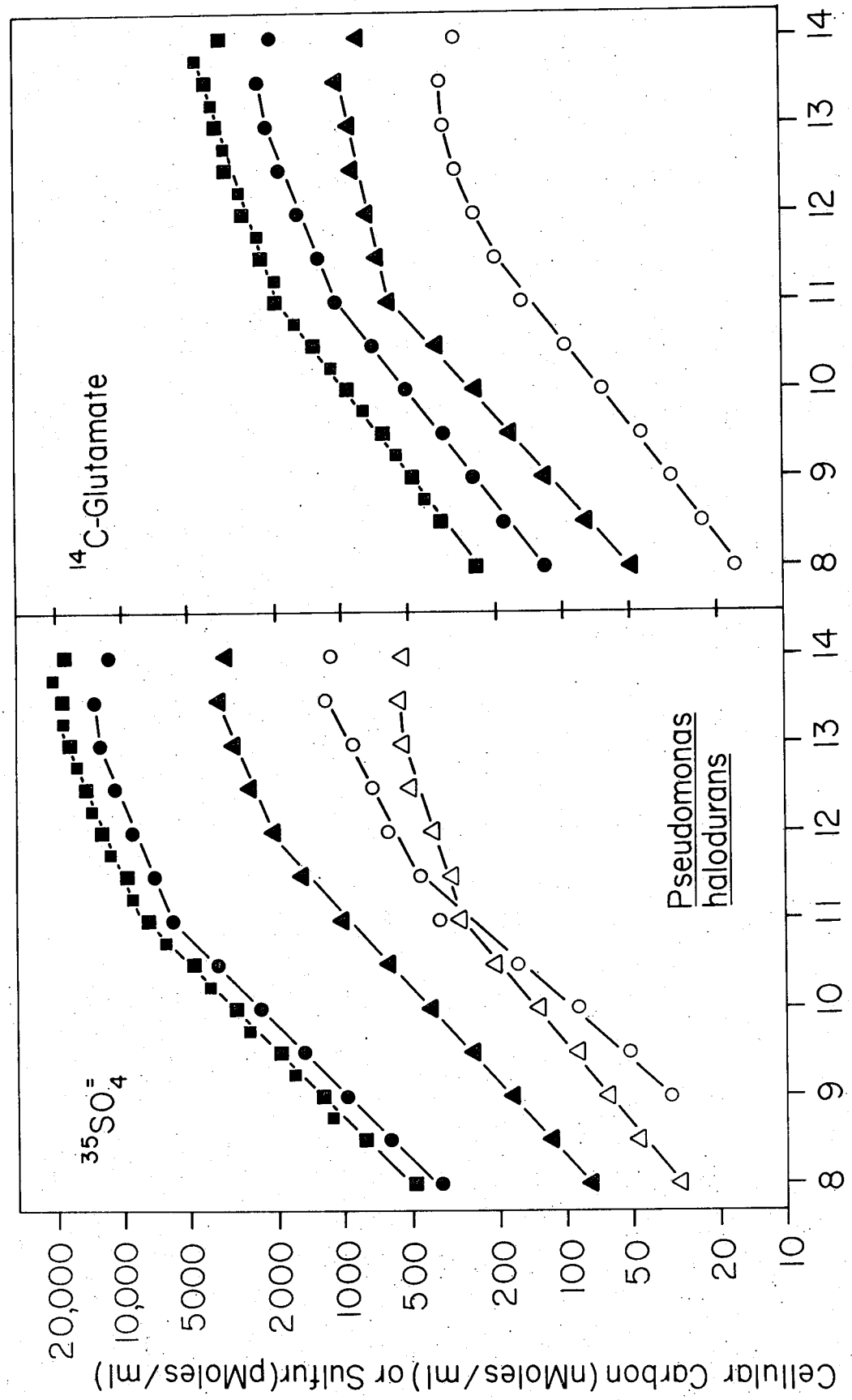

Per ml Culture: Carbon(nMoles); Sulfur(pMoles);
Direct Counts(10^6); or Protein(μg)
20,000
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
1
8
9
10
11
12
13
14
Incubation Time(Hours)
Pseudomonas
halodurans

A distinct break in carbon and sulfur assimilation and bulk protein synthesis occurs at 11 hours, at least one generation before any change in growth rate is detected in direct cell counts. This period of secondary exponential growth occurs at about half the initial growth rate and continues for an additional two hours. Less than 1% of the sulfate had been incorporated and only 20 % of the glutamate had been metabolized (incorporation + respiration) at 11 hours. A later experiment indicates that the glutamate concentration was not growth-limiting in this experiment.

The distribution of carbon and sulfur in major biochemical fractions is shown in Figure 4-2. Protein is the dominant sulfur-containing component, followed by low molecular weight (L.M.W.) organic sulfur compounds. The sum of these two fractions accounts for over 90 % of the total cellular sulfur. Alcohol soluble sulfur is less than 5% of the total, and is composed primarily of protein (Roberts et al., 1963; Chapter 5), and may consist of more hydrophobic proteins such as those found in membranes. Hot TCA soluble sulfur is usually found in low proportions (less than 5% of the total S) and follows virtually identical trends to the protein sulfur. Sulfur-containing RNA species have been identified in E. coli (Carbon et al., 1965; Lipsett, 1965) and would be found in this fraction. Later experiments suggest that this fraction also contains acid labile protein (Table A-10; Chapter 5). Inorganic sulfate is virtually absent (less than 1% of the total S), and no significant amount of sulfur is found in the lipid fraction (not shown for this experiment).

Protein-S, hot TCA soluble-S, and alcohol soluble-S follow the same pattern of incorporation with an abrupt rate decrease at 11 hours of incubation. In contrast, L.M.W. organic-S continues to increase at the in-

Figure 4-2. Distribution of carbon and sulfur in major biochemical fractions of P. halodurans during batch growth. Experimental details are found in Figure 4-1. Symbols, both panels: total uptake, ■ ; residue protein, ● ; L.M.W., ▲ . $SO_4^=$ panel: hot TCA soluble, △ ; alcohol soluble, ○ . Glutamate panel: lipid, ○ .


$^{35}SO_4^=$
^{14}C-Glutamate
Pseudomonas halodurans
Cellular Carbon (nMoles/ml) or Sulfur (pMoles/ml)
20,000
10,000
5000
2000
1000
500
200
100
50
20
10
8
9
10
11
12
13
14
Incubation Time (Hours)

itial rate for over one more generation, indicating a minor synthesis of precursors in excess of growth requirements during the onset of the stationary phase.

The distribution of carbon in P. halodurans is qualitatively similar to that of sulfur, with protein as the dominant component. Carbon is more widely distributed among the fractions, however. A substantial amount of carbon is found in lipids, but very little (less than 1%) in the alcohol soluble material. Hot TCA soluble carbon (not shown) decreased rapidly as a % of the total carbon and was not related to growth. This complex fraction contains both RNA and DNA as well as carbon storage polymers, and it is therefore not surprising that a large degree of variability is observed.

Protein carbon follows the same trend as protein sulfur, with a sharp rate break at 11 hours. Lipid carbon most closely resembles the direct cell count trend, as expected of a structural component more closely related to cell size than metabolic state. Cell size varies little in this organism except under extremes of salinity stress or sulfur starvation. In strict contrast to sulfur-labeled cells, the synthesis of L.M.W. carbon compounds occurs at a rate faster than the other parameters during early exponential growth, then decreases by over 5-fold at 11 hours.

The distribution of carbon and sulfur as a % of the total assimilated label for 9, 11, and 13 hours of growth is shown in Table 4-1. The entire data set is found in the Appendix (Tables A-1 and A-2). L.M.W. carbon reaches a maximum in the late exponential phase of growth, whereas L.M.W. organic sulfur increases towards the end of the growth cycle. The changes in the L.M.W. pools are reflected in the protein fraction, which

Table 4-1. Total Uptake of $^{35}SO_4^=$ and ^{14}C-Glutamate and Their Distribution in Biochemical Fractions of Pseudomonas halodurans During Batch Growth[a]

Hours of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		$^{35}SO_4^=$			
9	1245.1	13.8	2.7	5.1	78.4
11	7626.0	13.1	4.8	3.8	78.3
13	17,388.8	17.9	5.2	3.0	73.9

Hours of Incubation	nMoles C per ml	% of Total Radioactivity				
		L.M.W.	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
		^{14}C-Glutamate				
9	463.5	25.5	0	6.8	14.1	53.5
11	1945.9	30.5	0.4	7.7	8.8	52.7
13	3632.0	24.3	0.6	9.2	7.6	58.3

[a]Data from Figure 4-2.

accounts for a smaller proportion of the total sulfur during the late exponential phase when protein carbon is reaching its maximum value. The distribution of sulfur in the various fractions during unperturbed growth provides a basis for comparison with later experiments, as the proportion of sulfur in the different components is often a more sensitive indicator of stress than the total amount of sulfur assimilation.

Rate constants (expressed as doublings per hour, k) were calculated for the two apparently exponential growth periods for all parameters (Table 4-2). A small difference in the colony forming unit (CFU) rate can be attributed to decreasing numbers of dividing pairs (each pair forming one CFU), which declined from an average proportion during exponential growth of 9-15% to less than 2% at 14 hours, at which time the viable count: direct count ratio was 0.996. Although balanced growth was not obtained, a striking similarity is observed between the rates determined for incorporation of sulfate into protein (1.33 hr^{-1}) and bulk protein synthesis (1.35 hr^{-1}) during the primary exponential phase. The increase in protein carbon is considerably less (1.02 hr^{-1}). The same degree of reduction in rates occurred at 11 hours for bulk protein and protein-S, with similar secondary growth rates for protein-S (0.56 hr^{-1}), protein-C (0.54 hr^{-1}), and bulk protein (0.52 hr^{-1}). The dissimilarity of the rates of bulk protein synthesis and increase in cell numbers emphasizes the close relationship between sulfate incorporation into protein and bulk protein synthesis. This can be most clearly seen in the calculation of ratios among cell numbers, bulk protein, total and protein sulfur.

Table 4-2. Exponential Rate Constants During Batch Growth of *Pseudomonas halodurans*[a]

Parameter	Primary Exponential Period[b]				Secondary Exponential Period			
	n	k (hr^{-1})	r	Interval	n	k (hr^{-1})	r	Interval
				^{35}S-Sulfate				
Whole Cell S	12	1.31	0.9986	8 - 11	9	0.59	0.9953	11 - 13
LMW Organic S	8	1.23	0.9996	8 - 11.5	4	0.70	0.9907	11.5 - 13
EtOH Soluble S	6	1.60	0.9918	9 - 11.5	5	0.68	0.9935	11.5 - 13.5
Hot TCA Soluble S	7	1.08	0.9988	8 - 11	5	0.46	0.9870	11 - 13
Protein S	7	1.33	0.9997	8 - 11	5	0.56	0.9978	11 - 13
				^{14}C-Glutamate				
Whole Cell C	12	0.99	0.9988	8 - 11	12	0.44	0.9915	11 - 13.75
LMW C	7	1.16	0.9987	8 - 11	5	0.31	0.9862	11 - 13
Lipid C	7	1.06	0.9981	8 - 11	5	0.58	0.9903	11 - 13
Hot TCA Soluble C	5	0.73	0.9925	9 - 11	5	0.40	0.9645	11 - 13
Protein C	7	1.02	0.9994	8 - 11	5	0.54	0.9964	11 - 13
Acid Volatile C	7	0.95	0.9756	8 - 11	7	0.55	0.9984	11 - 14

Table 4-2. (Continued)

Parameter	Primary Exponential Period[b] n	k (hr^{-1})	r	Interval	Secondary Exponential Period n	k (hr^{-1})	r	Interval
				Biomass Parameters				
Direct Counts	9	1.17	0.9948	8 - 12	5	0.62	0.9846	12 - 14
Colony Forming Units	5	1.23	0.9963	8 - 12	3	0.68	0.9996	12 - 14
Bulk Protein	7	1.35	0.9993	8 - 11	5	0.52	0.9960	11 - 13
Mean (14 components)		1.16				0.55		
Standard Deviation		0.21				0.11		
C. V. (%)		18.3				20.7		

[a]Data from Figure 4-1 and 4-2.

[b]An abrupt break in growth rate at 11 hours was followed by a secondary exponential growth period of slower rate. Separate regressions for each period are presented. The correlation coefficient, r, of the least-squares linear regression of ln y vs. t is given for the interval (hours of growth) shown.

A small but significant amount (5-10 %) of the total protein is solubilized by the warm alcohol treatment, and affects the calculation of the weight % of S in the bulk protein. Since the amount of protein recovered in the protein residue is less than the total protein determined on samples of the cell suspension, the calculated weight % S in protein will be lower than the true value. However, a major aim of this work is to apply measurement of sulfur incorporation into protein to natural bacterial populations in seawater. Due to the high concentration of sulfate in seawater and the attendant low sensitivity of the method, it is unlikely that alcohol soluble protein will be detectable by labeling with sulfate. Therefore it is necessary to define an operational relationship between sulfate assimilation into residue protein and total protein synthesis as well as to find its absolute value. The fractionation procedure as described in Chapter 2 is quite useful with natural populations of marine bacteria (Chapter 5) and leads to an emphasis on the residue protein-S: total protein ratio as an operational definition. In most cases, the amount of protein in the fractionation residue is too small to be assayed conveniently, but examples are shown in Table 4-3 and other experiments where the assay of protein in the radio-labeled residue permitted calculation of the true weight % S in protein.

On a per cell basis, bulk protein increases throughout the primary exponential growth phase to reach a maximum of over 50 $\mu g/10^8$ cells at 11 hours. The decline in the bulk protein synthesis rate at this time leads to a continuing decrease in bulk protein per cell to the end of the experiment. This trend is identical for total sulfur. However, when sulfur incorporated into protein is normalized to either bulk protein or residue protein, the ratio is found to be constant throughout the growth

Table 4-3. Cell Number, Protein, and Sulfur Relationships for *Pseudomonas halodurans* During Batch Growth[a]

Time (Hours)	per ml Culture					per 10^8 cells		Weight % S	
	Cell Number	µg Protein		ng Sulfur		Protein (µg)	Total S (ng)	Protein S / Bulk	Protein S / Residue
		Bulk	Residue	Total	Residue				
8	5.17×10^6	1.4	1.2	15.3	11.6	26.9	296.8	0.84	0.97
8.5	6.53×10^6	2.5	1.8	25.5	19.9	38.0	390.2	0.80	1.08
9	1.04×10^7	3.7	3.1	39.9	31.3	35.2	383.9	0.86	1.02
9.5	1.38×10^7	6.2	5.2	61.4	48.6	44.8	444.6	0.79	0.93
10	2.23×10^7	9.7	8.2	96.7	76.9	43.5	433.5	0.79	0.94
10.5	3.94×10^7	14.9	12.3	152.0	120.5	37.7	385.9	0.81	0.98
11	4.27×10^7	24.0	18.8	244.5	191.4	56.1	572.6	0.70	0.80
11.5	8.83×10^7	27.4	24.0	304.5	231.6	31.1	344.9	0.66	0.81
12	1.21×10^8	35.1	28.5	392.4	293.8	29.0	324.3	0.71	0.84
12.5	1.47×10^8	41.4	34.8	473.6	349.7	28.2	322.2	0.72	0.84
13	2.10×10^8	48.3	41.6	557.6	412.2	23.0	265.5	0.77	0.95
13.5	2.28×10^8	53.4	43.6	602.6	430.3	23.4	264.3	0.81	0.99
14	2.82×10^8	56.4	41.2	582.8	422.2	20.0	206.7	0.75	1.03

Table 4-3. (Continued)

Time (Hours)	per ml Culture: Cell Number	µg Protein: Bulk	µg Protein: Residue	ng Sulfur: Total	ng Sulfur: Residue	per 10^8 cells: Protein (µg)	per 10^8 cells: Total S (ng)	Weight % S: Protein S / Bulk	Weight % S: Protein S / Residue
15	2.33×10^8	65.0	37.4	522.8	381.1	27.9	224.4	0.59	1.02
16	2.96×10^8	76.3	41.0	579.3	422.2	25.8	195.7	0.55	1.03
18	4.52×10^8	93.4	37.3	537.2	394.8	20.7	118.9	0.42	1.06
20	4.82×10^8	94.9	57.8	764.2	565.3	19.7	158.5	0.60	0.98
22	4.41×10^8	86.4	72.7	1145.1	854.7	19.6	259.7	0.99	1.18
24	5.01×10^8	82.9	80.3	1202.2	904.9	16.5	240.0	1.09	1.13
				8-14 hours	Mean (n=13)		356.6	0.77	0.94
					S. D.		95.0	0.06	0.09
					C. V. (%)		26.6	7.6	9.5
				All data	Mean (n=19)	29.8	307.0	0.75	0.98
					S. D.	10.4	111.5	0.15	0.10
					C. V. (%)	34.8	36.3	20.3	10.6

[a]Data from Figures 4-1 and 4-2.

curve. This indicates a constant protein composition in terms of the weight % S during unperturbed growth, even though the total protein per cell ranged over three-fold.

This experiment provides the first opportunity for practical application of the fractionation procedure to studies of microbial growth in this work. Although the data are drawn only for the period 8-14 hours in Figures 4-1 and 4-2, the experiment actually went on well into the stationary phase (24 hours of incubation). However, at the 14 hour point, a new box of Reeve Angel 984H filters from a new lot was opened for filtration of the fractionation samples. A separate dish contained the filters for whole cell isotope uptake, remaining from the old lot. After fractionation of the samples, it was clear that unusually low recovery based on whole cell uptake was achieved for most samples after 13.5 hours, and inspection of the new lot revealed a large proportion of apparently cracked filters. Since the total protein is determined on the whole cell suspension by direct precipitation, this resulted in artificially low weight % sulfur in protein when determined as residue protein-S:total protein in all but the last two samples, when recovery was normal (greater than 95%). In spite of this, the absolute value (residue protein-S:residue protein) and the distribution of carbon and sulfur in the samples was normal (Tables 4-3, A-1, and A-2), confirming the loss of whole cells (as would be expected of a cracked filter) rather than rupture of cells on the filter surface, which would disproportionately affect the L.M.W. organic component. A recovery factor could be rationalized under these circumstances, but was not applied. The company subsequently investigated their inventory, found a large proportion of similar

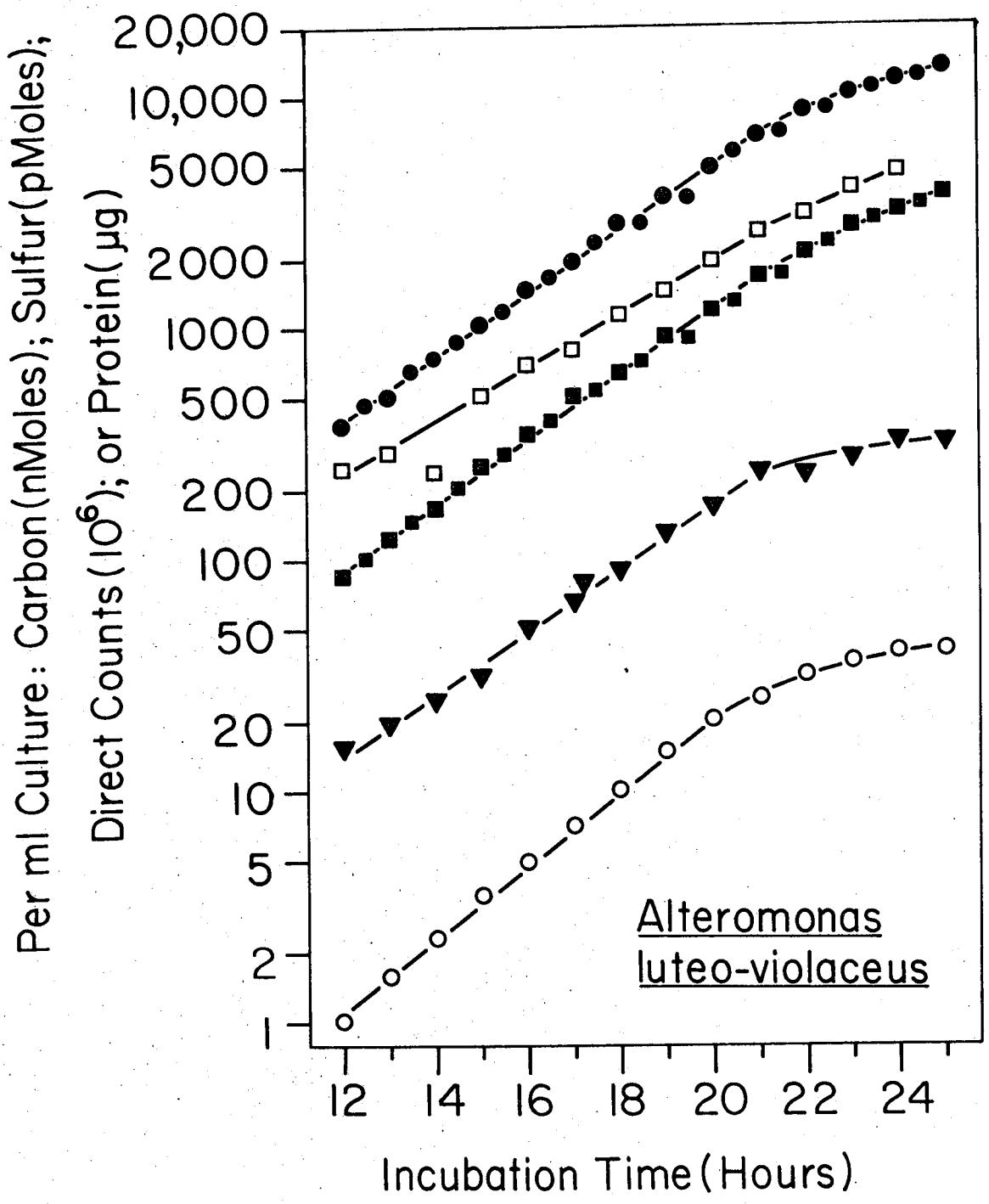
boxes, and discontinued the line in favor of the Whatman GF/F glass fiber filter, which turned out to be better in many respects and was used for most of the work in this and the next chapter.

Using only the data from the period 8-14 hours, the coefficient of variation around the mean for the operational value of residue protein-S: total protein is only 7.6% (0.77 ± 0.06 weight %). Furthermore, the mean value for the absolute weight % (residue protein-S:residue protein) is 0.94 ± 0.09, in good agreement with the value of 1.1% predicted by the "average protein" (Jukes et al., 1975; Reeck and Fisher, 1973). The absolute weight % carbon (residue protein-C:residue protein) was not determined, but must be higher than the residue protein-C:total protein ratio of 64.3 ± 17.1%, supporting the hypothesis that non-protein carbon is found in the residue. For comparison with other experiments, the mean ratios (with standard deviation and % variation around the mean) for pertinent parameters are: C:S, 103.5 ± 33.7 (32.5%); C:N, 3.0 ± 0.1 (29.4%); and carbon metabolism (incorporation + respiration):incorporation, 1.93 ± 0.23 (12.0 %).

Batch Growth Physiology of A. luteo-violaceus

A similar experiment performed with Alteromonas luteo-violaceus revealed several important differences in the basic growth habits of the two microorganisms. The data for whole cell carbon and sulfur assimilation, respiration, direct cell counts, and bulk protein synthesis are shown in Figure 4-3. Viable cell counts were not made during this experiment. The most notable difference is the absence of a break in any bio-

Figure 4-3. Growth, protein synthesis, carbon and sulfur assimilation by Alteromonas luteo-violaceus during batch growth. An overnight culture in complete medium containing 5mM glutamate and 500 μM sulfate was inoculated into 1500ml of fresh medium at a final density of about $2x10^5$cells/ml. After shaking for 10 minutes, the culture was aseptically divided into three portions: individual flasks received either $^{35}SO_4^=$ (final specific activity 4.5 DPM/pMole), UL-^{14}C-glutamic acid (final specific activity 55 DPM/nMole), or distilled water. Aliquots were withdrawn at intervals for total protein (○), direct cell counts (▼), acid volatile ^{14}C (□), total ^{14}C assimilated (■), and total ^{35}S assimilated (●). Larger symbols for assimilated ^{14}C and ^{35}S represent the sum of the biochemical fractions (Figure 4-4); smaller symbols are filtered cells counted unprocessed.


Per ml Culture: Carbon(nMoles); Sulfur(pMoles);
Direct Counts(10^6); or Protein(μg)
20,000
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
1
12
14
16
18
20
22
24
Incubation Time(Hours)
Alteromonas
luteo-violaceus

synthetic rate before the onset of the stationary phase determined from direct cell counts. Furthermore, all biosynthetic parameters increase at similar rates (mean 0.49 ± 0.07 hr^{-1}), although respiration is considerably slower (0.39 hr^{-1}).

Subcellular fractionation of the labeled cells demonstrates that the incorporation patterns for carbon and sulfur are very similar to those observed for P. halodurans (Figure 4-4). Protein is again the dominant component for both elements, followed by L.M.W. material. These two fractions account for 90 % of the total cellular sulfur and 70 % of the total carbon. The rate of increase (hr^{-1}) of alcohol soluble sulfur (0.66) is faster than the direct count rate (0.45), but is balanced by a slightly slower rate of increase in the hot TCA soluble fraction (0.42). The hot TCA soluble carbon of A. luteo-violaceus follows growth well and accounts for a substantial portion of the total carbon, being only slightly less than the L.M.W. component: it has been drawn one log scale lower for clarity.

The distribution of carbon and sulfur for 12, 18, and 24 hours are found in Table 4-4; the entire data set is appended in Tables A-3 and A-4. Very small amounts of sulfur are found as either inorganic sulfate or lipid material, as with P. halodurans. The beginning of the stationary phase is signalled by an increase in the proportion of L.M.W. organic sulfur and concommittant decrease in the relative proportion in protein, but no significant changes occur in the distribution of carbon.

Balanced growth in batch culture is not obtained with A. luteo-violaceus, but a much closer agreement among biosynthetic rates is observed (Table 4-5). In fact, the protein carbon and sulfur, L.M.W. carbon and sulfur, hot TCA soluble carbon and sulfur, and total carbon and sulfur

Figure 4-4. Distribution of carbon and sulfur in major biochemical fractions of A. luteo-violaceus during batch growth. Experimental details are found in Figure 4-3. Symbols, both panels: total uptake, ■ ; residue protein, ● ; L.M.W., ▲ ; hot TCA soluble, △ (shown x0.1 for glutamate). $SO_4^=$ panel: alcohol soluble, ○ . Glutamate panel: lipid, ○ .

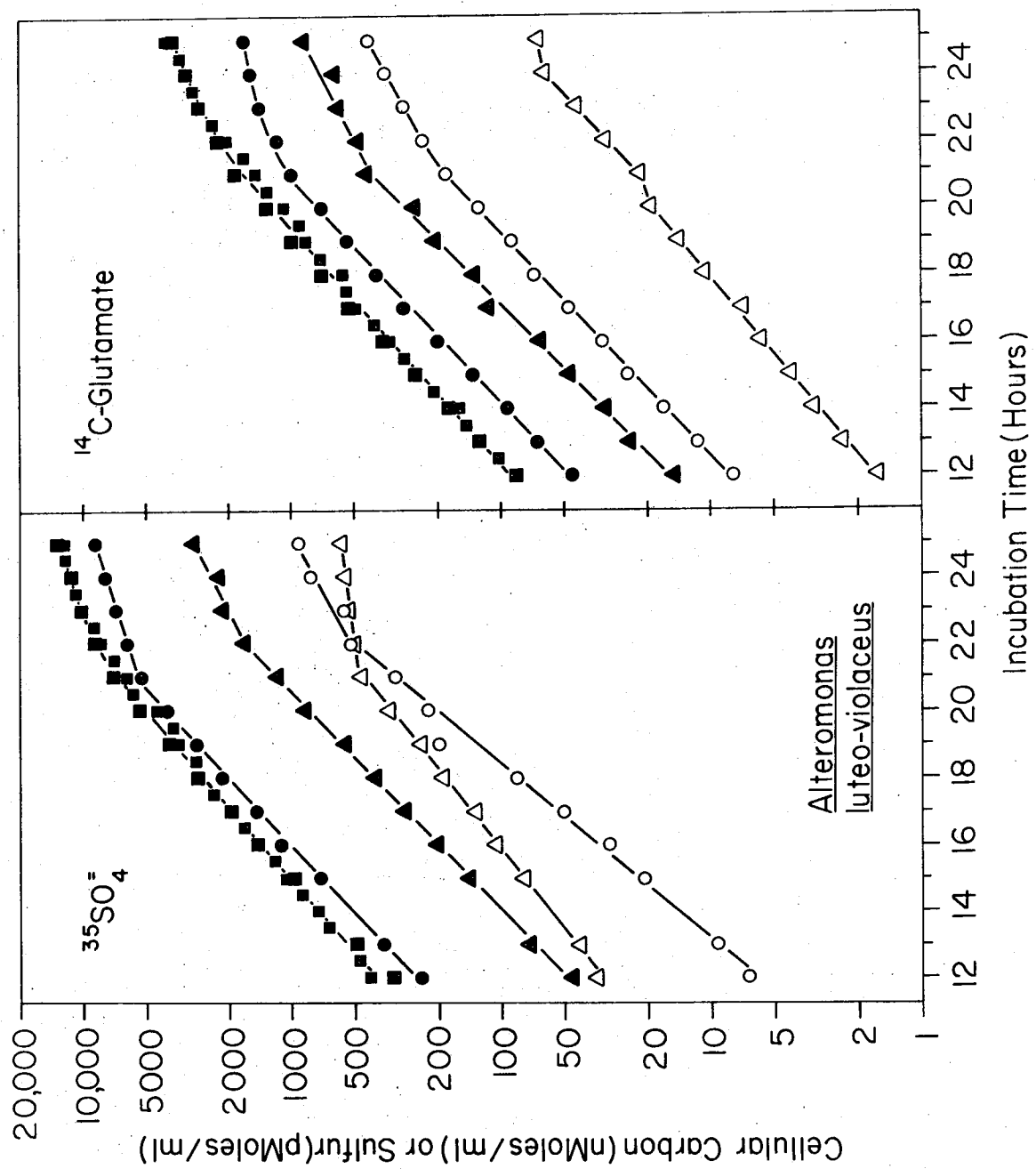

$^{35}SO_4^=$
^{14}C-Glutamate
Alteromonas luteo-violaceus
Cellular Carbon (nMoles/ml) or Sulfur (pMoles/ml)
Incubation Time (Hours)
20,000
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
1
12 14 16 18 20 22 24

Table 4-4. Total Uptake of $^{35}SO_4^=$ and ^{14}C-Glutamate and Their Distribution in Major Biochemical Fractions of Alteromonas luteo-violaceus During Batch Growth[a]

Hours of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		$^{35}SO_4^=$			
12	327.1	14.0	2.0	10.6	73.4
18	2801.2	14.2	3.0	6.8	76.0
24	11,485.6	19.6	7.0	4.8	68.6

Hours of Incubation	nMoles C per ml	% of Total Radioactivity				
		L.M.W.	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
		^{14}C-glutamate				
12	85.5	17.2	2.0	9.0	18.7	53.1
18	700.9	18.9	1.5	9.8	15.1	54.8
24	3181.0	19.3	2.0	11.1	19.2	48.4

[a]Data from Figure 4-4.

Table 4-5. Exponential Rate Constants During Batch Growth of *Alteromonas luteo-violaceus*[a]

Parameter	n	k (hr^{-1})	r
Direct counts	11	0.45	0.9971
Bulk Protein	10	0.52	0.9980
Whole Cell ^{35}S	28	0.46	0.9969
LMW Organic ^{35}S	9	0.51	0.9994
EtOH Soluble ^{35}S	9	0.66	0.9936
Hot TCA Soluble ^{35}S	9	0.42	0.9991
Protein ^{35}S	9	0.49	0.9988
Whole Cell ^{14}C	29	0.47	0.9963
LMW ^{14}C	10	0.52	0.9974
Lipid ^{14}C	10	0.50	0.9993
Hot TCA Soluble ^{14}C[b]	9	0.44	0.9986
Protein ^{14}C	10	0.49	0.9990
Acid Volatile ^{14}C[c]	9	0.39	0.9985
Mean (13 parameters)		0.49	
Standard Deviation		0.07	
C.V. (%)		13.5	

[a]Data from Figures 4-3 and 4-4 in the text; linear regression of ln y vs. t for the period 12-21 hours.

[b]12-20 hours only.

[c]14 hour point omitted.

assimilation agree with one another within an error of 5%. Most of the observed variation is due to low respiration and high alcohol soluble sulfur rates.

The similarity of biosynthetic rates for A. luteo-violaceus results in a relatively constant cellular composition, compiled in Table 4-6. Bulk protein and total assimilated sulfur per cell remain constant throughout exponential growth, but increase a little over 20 % in the stationary phase. The weight % sulfur in protein is likewise relatively constant for all samples. The operational weight % sulfur in protein (residue protein-S:total protein) is considerably lower for A. luteo-violaceus than for P. halodurans (0.66 vs. 0.77%), but the true weight % (residue protein-S:residue protein) is similar for the two bacteria (1.02 vs. 0.98). Other pertinent ratios derived from Figures 4-3 and 4-4 include: C:S (weight:weight), 90.0 ± 8.9 (9.9%), C:N (weight:weight), 4.49 ± 0.92 (20.5%), and M/I, 2.88 ± 0.42 (14.6%). The C:S ratio is slightly lower for A. luteo-violaceus, whereas the C:N ratio and the energetic cost of biosynthesis (M/I) are much higher.

In most cases, A. luteo-violaceus has a lower plating efficiency (viable count:direct count about 0.7) than P. halodurans, which may be a result of a protease which stimulates autolysis of cultures during stationary phases. The strong proteolytic activity increases the error associated with most measurements made with this organism, and has severe effects on the subcellular distribution of sulfur in some cases. This is demonstrated in the end-point samples (40 hours of incubation) shown at the bottom of Table A-3. Two samples were taken; one filtered in the usual way but stored at 4°C unfixed for two days. The other sample was centrifuged, rinsed with basal salts, and fixed with 10 % TCA. The re-

Table 4-6. Cell Number, Protein, and Sulfur Relationships for Alteromonas luteo-violaceus During Batch Growth[a]

Time (Hours)	per ml Culture					per 10^8 cells		Weight % S	
	Cell Number	µg Protein Bulk	µg Protein Residue	ng Sulfur Total	ng Sulfur Residue	Protein (µg)	Total S (ng)	Protein S / Bulk	Protein S / Residue
12	1.53×10^7	1.0	-	10.5	7.7	6.6	68.5	0.77	-
13	1.92×10^7	1.6	-	15.5	11.6	8.2	80.9	0.72	-
14	2.45×10^7	2.3	-	-	-	9.5	-	-	-
15	3.10×10^7	3.6	-	30.9	23.2	11.5	99.8	0.64	-
16	5.01×10^7	5.0	-	46.3	35.4	10.0	92.3	0.71	-
17	6.53×10^7	7.1	-	62.0	46.9	10.9	94.9	0.66	-
18	8.79×10^7	10.3	-	89.8	68.3	11.8	102.2	0.66	-
19	1.29×10^8	15.0	-	122.7	90.9	11.7	95.1	0.61	-
20	1.67×10^8	20.6	10.5	171.5	126.0	12.3	102.7	0.61	1.20
21	2.37×10^8	25.5	15.2	230.0	167.3	10.8	97.1	0.66	1.10
22	2.28×10^8	31.8	20.8	284.2	197.5	14.0	124.6	0.62	0.95
23	2.72×10^8	36.6	23.1	326.5	224.1	13.5	120.1	0.61	0.97
24	3.19×10^8	40.3	26.4	368.3	252.8	12.6	115.4	0.63	0.96
25	3.16×10^8	40.9	30.1	423.8	280.7	13.0	134.1	0.69	0.93
					Mean	11.2	102.1	0.66	1.02
					S.D.	2.1	17.9	0.05	0.11
					C.V. (%)	4.4	17.5	7.5	10.5

[a]Data from Figures 4-3 and 4-4.

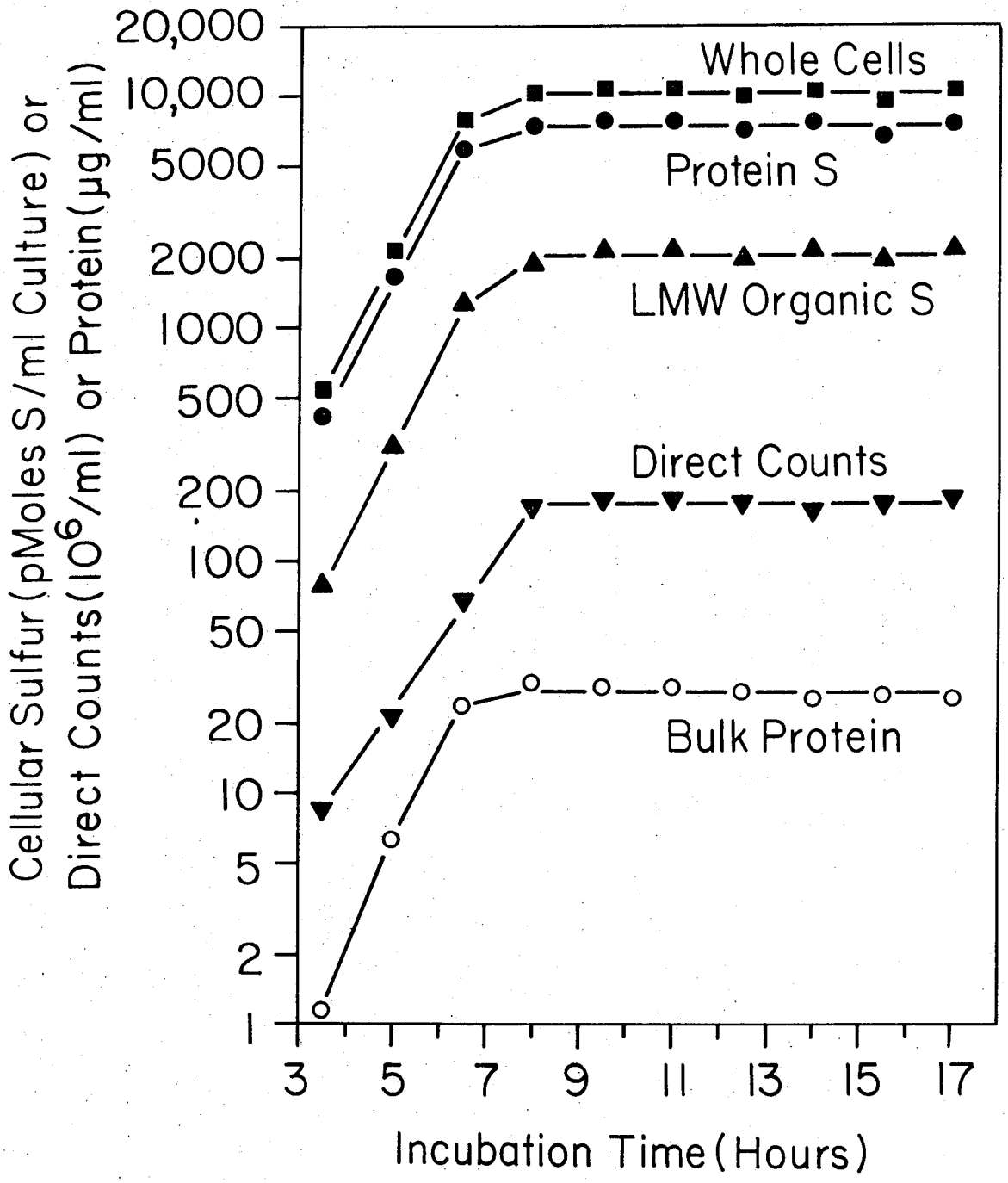
frigerated sample shows a nearly 4-fold decrease in the protein-S, all of which appeared in L.M.W. material. The centrifuged sample was similar, but the extent of protein degradation was substantially less. The short time required to spin the cells down was apparently sufficient for some proteolytic activity to manifest itself as the cells ceased biosynthetic reactions due to anaerobiosis.

Carbon Limited Growth of P. halodurans

Carbon and energy sources are often thought to be major growth-limiting factors for bacteria in marine environments due to the naturally low concentrations of labile dissolved organic compounds in seawater. During the preparation of ^{14}C-labeled bacterial protein destined for use as amphipod bait (Jannasch et al., 1980) the opportunity arose to investigate the effect of carbon depletion from the medium on the residue protein-S: total protein ratio. In order to obtain the highest yield of radio-labeled protein from ^{14}C-glutamate, a high specific activity medium is required, from which all of the glutamate carbon can be removed. An additional flask labeled with $^{35}SO_4^=$ permits the comparison of carbon and sulfur metabolism during this pertinent form of nutritional stress.

Figure 4-5 shows total, L.M.W., and protein-S as well as direct counts and bulk protein for three points in exponential growth and several hours of a stationary phase induced by carbon limitation. At 9.5 hours, the total carbon metabolized was 4.70mM (as carbon), equal to 93.9% of the total available glutamate. Since the L.M.W. organic sulfur

Figure 4-5. Growth, protein synthesis, and the total uptake and distribution of sulfur in major biochemical fractions of P. halodurans during a carbon-limited stationary phase. An overnight culture in complete medium containing 1mM glutamate and 250 μM sulfate was inoculated into 1500ml of fresh medium at a final density of about $2x10^6$cells/ml. After shaking for 10 minutes, the culture was aseptically divided into three portions: individual flasks received either $^{35}SO_4^=$ (final specific activity 5 DPM/pMole), UL-^{14}C-glutamic acid (final specific activity 285 DPM/nMole), or distilled water.


Cellular Sulfur (pMoles S/ml Culture) or
Direct Counts (10^6/ml) or Protein (µg/ml)
20,000
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
1
3
5
7
9
11
13
15
17
Incubation Time (Hours)
Whole Cells
Protein S
LMW Organic S
Direct Counts
Bulk Protein

and the protein sulfur together constitute over 90 % of the total sulfur, only these fractions will be shown in this and subsequent experiments. The complete fractionation data set is appended in Tables A-5 and A-6.

It is again observed that all sulfur-containing fractions and bulk protein synthesis decline in rate about 1-1.5 generations before the very abrupt cessation of cell division. Protein sulfur is the dominant sulfur-containing fraction, and the distribution of both carbon and sulfur in the major biochemical fractions is similar to that for normal batch growth. The number of dividing pairs of cells observed during the direct counts declined continuously from 11% at 3.5 hours of incubation to 0 % at 9.5 hours.

The most apparent feature of carbon limitation is the absolute lack of effect on any of the measured parameters. The total sulfur and the bulk protein per cell are much lower than the values for normal batch growth, but remain quite stable during more than 7 hours of the carbon-limited stationary phase (Table 4-7). In contrast, the weight % sulfur, both operational and true ratios, are very similar to the values reported in the previous experiment, and also show no influence of carbon limitation. Additionally, the C:S ratio (101.5 ± 76.7) and the C:N ratio (3.06 ± 0.20) are in agreement with the unperturbed batch growth experiment. The M/I ratio (2.39 ± 0.28) is 24% higher, possibly reflecting continued respiration to meet basal energy requirements at the expense of endogenous carbon compounds.

The results of this experiment verify that carbon limitation was not responsible for the cessation of growth in the normal batch culture, since the direct counts increase exponentially at the normal growth rate (about 1 hr^{-1}) until nearly all the glutamate is exhausted.

Table 4-7. Cell Number, Protein, and Sulfur Relationships for Pseudomonas halodurans During a Carbon-Limited Stationary Phase[a]

Time (Hours)	per ml Culture					per 10^8 cells		Weight % S	
	Cell Number	µg Protein		ng Sulfur		Protein (µg)	Total S (ng)	Protein S / Bulk	Protein S / Residue
		Bulk	Residue	Total	Residue				
3.5	8.59×10^6	1.2	-	17.3	13.2	13.4	201.4	1.14	-
5	2.16×10^7	6.3	6.1	69.2	53.8	29.2	320.4	0.85	0.88
6.5	6.77×10^7	23.6	19.1	255.1	188.7	34.9	376.8	0.80	0.99
8	1.70×10^8	29.5	25.0	333.0	236.9	17.4	195.9	0.80	0.95
9.5	1.83×10^8	28.5	25.9	345.3	248.8	15.6	188.7	0.87	0.96
11	1.81×10^8	28.3	25.4	345.7	245.6	15.6	191.0	0.87	0.97
12.5	1.79×10^8	27.3	23.3	317.6	225.1	15.3	177.4	0.83	0.97
14	1.64×10^8	25.5	26.5	338.3	242.5	15.5	206.3	0.95	0.91
15.5	1.75×10^8	26.4	23.6	301.6	213.2	15.1	172.3	0.81	0.90
17	1.85×10^8	25.5	24.8	336.7	240.3	13.8	182.0	0.94	0.97
					Mean	18.6	221.2	0.89	0.94
					S.D.	7.3	69.2	0.10	0.04
					C.V. (%)	39.3	31.3	11.7	4.0

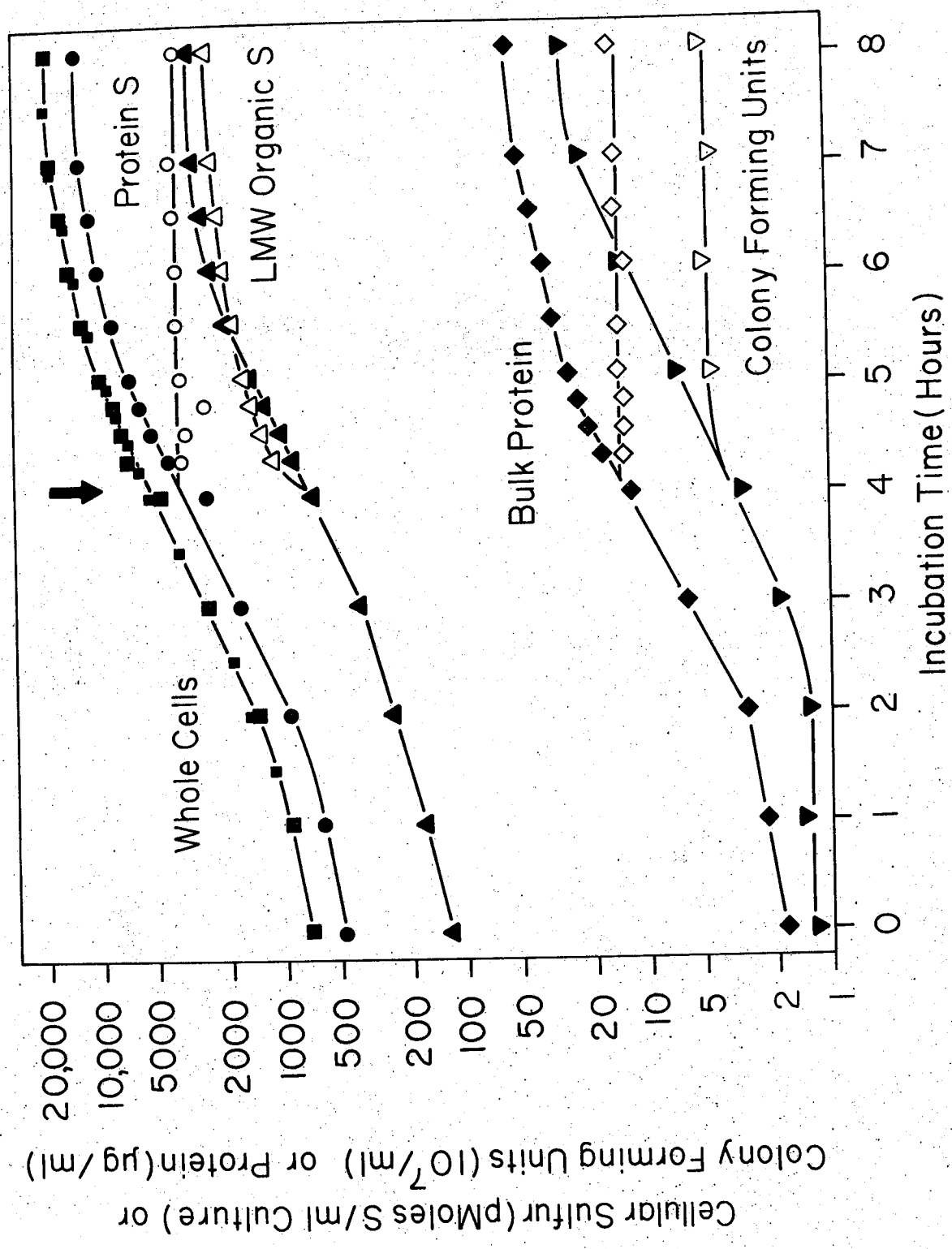
[a]Data from Figure 4-5.

Effects of Chloramphenicol on Growth, Protein Synthesis, and Sulfur Metabolism of P. halodurans and A. luteo-violaceus with a Note on Biosynthesis During Lag Phase Growth

The high percentage of the total cellular sulfur in protein indicates that this is the major sink for reduced sulfur. Abrupt termination of protein synthesis through the action of an external agent (e.g. antibiotic protein synthesis inhibitors) can provide information both on the incorporation of sulfur into protein and on the regulation of L.M.W. organic sulfur pools. Chloramphenicol (CAP) acts on the bacterial 50s ribosomal subunit, terminating protein chain elongation. As a bacteriostatic protein synthesis inhibitor with little effect on other cellular processes, CAP is an ideal agent for such an investigation.

The use of an inoculum previously labeled with $^{35}SO_4^{=}$ to equilibrium provides information on events during the lag phase of growth resulting from dilution of a stationary phase culture into fresh medium in addition to elucidation of the effects of CAP on growth, protein synthesis, and sulfur metabolism in P. halodurans. During the first two hours of incubation, no increase in viable or direct cell counts is observed, but protein synthesis and sulfate incorporation into all fractions indicate impending growth (Figure 4-6). Bulk protein increases by 60 % and total sulfur by 90 % during the lag phase. Sulfur incorporation and bulk protein synthesis then proceed at the exponential rate characteristic of growth in this medium, and increases in cell counts begin shortly thereafter.

Figure 4-6. Effects of chloramphenicol on growth, protein synthesis, and total uptake and distribution of sulfur in major biochemical fractions of P. halodurans. Cells were grown in complete medium containing 10mM glutamate and 1mM sulfate with $^{35}SO_4^{=}$ present (final specific activity 9 DPM/pMole) for 10 generations to obtain isotopic equilibrium. Shortly after the onset of the stationary phase, fresh medium of the same composition was inoculated to a final density of about $1x10^7$ cells/ml. Samples were withdrawn for direct and viable cell counts, total protein, and fractionation into major biochemical components. At 245 minutes, the culture was divided into two portions, one receiving CAP (25 μg/ml final concentration) and the other an equal amount of distilled water as a placebo; samples were taken for the same parameters for an additional 8 hours. Open symbols: CAP-treated; closed symbols: control culture.


Cellular Sulfur (pMoles S/ml Culture) or
Colony Forming Units (10^7/ml) or Protein (μg/ml)
20,000
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
1
Whole Cells
Protein S
LMW Organic S
Bulk Protein
Colony Forming Units
0
1
2
3
4
5
6
7
8
Incubation Time (Hours)

The control culture, grown in medium containing twice the amount of glutamate used in the medium for the batch growth experiment (Figure 4-1), closely resembles balanced growth for the period from 3-5 hours. Table 4-8 indicates the doubling times for the measured parameters and the correlation coefficients of the regressions used to determine them. The discrepancies among rates determined for bulk protein, direct counts, and sulfate incorporation into all fractions are much less pronounced than in experiments using lower substrate concentrations, and the pool size of the L.M.W. component is essentially constant for this period. However, the break in bulk protein synthesis rate characteristic of P. halodurans is observed, and the bulk protein and protein sulfur per cell are constant for only about one generation.

The culture was split in the mid-exponential phase and one portion was treated with chloramphenicol (25 μg/ml final concentration). An identical set of samples was taken from each flask at appropriate intervals. The data in Figure 4-6 reveal that an extremely rapid effect is exerted specifically on bulk protein synthesis and incorporation of sulfate into protein. Incorporation of sulfate into L.M.W. organic sulfur compounds does not respond in the same fashion. In fact the initial uptake of sulfate into this fraction is higher for CAP-treated cells than in the control culture. These effects are also manifest in the distribution of sulfur between the protein and L.M.W. fractions. Table 4-9 presents the proportion of the total S in the various fractions for the point just prior to CAP addition and for both control and CAP-treated cultures at 1 and 4 hours of incubation; the entire data set is appended in Table A-7. The proportion of the total sulfur in L.M.W. compounds doubles during the

Table 4-8. Exponential Rate Constants for Batch Growth of Pseudomonas halodurans: Control Culture for Chloramphenicol Inhibited Growth[a]

Parameter	n	k(hr^{-1})	r	Interval (min.)
Direct Counts	5	0.91	0.9970	180-425
Bulk Protein	7	1.06	0.9992	120-305
Whole cell ^{35}S	15	0.91	0.9971	120-305
LMW organic ^{35}S	7	0.93	0.9986	120-305
Protein ^{35}S	7	0.94	0.9888	120-305
Mean		0.95		
Standard Deviation		0.06		
C.V. (%)		6.6		

[a]Data from Figure 4-6.

Table 4-9. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans: Effects of Chloramphenicol[a]

Minutes of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		Batch Culture			
240	4484.2	14.8	24.0	4.3	56.9
		+ Placebo			
305	9672.7	14.8	14.1	2.5	68.6
485	19,136.0	16.4	13.0	2.6	68.0
		+Chloramphenicol			
305	6363.0	23.8	17.2	3.6	55.4
485	7562.6	31.9	16.5	2.9	48.7

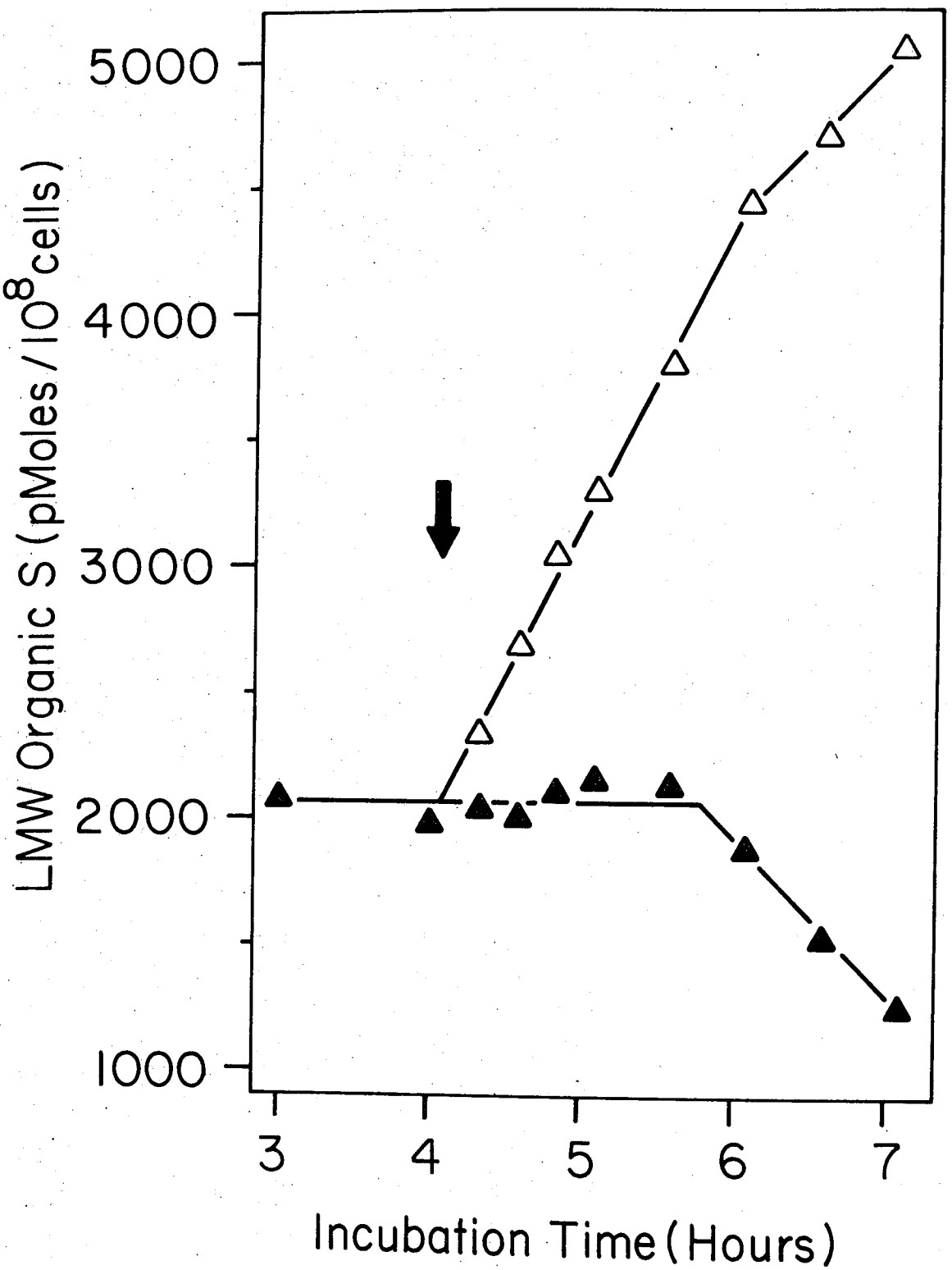
[a]Data from Figure 4-6. At 245 minutes the culture was split into two portions, one receiving CAP (25 µg/ml final concentration) and the other an equal amount of distilled water as a placebo.

course of CAP treatment, with a concommittant decline in residue protein sulfur. The amount of sulfur in the alcohol-ether soluble fraction is unusually high in this experiment, both in control and CAP-treated cultures, but is probably due to a slightly different pH of the extraction solution (Chapter 2)

It was not practical to take samples for direct counts and bulk protein at the exact time of CAP addition, but the onset of inhibition may be deduced from the intersection of the line describing the mean value of the CAP-treated bulk protein and residue protein-S with the regression line delineating the same parameters in the control culture. The line drawn for the mean value of residue protein-S in the CAP-treated culture intercepts the regression line for the control culture at 2 minutues after CAP addition. The effects of CAP on bulk protein synthesis also take place 2 minutes after addition. In contrast, the colony forming unit average intercepts its control at 24 minutes, suggesting that cells near to division are competent to complete the process. The fact that the CFU remains constant verifies the reversible, bacteriostatic nature of this antibiotic.

The data for the L.M.W. organic sulfur component shown in Figure 4-6 and Table 4-9 demonstrate a rapid initial pooling in this fraction. A much clearer view of the phenomenon may be obtained by comparing the size of the L.M.W. organic sulfur pool of control and CAP-treated cultures on a per cell basis, as shown in Figure 4-7. The L.M.W. organic sulfur content of P. halodurans in the control culture remains relatively constant during the exponential growth phase, but addition of CAP results in a linear increase in pool size to a value over twice that of the control

Figure 4-7. Effects of chloramphenicol on the L.M.W. organic sulfur pool in P. halodurans. Experimental details in Figure 4-6. Cell density was taken from the regression line through the direct cell count data when no actual count was made. The arrow indicates the time of CAP addition. Open symbols: CAP-treated; closed symbols: control culture.


5000
4000
3000
2000
1000
LMW Organic S (pMoles / 10^8 cells)
3
4
5
6
7
Incubation Time (Hours)

cells before any regulation of synthesis becomes manifest. The synthesis of the L.M.W. organic sulfur compounds is usually well coupled to cellular requirements for protein synthesis, but the termination of their utilization requires either several generation times or, more likely, the attainment of a certain intracellular concentration to stimulate regulatory processes.

If only whole cell uptake data had been taken, the action of CAP would have appeared to be rapid but incomplete (Figure 4-8); during the treatment period a net increase in total cellular sulfur of 68% occurred in CAP-treated cells. This represents only 79% inhibition relative to the control culture. The reason for the continued uptake of sulfate is, however, clear when samples from the control and CAP-treated cultures are fractionated into the major biochemical components.

The ratios of protein and total sulfur per cell for the control culture are similar to the previous experiments. CAP has no effect on the operational relationship between residue protein-S and total protein (Table 4-10). Fluctuations in bulk protein per cell are prevented by CAP treatment, but the total sulfur per cell increases as a result of the inhibition of protein synthesis. The whole cell uptake of sulfate has thus been uncoupled from growth, which demonstrates that the use of whole cell sulfate uptake is not necessarily an accurate indicator of bacterial growth.

The response of A. luteo-violaceus to chloramphenicol is strikingly different from that of P. halodurans and verifies the presence of a powerful extracellular proteolytic activity associated with this microorganism. Figure 4-9 shows that the addition of CAP to a mid-exponential

Figure 4-8. Effect of chloramphenicol on total sulfate uptake by P. halodurans. Experimental details in Figure 4-6. At the arrow, CAP (25 µg/ml final concentration) was added to one half of the culture. Open symbols indicate CAP-treated cells, closed symbols are from the control culture.

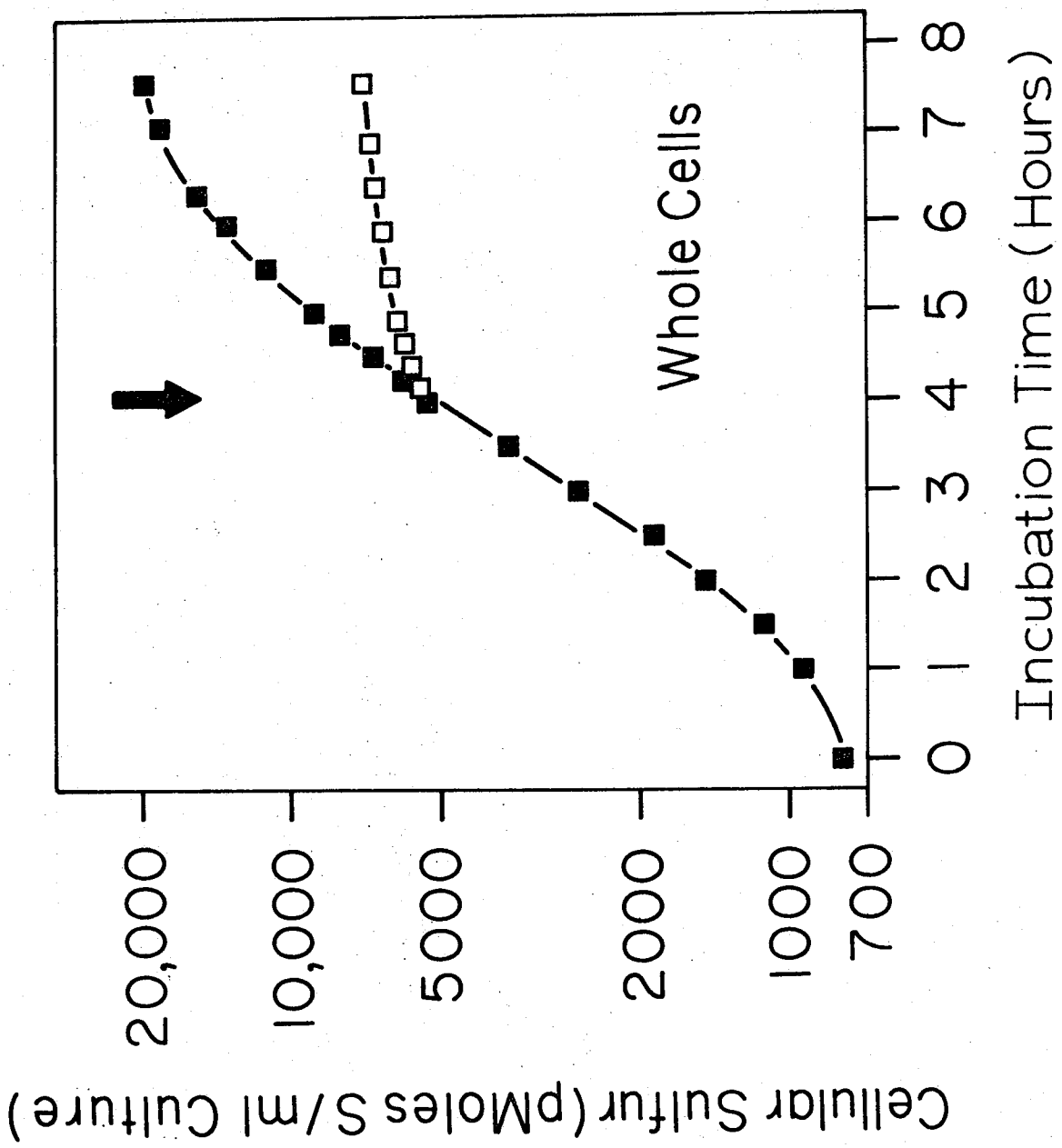

Cellular Sulfur (pMoles S/ml Culture)
20,000
10,000
5000
2000
1000
700
0 1 2 3 4 5 6 7 8
Incubation Time (Hours)
Whole Cells

Table 4-10. Cell Number, Protein, and Sulfur Relationships for Pseudomonas halodurans: Effects of Chloramphenicol[a]

Minutes of Incubation	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
				Batch Culture			
0	1.22×10^7	1.8	23.1	15.2	14.8	188.9	0.85
60	1.40×10^7	2.3	29.3	19.4	16.4	209.0	0.85
120	1.32×10^7	2.9	44.5	29.7	22.0	336.8	1.02
180	1.89×10^7	6.1	81.9	54.5	32.3	433.2	0.89
240	3.02×10^7	12.2	145.6	81.8	40.4	482.2	0.67
				+ Placebo			
260	-	17.2	222.6	136.8	-	-	0.80
275	-	20.1	238.0	162.5	-	-	0.81
290	-	23.5	262.3	188.1	-	-	0.80
305	6.84×10^7	27.4	311.4	212.9	40.1	455.3	0.78
335	-	32.0	392.6	265.4	-	-	0.83
365	1.36×10^8	36.9	466.2	316.7	27.1	342.8	0.86
395	-	42.2	525.4	355.1	-	-	0.84
425	2.23×10^8	48.8	596.4	407.4	21.9	267.5	0.83
485	2.82×10^8	56.6	613.9	417.3	20.1	217.7	0.74

Table 4-10. (Continued)

Minutes of Incubation	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
				+ Chloramphenicol			
260	-	13.2	188.4	112.1	-	-	0.85
275	-	13.3	224.6	105.8	-	-	0.80
290	-	13.0	172.3	83.3	-	-	0.64
305	4.34×10^7	14.0	206.8	113.1	32.3	476.4	0.81
335	-	14.1	216.3	118.2	-	-	0.84
365	4.86×10^7	13.2	228.0	117.2	27.2	469.2	0.89
395	-	14.7	228.3	122.1	-	-	0.83
425	4.33×10^7	14.7	240.7	126.6	34.0	556.0	0.86
485	4.85×10^7	15.3	244.6	118.0	31.3	504.4	0.77
				Mean	27.7	380.0	0.82
				S.D.	8.3	126.0	0.07
				C.V. (%)	30.0	33.2	9.1

[a]Data from Figure 4-7.

phase culture results not only in rapid blockage of protein synthesis but also in cell autolysis and protein hydrolysis. During the first 30 minutes of CAP treatment residue protein sulfur and bulk protein remain constant, whereas a rapid incorporation of sulfate into L.M.W. organic compounds occurs, as observed for P. halodurans. By 60 minutes of CAP treatment, however, all components had begun to decline, and the viable counts had dropped to less than $5x10^5$cells/ml. Autolysis is indicated by a decrease in direct cell counts and an increase in the viscosity of the culture medium, but cannot explain the loss of bulk protein from the culture because the analysis of protein is carried out on TCA-precipitable material in the cell suspension rather than on filter-retainable material. The percentage of the total sulfur incorporated into protein remains high throughout CAP treatment (Table 4-11 and appended Table A-8); the loss of sulfur-containing compounds is uniform among the fractions. This implicates an extracellular protease, since intracellular proteolytic activity would be expected to alter the relative distribution of sulfur in favor of L.M.W. organic sulfur components. The sulfur distribution at 15 and 60 minutes of CAP treatment are identical, although the total sulfur is over 30 % less by 60 minutes. After this time, the amount of L.M.W. organic sulfur declines to less than one-third of the value at 15 minutes and half that of the control culture.

Peculiar patterns of sulfur metabolism in A. luteo-violaceus are observed during the lag phase resulting from inoculation with a stationary phase culture. It was shown in the batch growth experiment (c.f. Table 4-3) that the proportion of sulfur in L.M.W. organic compounds increases during the stationary phase. In this experiment, the phenomenon is quite

Figure 4-9. Effects of chloramphenicol on growth, protein synthesis, and total uptake and distribution of sulfur in major biochemical fractions of A. luteo-violaceus. Cells were grown in complete medium containing 10mM glutamate and 100 μM sulfate with $^{35}SO_4^=$ present (final specific activity 10 DPM/pMole) for 10 generations to obtain isotopic equilibrium. Shortly after the onset of the stationary phase, fresh medium of the same composition was inoculated to a final density of about $9x10^6$cells/ml. Samples were withdrawn for direct and viable cell counts, total protein, and fractionation into major biochemical components. At 460 minutes, the culture was divided into two portions, one receiving CAP (25 μg/ml final concentration) and the other an equal amount of distilled water as a placebo; samples were taken for the same parameters for an additional 8 hours. Open symbols: CAP-treated; closed symbols: control culture.

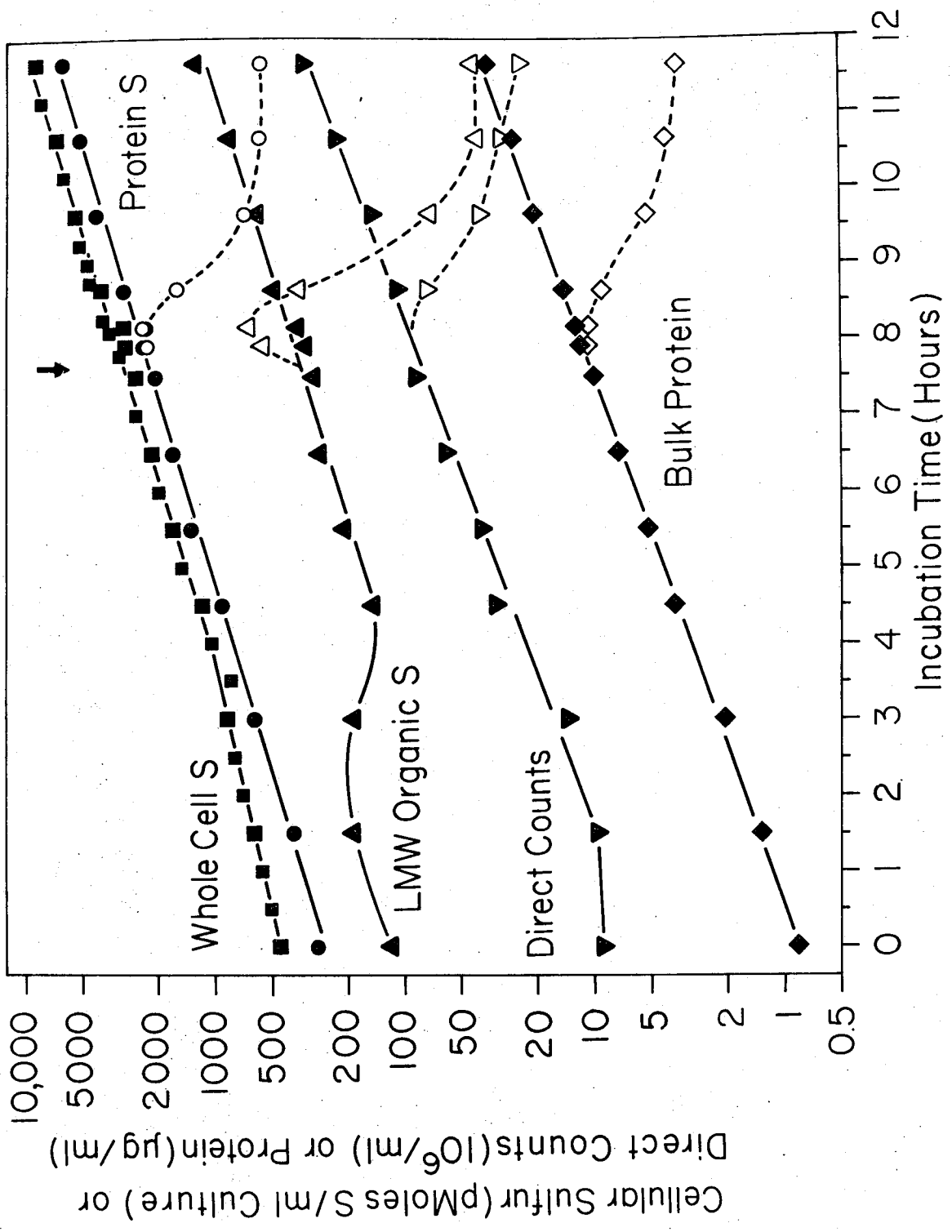

Cellular Sulfur (pMoles S/ml Culture) or
Direct Counts (10^6/ml) or Protein (µg/ml)
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
Incubation Time (Hours)
Whole Cell S
Protein S
LMW Organic S
Direct Counts
Bulk Protein

Table 4-11. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus: Effects of Chloramphenicol[a]

Minutes of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		Batch Culture			
450	2505.4	11.7	3.3	4.7	80.2
		+Placebo			
475	2845.6	11.5	2.7	5.3	80.5
520	3827.5	12.5	5.2	4.5	77.8
700	8770.7	14.5	9.3	3.3	72.9
		+Chloramphenicol			
475	3055.3	17.8	3.1	5.0	74.2
520	2127.1	17.2	4.3	5.3	73.3
700	751.7	5.7	9.2	8.8	76.3

[a]Data from Figure 4-9.

pronounced: nearly 30 % of the total sulfur was comprised of L.M.W. organic material at inoculation. Both bulk protein synthesis and incorporation of sulfur into protein begin immediately, but there is no increase in the amount of L.M.W. organic material. The use of previously pooled precursors results in an increase in total sulfur at a rate only 60 % of the incorporation of sulfur into protein for the first two generations. Initial synthetic reactions take place partly at the expense of endogenous pools. The smaller amount of total sulfate uptake relative to sulfur incorporation into protein observed here is in the opposite sense to the uncoupling found with P. halodurans in the previous experiment, when cellular sulfate uptake rates during CAP inhibition were greater than sulfur incorporation into protein. After 4.5 hours of incubation, all measured parameters attained a state of exponential increase, with very similar growth rates for each of the parameters to the end of the experiment (Table 4-12).

The ratios of bulk protein and total sulfur per cell and residue protein-S:total protein are found in Table 4-13. After 4.5 hours the control culture reached a constant composition, although a large degree of variability exists. Less error than expected is associated with autolysis and protein degradation in the CAP-treated culture; the bulk protein and total sulfur per cell are not substantially different, indicating that the rates of degradation of whole cells and their protein component are very similar. Total and protein sulfur decline at rates faster than bulk protein, however, and the mean weight % S in protein (protein-S:total protein) for the CAP-treated culture, 0.60, is less than the mean for all control data, 0.74. In this experiment the weight % sulfur in protein

Table 4-12. Exponential Rate Constants for Batch Growth of Alteromonas luteo-violaceus: Control Culture for Chloramphenicol Inhibition Experiment[a]

Parameter	n	k (hr^{-1})	r	Interval (Minutes)
Direct counts	10	0.50	0.9958	90 - 700
Bulk Protein	13	0.47	0.9994	0 - 700
Whole Cell ^{35}S	25	0.42	0.9908	270 - 700
LMW Organic ^{35}S	10	0.41	0.9878	270 - 700
Protein ^{35}S	12	0.40	0.9976	90 - 700
Mean		0.44		
Standard Deviation		0.04		
C.V. (%)		9.8		

[a]Data from Figure 4-9.

Table 4-13. Cell Number, Protein, and Sulfur Relationships for Alteromonas luteo-violaceus: Effects of Chloramphenicol[a]

Minutes of Incubation	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
			Batch Culture				
0	8.89×10^6	0.8	14.4	9.2	9.5	162.0	1.09
90	9.41×10^6	1.3	19.7	12.2	13.9	209.4	0.93
180	1.37×10^7	2.1	27.3	19.5	15.0	199.2	0.95
270	3.27×10^7	3.8	37.2	29.2	11.6	113.8	0.77
330	3.87×10^7	5.2	53.3	42.2	13.5	137.8	0.81
390	5.98×10^7	7.4	67.5	52.3	12.3	112.8	0.71
450	8.53×10^7	9.7	82.2	64.5	11.4	96.3	0.66
			+ Placebo				
475	-	11.5	93.4	73.4	-	-	0.64
490	-	12.1	95.2	71.6	-	-	0.59
520	1.07×10^8	14.0	126.0	95.4	13.1	117.7	0.68
580	1.45×10^8	20.3	173.8	133.8	14.0	119.9	0.66
640	2.24×10^8	26.3	222.4	163.7	11.7	99.3	0.62
700	3.34×10^8	36.2	285.3	205.0	10.8	85.4	0.57

Table 4-13. (Continued)

Minutes of Incubation	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
				+ Chloramphenicol			
475	-	10.6	100.1	72.6	-	-	0.69
490	-	10.3	106.0	74.3	-	-	0.72
520	7.41×10^7	8.8	70.3	50.0	11.9	94.9	0.57
580	3.90×10^7	5.1	31.5	22.2	13.2	80.8	0.43
640	2.96×10^7	4.1	26.6	18.4	13.9	89.7	0.45
700	2.39×10^7	3.6	27.4	18.4	15.1	114.6	0.76
				Mean	12.4	132.1	0.74
			- CAP	S.D.	1.6	41.2	0.16
				C.V. (%)	13.1	31.2	21.3
				Mean	12.7	122.2	0.70
			All Data	S.D.	1.6	39.3	0.16
				C.V. (%)	12.5	32.2	23.4

[a]Data from Figure 4-9.

by the operational definition decreased continuously throughout the experiment, over a range of nearly 2-fold. This is accompanied by a rate of sulfate incoporation into alcohol soluble material at a rate nearly twice that of the other components (0.85 hr^{-1}).

The autolysis of cells and rapid protein degradation introduce a serious artifact into the measurement of biomass parameters in A. luteoviolaceus. The cells become increasingly more fragile and susceptible to filtration rupture as they approach stationary phases as emphasized in this experiment. Therefore the relationship between residue protein-S trapped on filters and bulk protein precipitated directly out of the culture medium may be in error. In this context it is noteworthy that the weight % S determined as residue protein-S:total protein is only 50-60 % of the true weight % S determined on the residue protein (Table 4-6), whereas it is greater than 85% for P. halodurans (Tables 4-3 and 4-7) indicating a much higher recovery of protein in the residue for P. halodurans.

Effects of Sulfur Starvation on Sulfur Distribution in A. luteoviolaceus and P. halodurans

It was shown in Chapter 3 that the increase in sulfate uptake rate during sulfur starvation is mirrored by a decrease in the size of the L.M.W. organic sulfur pool for both bacteria. If the residue protein-S: total protein relationship is constant, the material lost from the L.M.W. pool should appear in the protein fraction in proportion to the amount of new protein synthesized. Sulfur starvation may provide a means of varying

the residue protein-S:total protein ratio and demonstrate potential variability of protein composition with respect to sulfur. The microorganisms in this study are unlikely to have ever experienced sulfur starvation, hence much information on the regulation and accessibilty of L.M.W. organic sulfur pools may be gained.

The distribution of cellular sulfur, bulk protein, and direct cell counts is shown for A. luteo-violaceus in Figure 4-10. A decrease of 69% in L.M.W. pool material occurs during the first hour of sulfur starvation, and a corresponding increase in protein sulfur begins after 5 minutes, reaching a maximum at 30 minutes of sulfur starvation. There is an initial decrease in the free sulfate pool of small magnitude, but this pool is so small initially that its utilization is insignificant in the overall sulfur budget. From 2-5 hours of sulfur starvation, all parameters remain constant, after which cellular autolysis begins. An unfortunate loss of total cellular sulfur of about 5% during the first 10 minutes obscurs interpretation of some events during this early period. It is not known whether this material is excreted or a result of poor recovery.

Table 4-14 shows the sulfur distribution for selected points (see also appended Table A-9), with the distribution before harvesting for comparison. Virtually no change occurs as a result of washing the cells, but within 5 minutes of resuspension in sulfur-free medium significant movement of label from L.M.W. organic sulfur to protein sulfur components can be seen. The lipid fraction contains very little sulfur, and changes in the other fractions are undetectable.

Figure 4-10. Metabolism of endogenous sulfur-containing pools during sulfur starvation of Alteromonas luteo-violaceus. Cells were grown in complete medium containing 10mM glucose and 100 μM sulfate, with $^{35}SO_4^{=}$ at a specific activity of 11 DPM/pMole. After more than ten generations in this medium, late exponential phase cells were harvested by centrifugation, washed with basal salts, and resuspended in complete medium minus sulfate. Samples were taken for direct and viable cell counts, total protein, and distribution of ^{35}S in major biochemical fractions.

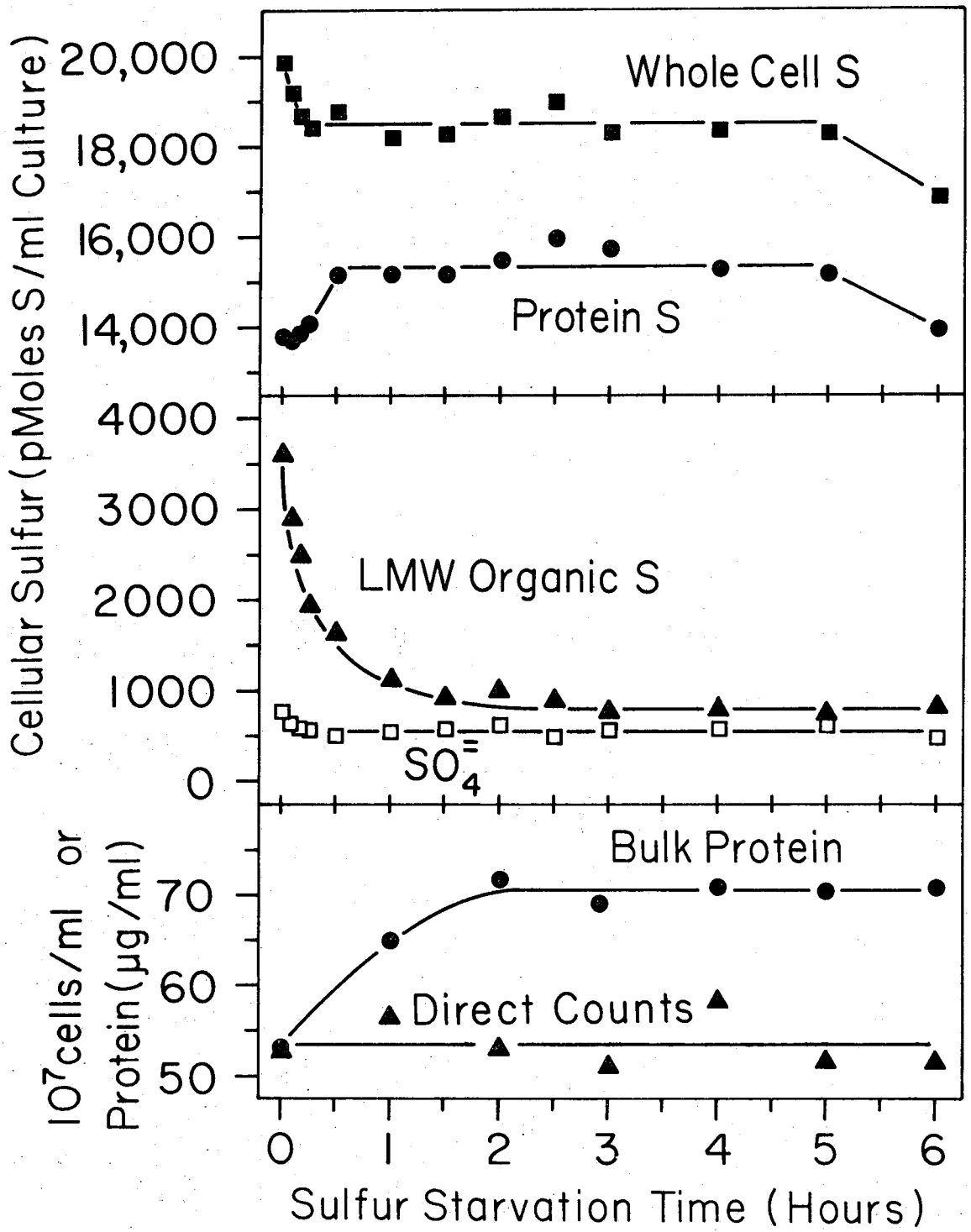

Cellular Sulfur (pMoles S/ml Culture)
20,000
18,000
16,000
14,000
Whole Cell S
Protein S
4000
3000
2000
1000
0
LMW Organic S
$SO_4^=$
10^7cells/ml or Protein (µg/ml)
70
60
50
Bulk Protein
Direct Counts
0
1
2
3
4
5
6
Sulfur Starvation Time (Hours)

Table 4-14. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus During Sulfur Starvation[a]

Minutes of Sulfur Starvation	pMoles S per ml	% of Total Radioactivity					
		$SO_4^{=}$	L.M.W. Organic	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
Batch[b]	24,018.5	3.2	17.9	3.0	0.9	4.9	70.1
0	19,880.5	3.8	18.1	3.4	0.8	4.8	69.1
10	18,612.6	3.2	13.4	2.5	0.7	5.9	74.3
30	18,741.0	2.7	8.7	1.6	0.6	5.8	80.7
60	18,169.3	3.0	6.2	1.0	0.4	6.1	83.3
180	18,292.9	3.1	4.1	0.8	0.4	5.8	85.9

[a]Data from Figure 4-10.

[b]Sample of the culture prior to harvesting and resuspension in sulfur-free medium.

Direct counts remained constant over the course of the experiment, but bulk protein increased during the first two hours to a plateau 34% higher than at the beginning of sulfur starvation, leading to an increase in the bulk protein per cell which is two-fold greater than the increase in residue protein-S (Table 4-15). Viable counts (not shown) remain constant for the first 2 hours (viable:direct count ratio = 0.65), then begin a steady decline coincident with the time at which the L.M.W. organic sulfur pool reaches a minimum and all apparent sulfur metabolism has ceased. The total sulfur per cell remains constant after the initial loss, but the residue protein-S:total protein ratio decreases nearly 20 % during the 6 hour period.

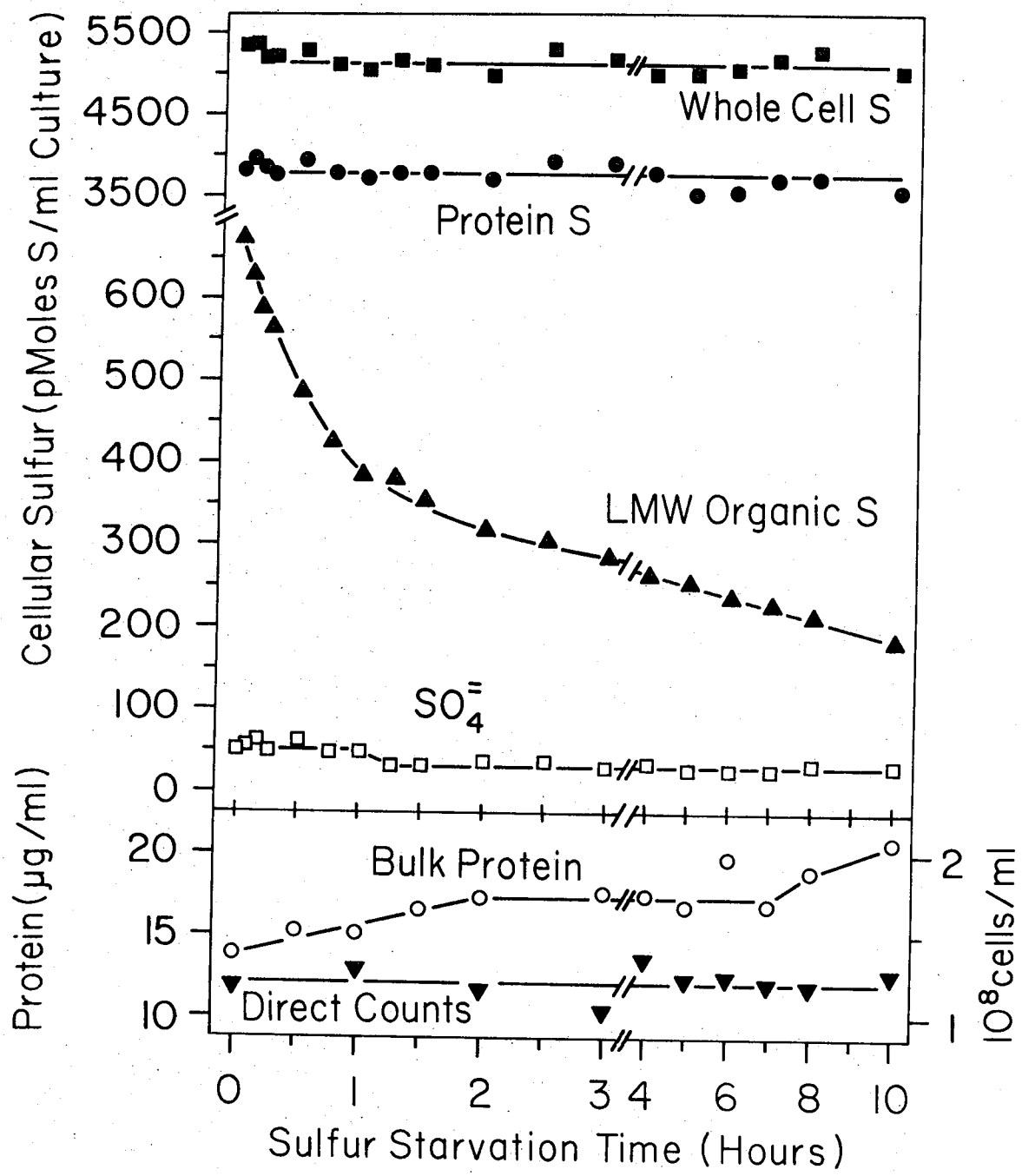
The initial response of Pseudomonas halodurans to sulfur starvation is very similar to that of Alteromonas luteo-violaceus. However, the initial size of the L.M.W. organic sulfur pool is smaller (12.6 vs. 18.1% of the total sulfur at 0 time) and the rate of its utilization is slower, decreasing by only 43% in the first hour, after which it decreases in a linear fashion to the end of the experiment. Figure 4-11 shows that the total sulfur and residue protein sulfur remain constant within experimental error. The inorganic sulfate pool, initially only about 1% of the total sulfur, remains constant for the first hour, then drops by about 30 % to a level which is unchanged for the duration of sulfur starvation. Direct cell counts do not increase, but bulk protein increases for 2 hours to a plateau, then increases again after 7 hours of sulfur starvation to a level 50 % above the initial protein concentration. The average viable:direct cell count was 0.88.

Table 4-15. Cell Number, Protein, and Sulfur Relationships for Alteromonas luteo-violaceus During Sulfur Starvation[a]

Hours of Sulfur Starvation	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
Batch	8.11×10^8	78.3	770.1	540.1	9.7	95.0	0.69
0	5.31×10^8	52.9	637.4	440.5	10.0	120.0	0.83
1	5.63×10^8	64.9	582.6	485.3	11.5	103.5	0.75
2	5.30×10^8	71.6	597.9	496.1	13.5	112.8	0.69
3	5.10×10^8	69.0	586.5	503.6	13.5	115.0	0.70
4	5.83×10^8	70.8	587.6	490.1	12.1	100.8	0.69
5	5.17×10^8	70.3	585.1	486.4	13.6	113.2	0.69
6	5.14×10^8	70.8	540.5	446.8	13.8	105.1	0.63
				Mean	12.2	108.2	0.71
				S.D.	1.7	8.4	0.06
				C.V. (%)	13.8	7.8	8.3

[a]Data from Figure 4-10. A sample of the culture was taken before harvesting and resuspension in sulfur-free medium for comparison with 0 starvation time.

Figure 4-11. Metabolism of endogenous sulfur-containing pools during sulfur starvation of Pseudomonas halodurans. Cells were grown in complete medium containing 10mM glucose and 100 μM sulfate, with $^{35}SO_4^=$ at a specific activity of 19 DPM/pMole. After more than ten generations in this medium, late exponential phase cells were harvested by centrifugation, washed with basal salts, and resuspended in complete medium minus sulfate. Samples were taken for direct and viable cell counts, total protein, and distribution of ^{35}S in major biochemical fractions.


Cellular Sulfur (pMoles S/ml Culture)
5500
4500
3500
Whole Cell S
Protein S
600
500
400
300
200
100
0
LMW Organic S
$SO_4^=$
Protein (μg/ml)
20
15
10
Bulk Protein
Direct Counts
2
1
10^8cells/ml
0
1
2
3
4
6
8
10
Sulfur Starvation Time (Hours)

There is no change in the proportion of sulfur in either the alcohol-ether or hot TCA soluble fractions (Table 4-16) for the first 3 hours. As with A. luteo-violaceus, metabolic events are confined to the L.M.W. organic and protein sulfur fractions. The loss of small molecules is reflected in an increase in the proportion of sulfur in protein, but the error involved in fractionation obscurred possible increases in the absolute amount of sulfur in the protein fraction.

A potential artifact of the fractionation procedure is observed in the distribution of sulfur at 480 minutes (Table 4-16). At the time of this experiment, it was only possible to fractionate 16 samples in a day, and hence the samples from 300-600 minutes were refrigerated, fixed in 10 % TCA. The processing of these samples 12 days later revealed that prolonged, mild acid hydrolysis shifts sulfur from protein , and to a lesser extent alcohol soluble material, to the hot TCA soluble fraction. Thus it is important to process samples which have been fixed with TCA rapidly, or freeze the filters unfixed for later manipulation. It will be shown in a later experiment (Chapter 5) that a portion of the bulk protein also is found in the hot TCA soluble fraction under certain circumstances.

A substantial amount of protein is produced by P. halodurans during sulfur starvation. It is apparent that much of this protein is impoverished in sulfur, and demonstrates that the protein-S:total protein ratio is not constant under sulfur starvation stress. Using a mean protein-S: total protein ratio of 0.8 (Tables 4-3, 7, and 10), the 6.9 μg of protein produced during the experiment would require about 1700 pMoles of sulfur, almost three times the amount available in the L.M.W. organic sulfur pool

Table 4-16. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans During Sulfur Starvation[a]

Minutes of Sulfur Starvation	pMoles S per ml	% of Total Radioactivity				
		$SO_4^{=}$	L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
Batch	6904.4	0.9	13.3	5.8	8.6	71.4
0	5337.7	0.9	12.6	5.8	9.1	71.5
10	5198.3	1.2	11.3	4.7	8.9	73.9
30	5280.8	1.2	9.2	5.6	9.8	74.3
60	5035.7	0.9	7.6	7.3	10.4	73.8
180	5184.2	0.5	5.5	9.4	9.3	75.3
480	5293.2	0.6	4.0	4.8	20.1	70.5

[a]Data from Figure 4-11. A sample of the culture (Batch) was taken before harvesting and resuspension in sulfur-free medium for comparison with the 0 starvation time sample.

[b]Samples from 300-600 minutes were fractionated 12 days after the experiment, all previous samples being done the second day. During this period they were stored at 4°C in 10 % TCA.

and 3.5 times the amount actually metabolized. As a result, the protein-S:total protein ratio decreases throughout the 10 hour period, shown in Table 4-17. In contrast, the protein synthesized by A. luteo-violaceus in the previous experiment requires only 14% more sulfur than is available (using 0.7 weight % S in protein; Tables 4-6 and 13), and the weight % S in protein does not change more than can be accounted for by the initial loss of total sulfur. The potential varibility of protein composition with respect to sulfur content is therefore much higher in P. halodurans. The results of this experiment are not pertinent to the hypothesis that the residue protein-S:total protein is constant enough to use as a measure of microbial protein synthesis in seawater because sulfate is never absent in marine habitats. However, the experiment demonstrates that the composition of protein can be regulated by nutritional stresses.

Short Term Sulfate Incorporation Patterns in Sulfur-Starved Alteromonas luteo-violaceus

The de novo synthesis of bulk protein by A. luteo-violaceus during sulfur starvation and the retention of viability until L.M.W. organic sulfur pools are exhausted raises questions about the recovery of the organism from sulfur starvation and the filling of previously depleted L.M.W. organic sulfur pools. Additionally, inorganic sulfate has not been observed as an important component of the total cellular sulfur under any conditions, and it is of value to know whether sulfate uptake can exceed metabolic demands for sulfur. To answer these questions, a culture of A. luteo-violaceus was washed in RLC-water and resuspended in complete med-

Table 4-17. Cell Number, Protein, and Sulfur Relationships for Pseudomonas halodurans During Sulfur Starvation[a]

Hours of Sulfur Starvation	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
Batch	1.40×10^8	20.2	221.4	158.0	14.4	158.1	0.78
0	1.19×10^8	13.8	171.1	122.4	11.6	143.8	0.89
0.5	-	15.1	169.3	125.8	-	-	0.83
1	1.28×10^8	15.0	161.5	119.1	11.7	126.1	0.79
1.5	-	16.5	163.8	121.5	-	-	0.74
2	1.16×10^8	17.2	159.1	118.9	14.8	137.2	0.69
3	1.03×10^8	17.5	166.2	125.2	17.0	161.4	0.72
4	1.35×10^8	17.3	160.4	121.4	12.8	118.8	0.70
5	1.22×10^8	16.7	160.3	113.3	13.7	131.4	0.68
6	1.24×10^8	19.7	162.3	114.4	15.9	130.9	0.58
7	1.20×10^8	16.8	166.4	119.2	14.0	138.7	0.71
8	1.18×10^8	18.9	169.7	119.6	16.0	143.8	0.63
10	1.26×10^8	20.7	160.9	114.7	16.4	127.7	0.55
				Mean	14.4	138.0	0.71
				S.D.	1.8	13.2	0.10
				C.V. (%)	12.8	9.5	13.3

[a]Data from Figure 4-11.

ium minus sulfur. After two hours of sulfur starvation, $^{35}SO_4^{=}$ was added and the distribution of sulfur in the major fractions analyzed. Figure 4-12 shows a one hour lag in growth determined by direct counts, during which time L.M.W. organic sulfur compounds dominate the newly transported sulfur. A substantial amount of sulfate is incorporated into protein within 5 minutes, although it is a small percent of the total. Bulk protein synthesis also begins rapidly but requires 30 minutes to reach its maximum rate.

Table 4-18 presents the distribution data for representative points (see also appended Table A-11) and demonstrates that inorganic sulfur can comprise a significant proportion of the total sulfur. The initial high proportion of L.M.W. organic sulfur declines rapidly, the difference being recovered in the residue protein fraction, with very little activity in alcohol-ether or hot TCA soluble material. By 30 minutes of incubation with sulfate, the inorganic sulfate pool has returned to its normal level of about 2% of the total sulfur. The L.M.W. organic and protein fractions are equivalent at 60 minutes.

In the sulfur starvation experiment with A. luteo-violaceus, the 0 time L.M.W. pool size was 677 pMoles S/10^8cells, of which 487 were utilized during the first two hours. This deficit is recovered in slightly over 10 minutes of incubation with sulfate. The pool size continues to increase to a maximum at 60 minutes of 1365 pMoles S/10^8cells, about twice the normal value, after which it abruptly declines as a result of the burst of cell division (946 pMoles S/10^8cells at 90 minutes) and continues to decrease to the end of the experiment.

Figure 4-12. Sulfate incorporation patterns by sulfur-starved Alteromonas luteo-violaceus. Cells were grown in complete medium containing 10mM glucose and 100 μM $SO_4^=$ were harvested by centrifugation, washed with RLC-water, and resuspended in complete medium minus sulfur. After two hours, $^{35}SO_4^=$ (final concentration, 100 μM; specific activity 20 DPM/pMole) was added and samples removed for direct counts, total protein, and distribution of sulfur in major biochemical fractions. The zero time isotope adsorption blank has been subtracted.

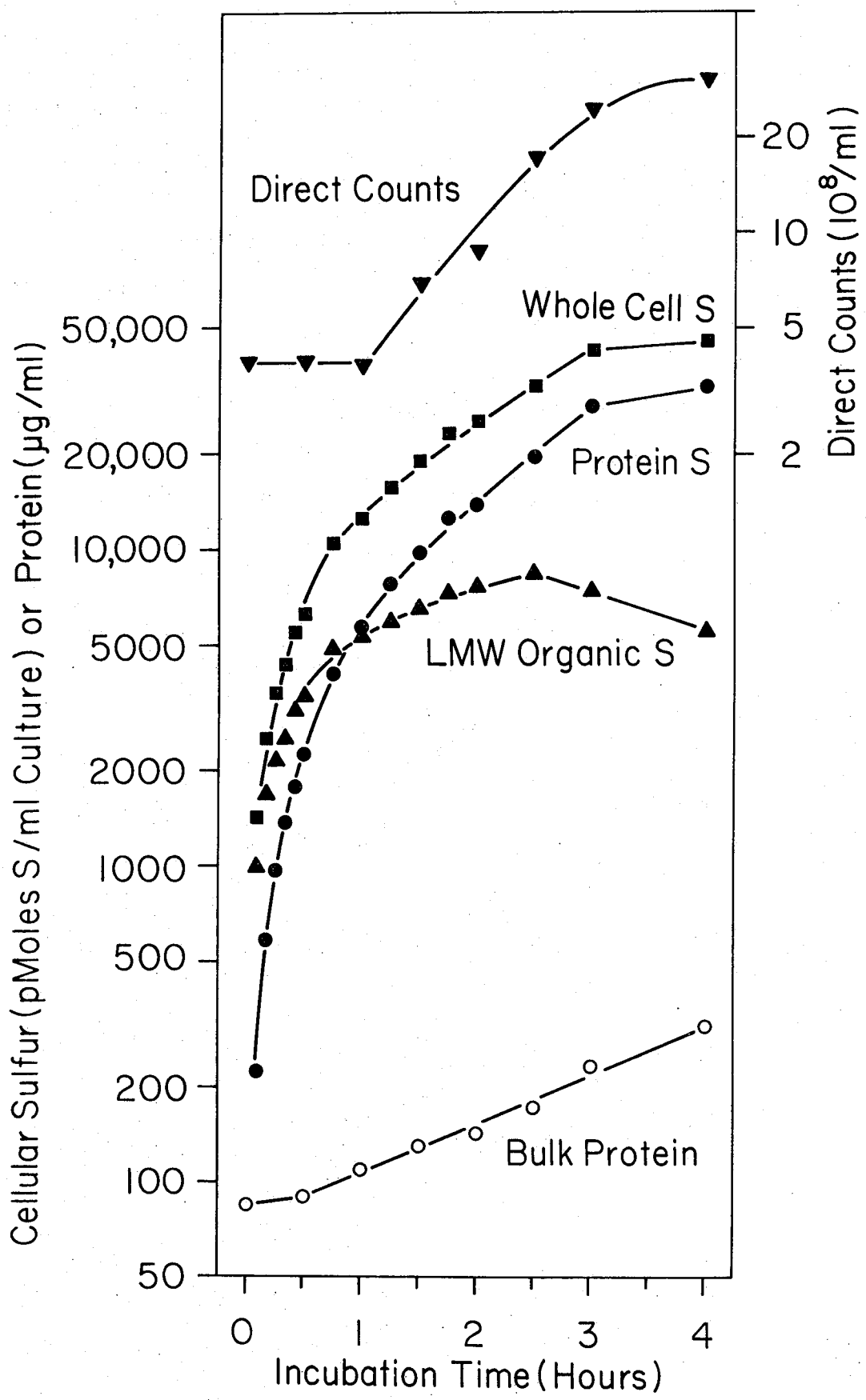

Direct Counts
Whole Cell S
Protein S
LMW Organic S
Bulk Protein
Cellular Sulfur (pMoles S/ml Culture) or Protein (µg/ml)
50,000
20,000
10,000
5000
2000
1000
500
200
100
50
Direct Counts (10⁸/ml)
20
10
5
2
0
1
2
3
4
Incubation Time (Hours)

Table 4-18. Total Uptake of $^{35}SO_4^=$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus During Recovery from Sulfur Starvation[a]

Minutes of Incubation	pMoles S per ml	% of Total Radioactivity				
		$SO_4^=$	L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
5	1409.5	6.6	70.3	1.7	4.1	17.4
10	2531.4	4.2	66.4	2.0	4.3	23.1
30	6234.4	2.5	55.2	3.4	2.8	36.1
60	12,448.7	1.9	42.8	3.3	6.4	45.7
120	25,209.7	2.3	30.3	5.7	7.1	54.6
180	42,433.0	2.4	17.6	5.5	7.7	66.9

[a]Data from Figure 4-12.

The synthesis of sulfur-deficient protein during sulfur starvation may continue during the early stages of recovery, but the rapid incorporation of sulfur into protein as the L.M.W. organic pools are being replenished suggests that this is not so. Calculation of the residue protein-S:total protein ratio, based on the difference between the sample and the 0 time protein level shows that the normal weight % S is found in the newly synthesized protein (Table 4-19), but drops at very high cell density at the end of the experiment.

Influence of sulfate concentration on growth and sulfur distribution in Pseudomonas halodurans and Alteromonas luteo-violaceus

Previous experiments demonstrate that both bacteria in this study are capable of synthesizing protein in excess of that predicted by the amount of sulfur available in the L.M.W. organic sulfur pool. Since sulfur-deficient protein is produced under the influence of sulfur starvation, the concentration of sulfate in the medium may be an important component in the regulation of protein content and composition. In addition, the high K_m for sulfate relative to other microorganisms raises the question of their ability to grow at low sulfate concentrations. The salinity requirement for P. halodurans requires that even this estuarine bacterium cannot be exposed to sulfate concentrations less than 3mM (one-fifth seawater concentration), but the results of growth at varying sulfate concentrations may explain some of the results of the sulfur starvation study.

Table 4-19. Protein-Sulfur:Total Protein Relationship for Newly Synthesized Protein by Alteromonas luteo-violaceus During Recovery from Sulfur Starvation[a]

Minutes of Incubation	Per ml Culture: Bulk Protein (µg)	New Protein[b] (µg)	Protein-S (ng)	Weight %[c]
0	85.3 ± 2.5	-	-	-
30	89.6 ± 4.1	4.3 ± 4.8	72.2	1.68
60	109.7 ± 3.5	24.4 ± 4.3	182.4	0.75
90	130.6 ± 1.7	45.3 ± 3.0	312.7	0.69
120	142.5 ± 4.5	57.2 ± 5.1	441.5	0.77
150	173.3 ± 9.2	88.0 ± 9.5	629.8	0.72
180	234.3 ± 5.0	149.0 ± 5.6	909.8	0.61
240	313.1 ± 9.9	227.8 ± 10.2	1046.6	0.46
			Mean	0.81
		All data	S.D.	0.39
			C.V. (%)	49.0
			Mean	0.67
		60-240 minutes	S.D.	0.12
			C.V. (%)	17.4

[a]Data from Figure 4-12.

[b]Sample value minus 0 time protein.

[c]Based on newly synthesized protein.

A lag in growth is observed for cultures with sulfate concentrations below 500 μM. Table 4-20 shows an influence of sulfate concentration on both growth rate and sulfur content at 50 and 100 μM sulfate, whereas the culture containing 250 μM sulfate lags in growth but attains the normal growth rate. The growth rate determined by direct counts agrees well with that from ^{35}S incorporation. All cultures exhibit increasing sulfur content per cell until about $5x10^7$ cells/ml followed by a steady decrease characteristic of P. halodurans.

There is much less variability in the distribution of sulfur as a function of external sulfate concentration. Table 4-21 shows that only the 50 μM sulfate concentration affected the proportion of sulfur in the major biochemical fractions, having a reduced amount of L.M.W. organic S at either cell density. Sampling at low cell densities eliminated any problem arising from medium depletion resulting from sulfate assimilation (less than 8% at 50 μM), but even at the high cell density, where over 50% of the sulfate has been incorporated at 50 μM, the same trends are apparent. The early stationary phase decrease in residue protein-S as a percent of the total sulfur and concommittant increase in the proportion of L.M.W. organic compounds usually observed with P. halodurans occurs at all sulfate concentrations to the same extent. The alcohol soluble sulfur increased by two-fold for all cultures at higher density for unknown reasons.

The influence of external sulfate concentration on the total cellular sulfur is reflected well in the sulfur content of the protein in P. halodurans. Table 4-22 presents calculations of the weight % sulfur in protein for both protein-S:total protein and protein-S:residue protein. As

Table 4-20. Growth Rate and Sulfur Content of Pseudomonas halodurans Grown at Varying Sulfate Concentrations[a]

Added Sulfate (μM)	Direct Counts k (hr^{-1})	Direct Counts Hours to $1x10^7$/ml	^{35}S Incorporation k (hr^{-1})	^{35}S Incorporation Hours to 3000 pM S/ml	pMoles S per 10^8cells[b]
50	0.36	33.7	0.33	39.8	9060 ± 1113(4)
100	0.68	20.9	0.64	23.6	9808 ± 1646(5)
250	0.96	15.2	0.95	16.4	14,483 ± 3274(4)
500	0.94	13.2	0.98	14.3	13,902 ± 729(4)
1000	0.94	12.8	0.95	13.8	14,938 ± 760(4)
2000	0.94	13.0	0.99	14.0	14,217 ± 3535(4)

[a]Complete medium containing 10mM glutamate and 0.5 μCi $^{35}SO_4^{=}$/ml but without added sulfur was inoculated with P. halodurans at $2x10^4$cells/ml final density. Aliquots were withdrawn into sterile flasks and supplemented with 50, 100, 250, 500, 1000, or 2000 μM sulfate (final concentration). Direct counts and whole cell ^{35}S uptake were determined at intervals; at about 2-6x10^7cells/ml and 2-4x10^8cells/ml samples were taken for bulk protein and ^{35}S distribution. Growth rate is determined using the least-squares linear regression of ln x vs. t; time to reach $1x10^7$cells/ml or 3000 pMoles S/ml is interpolated from the regression line.

[b]Calculated using data up to $1x10^8$cells/ml, after which the sulfur content per cell drops sharply.

Table 4-21. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans Grown at Varying Sulfate Concentrations

Added Sulfate (μM)	pMoles S per ml	% of Total Radioactivity				
		$SO_4^{=}$	L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
Late Exponential Cells ($<6x10^7$ cells/ml)						
50	3785.3	1.6	8.5	4.9	4.8	80.3
100	5298.8	1.3	14.2	4.5	4.2	75.7
250	4499.1	1.2	12.0	4.6	4.3	77.8
500	3989.6	1.4	11.8	2.8	4.8	79.3
1000	4906.7	1.6	11.5	3.8	7.3	75.7
2000	3481.8	0.0	12.4	1.5	5.1	81.0
Early Stationary Cells ($>2x10^8$ cells/ml)						
50	27,218.2	1.4	14.8	11.3	3.2	69.2
100	20,908.9	1.0	18.9	10.1	3.5	66.6
250	17,473.7	1.2	19.3	9.4	3.7	66.4
500	21,183.1	1.0	18.4	9.1	3.9	67.6
1000	22,903.2	1.9	16.3	8.8	3.3	69.7
2000	18,155.7	2.3	18.5	10.0	3.7	65.5

[a]Experimental details in Table 4-20.

Table 4-22. Cell Number, Protein, and Sulfur Relationships for Pseudomonas halodurans Grown at Varying Sulfate Concentrations[a]

Added Sulfate (μM)	per ml Culture					per 10^8 cells		Protein Weight % S	
	Cell Number	μg Bulk	Protein Residue	ng Sulfur Total	ng Sulfur Residue	Protein (μg)	Total S (ng)	Protein S / Bulk	Protein S / Residue
50	4.84×10^7	14.9	-	121.4	97.5	30.8	250.8	0.65	-
	3.44×10^8	91.8	75.8	872.7	603.7	26.7	253.7	0.66	0.80
100	5.85×10^7	17.5	-	169.9	128.6	29.9	290.4	0.74	-
	2.91×10^8	64.8	51.5	670.4	446.6	22.3	230.4	0.69	0.87
250	3.87×10^7	14.6	-	144.3	112.2	37.8	372.8	0.77	-
	1.07×10^8	52.5	40.3	560.3	371.9	49.1	523.6	0.71	0.92
500	2.81×10^7	12.0	-	127.9	101.5	42.8	455.2	0.84	-
	2.99×10^8	59.8	48.1	679.2	459.1	20.0	227.2	0.77	0.95
1000	3.49×10^7	14.6	-	157.3	119.1	41.7	450.8	0.82	-
	2.96×10^8	56.7	46.3	734.4	512.2	19.2	248.1	0.90	1.11
2000	2.35×10^7	10.3	-	111.6	90.4	43.6	475.1	0.88	-
	2.95×10^8	58.8	36.7	582.1	381.4	19.9	197.3	0.65	1.04
					Mean	32.0	331.3	0.76	0.95
					S.D.	10.7	116.4	0.09	0.11
					C.V. (%)	33.4	35.1	11.7	11.9

[a]Experimental details in Table 4-20.

with the total cellular sulfur, the weight % S in protein is dependent on the sulfate concentration up to 500 μM. The mean and standard deviation of the weight % S in protein are not applicable; they are shown to compare with the variability observed in cultures at a single concentration in other experiments. The similarity in variation around the mean for bulk protein and total sulfur on a per cell basis indicates that the two parameters are well related. The values for the weight % S at the higher sulfate concentrations are in good agreement with previous experiments.

The same medium was used for the concurrent study of sulfate concentration effects on the growth and sulfur metabolism of Alteromonas luteo-violaceus. Since this organism grows well at low sulfate concentrations (it was responsible for the development of RLC-water; see Chapter 2), the amounts of added sulfate were 20, 50, 100, 250, 500, and 1000 μM final concentration. The procedures were identical to the previous experiment, except that the first samples for protein and sulfur distribution were taken in the late exponential phase (about 1×10^8 cells/ml) due to the lower protein and total sulfur per cell in A. luteo-violaceus.

Sulfur-limited growth does not occur at the concentrations used in this experiment. The mean growth rate (hr^{-1}) of all cultures is 0.47 ± 0.03 by direct counts and 0.43 ± 0.02 by ^{35}S incorporation, with no variation relative to the sulfate concentration and no lag in either parameter. There is a direct relationship between the total cellular sulfur and the external sulfate concentration: in pMoles S/10^8 cells, the values for the respective concentrations are 1930 ± 178 (20 μM), 2676 ± 49 (50 μM), 3056 ± 473 (100 μM), 3151 ± 207 (250 μM), 3879 ± 371 (500 μM), and 4284 ± 1017 (1mM).

The distribution of sulfur obeys exactly the same pattern in A. luteo-violaceus as found for P. halodurans (Table 4-23). A slight decrease in the proportion of L.M.W. organic sulfur is observed at the lowest sulfate concentration at the lower cell density, the rest being identical within experimental error. The higher cell density again reveals an increase in the alcohol soluble sulfur and L.M.W. organic fractions at the expense of protein, but the 20 μM sulfate concentration is indistinguishable from the others.

The same pattern of sulfate concentration effect on the residue protein sulfur:total and residue protein ratios is followed by A. luteo-violaceus. In keeping with its ability to grow well at low sulfate concentrations, the external sulfate concentration at which the maximum sulfur content in protein is found is 250 μM, with significant reduction in protein-S below 100 μM. The weight % S in the residue protein is 20 % less than that of P. halodurans, as shown in Table 4-24. The residue protein-S:total protein ratio consistently decreases at higher cell density. This is an artifact due to the fragility of these cells in the stationary phase with loss or rupture of cells during filtration, since the recovery of 5ml fractionation samples is an average of 78% relative to untreated 1ml filters whereas the recovery of exponential phase cells in this experiment is greater than 95%. The residue protein-S:residue protein ratio is independent of recovery and is valid in any case. The variable recovery of A. luteo-violaceus by filtration in later stages of growth has been a problem throughout the course of this work.

Table 4-23. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus Grown at Varying Sulfate Concentrations[a]

Added Sulfate (μM)	pMoles S per ml	% of Total Radioactivity				
		$SO_4^{=}$	L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
Late Exponential Cells ($<2x10^8$cells/ml)						
20	2027.3	2.8	9.7	3.6	5.2	78.8
50	2949.5	3.3	11.6	3.7	4.8	76.6
100	2386.2	2.9	11.9	2.7	4.6	77.9
250	6452.0	2.9	13.5	4.7	4.1	74.8
500	5578.8	2.9	13.5	5.4	3.9	74.2
1000	4471.0	4.2	12.4	3.6	4.3	75.5
Early Stationary Cells ($>2x10^8$cells/ml)						
20	11,880.4	2.1	19.0	8.7	2.9	67.3
50	13,461.3	1.9	22.6	7.7	3.6	64.2
100	12,454.7	2.8	17.6	8.1	3.7	67.9
250	17,085.6	3.4	23.9	8.6	3.1	61.0
500	14,661.3	3.2	20.0	8.4	3.6	64.8
1000	15,291.3	3.3	21.4	9.3	3.5	62.6

[a]Complete medium containing 10mM glutamate and 0.5 μCi $^{35}SO_4^{=}$/ml but without added sulfur was inoculated with A. luteo-violaceus at $2x10^4$cells/ml final density. Aliquots were withdrawn into sterile flasks and supplemented with 20, 50, 100, 250, 500, or 1000 μM sulfate (final concentration). Direct counts and whole cell ^{35}S uptake were determined at intervals; at about $1x10^8$cells/ml and $2-4x10^8$cells/ml samples were taken for bulk protein and ^{35}S distribution.

Table 4-24. Cell Number, Protein, and Sulfur Relationships for Alteromonas luteo-violaceus Grown at Varying Sulfate Concentrations[a]

Added Sulfate (µM)	per ml Culture					per 10^8 cells		Protein Weight % S	
	Cell Number	µg Protein Bulk	µg Protein Residue	ng Sulfur Total	ng Sulfur Residue	Protein (µg)	Total S (ng)	Protein S / Bulk	Protein S / Residue
20	1.14×10^8	11.9	-	65.0	51.2	10.5	57.0	0.43	-
	3.28×10^8	61.1	44.8	380.9	256.3	18.6	116.1	0.42	0.57
50	1.11×10^8	14.4	-	94.6	72.5	12.9	85.2	0.51	-
	3.44×10^8	58.9	40.6	431.6	277.2	17.1	125.5	0.47	0.68
100	9.42×10^7	9.9	-	76.5	59.6	10.5	81.2	0.60	-
	3.20×10^8	56.8	36.3	399.3	271.3	17.7	124.8	0.48	0.75
250	2.14×10^8	24.9	-	206.9	154.7	11.6	96.7	0.62	-
	2.72×10^8	60.0	42.2	547.8	334.1	22.0	201.4	0.56	0.79
500	1.64×10^8	21.0	-	178.9	132.8	12.8	109.1	0.63	-
	2.51×10^8	59.9	38.3	470.1	304.6	23.9	187.3	0.51	0.80
1000	1.41×10^8	16.6	-	143.4	108.3	11.8	101.7	0.65	-
	3.12×10^8	55.4	38.6	490.3	306.8	17.8	157.1	0.55	0.80
					Mean	15.6	120.3	0.54	0.73
					S.D.	4.6	42.9	0.08	0.09
					C.V. (%)	29.2	35.7	14.6	12.5

[a]Experimental details in Table 4-23.

Influence of the Carbon and Energy Source for Growth on the Protein Content and Composition of Pseudomonas halodurans, with a Note Concerning Nitrogen Limitation

In the natural environment, microorganisms are exposed to a large array of dissolved organic carbon compounds which may be used for growth and energy requirements. It is therefore pertinent to determine the influence of the growth substrate on the sulfur content of protein if the measurement of protein sulfur is to be used as an assay of protein synthesis in marine ecosystems. Ten substrates were used, of which 8 supported growth of the organism; lactose and mannose were ineffective. Samples were taken for whole cell ^{35}S to determine the growth rate, and samples were filtered for fractionation and prepared for bulk protein analysis. The distribution of sulfur during growth on acetate, citrate, ethanol, fructose, glucose, glutamate, lactate, and pyruvate were identical within experimental error (see appended Table A-12). The growth rates ranged from 0.44-1.03 hr^{-1}. Table 4-25 reveals no consistent influence of either growth substrate or cell density on the residue protein-S:total protein relationship, the mean value (0.92 %) being similar to previous values and having a small coefficient of variation (6.1%).

A surprising result of the growth of P. halodurans on various carbon and energy sources is that the final bulk protein content of all cultures were strikingly similar (23.1 ± 1.4 µg/ml culture), as were the final values for total sulfur (296.6 ± 9.4 ng S/ml culture). Routine culture of P. halodurans is carried out in medium containing 500 µM ammonia and 10mM

Table 4-25. Cell Number, Protein, and Sulfur Relationships for Pseudomonas halodurans Grown on a Variety of Individual Carbon and Energy Sources[a]

Substrate	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
Acetate (10mM)	1.55×10^8	20.8	266.9	197.1	13.4	172.2	0.95
$k=0.47hr^{-1}$	2.49×10^8	22.9	278.1	211.7	9.2	111.7	0.92
Citrate (10mM)	5.81×10^7	16.9	193.2	140.6	29.1	332.5	0.83
$k=1.03hr^{-1}$	1.82×10^8	22.8	300.3	218.6	12.5	165.0	0.96
Ethanol (207mM)	1.34×10^8	22.8	280.3	211.1	17.0	209.2	0.90
$k=0.82hr^{-1}$	2.42×10^8	20.7	292.5	226.5	8.6	120.9	1.04
Fructose (10mM)	4.27×10^7	14.9	174.5	128.7	34.8	408.7	0.87
$k=0.76hr^{-1}$	1.86×10^8	23.5	304.2	217.1	12.6	163.5	0.92
Glucose (10mM)	3.49×10^7	7.0	88.7	68.8	20.0	254.2	0.98
$k=0.44hr^{-1}$	2.59×10^8	25.3	295.1	222.3	9.8	113.9	0.88
Lactate (10mM)	6.67×10^7	13.6	167.6	125.5	20.5	251.3	0.92
$k=0.70hr^{-1}$	1.87×10^8	22.3	305.8	224.6	11.9	163.5	1.01

Table 4-25. (Continued)

Substrate	per ml Culture				per 10^8 cells		Weight % S
	Cells	Protein (µg)	Total S (ng)	Protein S (ng)	Protein (µg)	Total S (ng)	Protein S / Bulk
Pyruvate (10mM)	4.08×10^7	13.7	167.3	121.7	33.5	410.0	0.89
$k=0.70 hr^{-1}$	1.13×10^8	24.1	299.9	211.0	21.3	265.4	0.88
Glutamate[c] (10mM)	1.08×10^8	24.4	312.8	215.7	22.6	289.6	0.89
				Mean (n=15)	18.5	228.8	0.92
				Standard Deviation	8.6	98.9	0.06
				C. V. (%)	46	43	6.1

[a] 1 liter of basal salts medium containing inorganic nutrients, trace metals, and $^{35}SO_4^{=}$ (final concentration, 1mM, 2 DPM/pMole) was inoculated with exponentially growing cells to a final density of about 3×10^4/ml and the culture was shaken for 10 minutes. Aliquots of 100ml were transferred into sterile flasks and supplemented with sterile substrates to give the final concentrations indicated. The cultures were shaken at 250 RPM at 21°C. 1ml samples were withdrawn at intervals and filtered for whole cell ^{35}S, from which an approximate growth rate k (doublings per hour) was determined. During late exponential and early stationary growth, samples were collected for total ^{35}S (1ml), distribution of 35S (5ml), total protein (10ml), and cell counts (1ml). Samples were processed as described in Chapter 2. Reported values for protein are the average of duplicate samples.

[b] Growth rate not determined. The average growth rate on glutamate is 1 hr^{-1}.

glutamate, with an effective nitrogen concentration of 10.5 mM. Using a protein-N content of 17% by weight (Jukes et al., 1975), the final protein in the low nitrogen cultures contains almost 60 % of the added ammonia-N. Since nitrogen is an important component of nucleic acids and L.M.W. compounds, it is likely that the second sample of cultures grown with 500 μM available N had reached a nitrogen-limited stationary phase. For example, particulate nitrogen analyses from the batch culture experiment indicate the utilization of 640 μM N at a cell density of $2x10^8$cells/ml. A later experiment using glucose as the carbon and energy source verified that nitrogen limits the amount of protein production at 500 μM N, although the cell numbers attain nearly the normal stationary phase density, leading to markedly decreased protein per cell (data not shown). Thus neither the carbon and energy source nor nitrogen limitation affect the residue protein-S:total protein ratio in P. halodurans, an important consideration for natural population studies.

Effects of Low Concentrations of Compounds Containing Reduced Sulfur on Sulfate Incorporation by Pseudomonas 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 biosynthetic 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 concen-

tration L.M.W. organic sulfur compounds affect the incorporation of sulfate sulfur into protein.

P. halodurans was grown in complete medium containing 1mM 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. At the low cell density sampling point less than 10 μM sulfate is assimilated, so if the reduced sulfur compounds are utilized preferentially to sulfate an effect should be discernable even at 1 μM levels of added sulfur. This is not the most sensitive approach, but it is amenable to use as a bioassay for L.M.W. organic sulfur compounds in natural seawater samples.

The distribution of sulfur in the major biochemical fractions is shown in Table 4-26. There is no effect of the supplements on the growth rate. At low cell density methionine and cystine clearly affect the amount of sulfate-S appearing in L.M.W. organic pools and residue protein-S. Methionine causes a redistribution of sulfur from residue protein-S to the L.M.W. organic-S fraction, and the effect is proportional to the concentration of added sulfur. In contrast, cystine apparently suppresses incorporation of sulfate into the L.M.W. organic-S pool, thus increasing the proportion of sulfate-S in residue protein. Thiosulfate and glutathione are without appreciable effect at these concentrations.

The higher cell density samples exhibit the same pattern of effect with cystine and methionine, but only at the higher level of added sulfur. Glutathione and thiosulfate remain ineffective.

Table 4-26. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans: Effects of Organic Sulfur Sources on Sulfate Incorporation[a]

Added S (μM)	pMoles S per ml[b]	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
Low Density ($<1x10^8$ cells/ml)					
		Sulfate (Control)			
	4399.0	11.9	5.9	3.7	78.5
		Thiosulfate			
2	4380.0	12.1	5.0	3.8	79.0
20	3733.4	11.4	3.1	4.3	81.2
		Methionine			
1	6860.1	16.1	7.8	3.3	72.8
10	2718.3	35.9	4.2	2.8	57.1
		Cystine			
1	6286.4	9.6	7.9	3.7	78.8
4.5	5573.6	8.4	5.8	3.5	82.4
		Glutathione			
1	5710.0	13.2	5.6	3.6	77.7
10	8676.0	15.3	5.4	3.1	76.2

Table 4-26. (Continued)

Added S (μM)	pMoles S per ml[b]	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		High Density (>1x10^{8}cells/ml)			
		Sulfate (Control)			
	10,586.6	18.0	9.1	3.4	69.5
	18,715.4	18.4	10.7	3.1	67.7
		Thiosulfate			
2	16,562.2	19.1	9.6	2.9	68.5
20	15,175.3	18.6	9.4	2.8	69.2
		Methionine			
1	17,949.9	19.2	9.8	2.6	68.3
10	12,676.1	26.4	8.9	2.5	62.3
		Cystine			
1	16,668.7	17.4	10.7	2.8	69.1
4.5	13,382.8	13.9	10.0	2.9	73.2
		Glutathione			
1	17,192.0	18.7	10.1	2.8	68.5
10	18,935.5	18.4	9.6	2.7	69.3

[a]One liter of complete medium containing 10mM glutamate and 1mM $^{35}SO_4^{=}$ (specific activity 2 DPM/pMole) was inoculated with late exponential phase cells at a final density of about 3x10^{4}cells/ml and shaken for 10 minutes. Aliquots were transferred into sterile flasks and supplemented with filter-sterilized sulfur sources at the indicated centrations, and samples were taken at intervals for direct counts. During the mid to late exponential phase and again in the early stationary phase of growth samples were prepared for bulk protein analysis and biochemical fractionation as described.

[b]Derived from sulfate.

The observed differences in the distribution of sulfur in the methionine and cystine-supplemented cultures is amplified by analysis of the sulfate-sulfur content of the bulk and residue protein. Table 4-27 emphasizes the effect of methionine on sulfate incorporation; at the 10 μM concentration over two-thirds of the residue protein sulfur is derived from methionine early in growth, and the impact remains, although damped, at the higher cell density. A proportional reduction in the residue protein-S:residue protein ratio is observed. The lower concentration of methionine exerts a barely detectable effect, but based on the mean residue protein-S:bulk protein ratio for the controls (0.75%), the apparent sulfur depletion based on sulfate incorporation of 5.6% is in agreement with the 5.7% loss of sulfate-S from the residue protein fraction to the L.M.W. organic-S pool. At the higher methionine concentration the residue protein-S:bulk protein ratio is more sensitive to methionine replacement of sulfate than the sulfur distribution because of decreased total sulfate uptake.

The cystine-mediated depletion of residue protein-S derived from sulfate decreases with cell density at the low concentration, indicating substantial utilization. The effect of cystine at 1 μM is more pronounced in the depletion of residue protein-S than in the distribution of sulfate-S, and is greater than the effect of methionine at the same concentration. However, the higher concentration has a similar effect at both low and high cell densities which suggests that the cystine concentration has not been seriously altered. Based on the residue protein-S:residue protein ratio, the apparent sulfate-S depletion of the methionine and cystine residues at high cell density indicate the incorporation of

Table 4-27. Cell Number, Protein, and Sulfur Relationships for Pseudomonas halodurans: Effects of Organic Sulfur Compounds on Sulfate Incorporation into Protein[a]

Added Sulfur	per ml Culture					per 10^8 cells		Protein Weight % S[b]	
	Cell Number	µg Protein Bulk	µg Protein Residue	ng Sulfur[b] Total	ng Sulfur[b] Residue	Protein (µg)	Total S[b] (ng)	Protein S / Bulk	Protein S / Residue
None									
	2.53×10^7	15.1	10.9	141.0	110.7	59.7	557.5	0.73	1.02
	1.08×10^8	31.1	25.2	339.4	236.0	28.8	314.3	0.76	0.94
	2.45×10^8	53.3	41.9	600.1	406.2	21.8	244.9	0.76	0.97
Thiosulfate									
1 µM	2.48×10^7	14.2	-	140.4	110.9	57.3	566.3	0.69	-
	2.01×10^8	49.4	38.2	531.1	363.5	24.6	264.2	0.69	0.87
10 µM	2.12×10^7	14.0	-	119.7	97.2	66.0	564.7	0.71	-
	1.85×10^8	48.8	38.6	486.6	336.8	26.4	263.0	0.74	0.89
Methionine									
1 µM	6.30×10^7	22.6	-	220.0	160.2	35.9	349.1	0.71	-
	1.85×10^8	53.0	44.0	575.5	393.2	28.6	311.1	0.74	0.89
10 µM	4.27×10^7	22.0	-	87.2	49.8	46.6	204.1	0.23	-
	2.40×10^8	50.2	43.1	406.4	253.0	20.9	169.4	0.50	0.59

Table 4-27. (Continued)

Added Sulfur	per ml Culture: Cell Number	µg Protein: Bulk	µg Protein: Residue	ng Sulfur[b]: Total	ng Sulfur[b]: Residue	per 10^8 cells: Protein (µg)	per 10^8 cells: Total S[b] (ng)	Protein Weight % S[b]: Protein S / Bulk	Protein Weight % S[b]: Protein S / Residue
Cystine									
1 µM	7.12×10^7	24.6	–	201.6	158.8	34.6	283.1	0.65	–
	2.33×10^8	52.2	44.3	534.5	369.5	22.4	229.4	0.71	0.83
4.5 µM	1.14×10^8	25.0	–	178.7	147.2	21.9	156.8	0.59	–
	2.53×10^8	51.8	42.1	429.1	314.1	20.5	169.6	0.61	0.75
Glutathione									
1 µM	3.25×10^7	18.9	–	183.1	142.2	58.2	563.3	0.75	–
	2.10×10^8	49.4	39.8	551.2	377.7	23.5	262.5	0.76	0.95
10 µM	6.89×10^7	25.7	–	278.2	211.9	37.3	403.8	0.83	–
	2.30×10^8	54.9	44.9	607.1	421.0	23.9	264.0	0.77	0.94

[a] Experimental details in Table 4-26.

[b] Derived from sulfate.

110 % of the methionine at 1 μM and 52% at 10 μM, compared to 97% of the cystine at 1 μM but only 33% at 4.5 μM. Thus methionine is more effective than cystine in replacing sulfate in protein synthesis, but higher concentrations are required to initiate an effect.

Glutathione is without effect on any measured parameter at either concentration or sampling density. The effect of thiosulfate is more difficult to interpret, as there is a greater reduction of sulfate-derived sulfur in residue protein at the lower concentration at both sampling densities.

Application of sulfate incorporation into protein as a method for measuring bacterial growth requires that there be little lag in the incorporation of sulfate into protein due to the equilibration of radiosulfate with endogenous pools (c.f. Karl, 1979). If a substantial amount of equilibration takes place, there will be a delay in the incorporation of the label into residue protein which can be measured using isotope uptake kinetics. Equation 4-1 is derived from Appendix 1 of Roberts et al. (1963) and describes the theoretical whole cell uptake curve of radioisotope by a culture in balanced growth which is growing exponentially at k doublings hr^{-1}:

$$\text{Total Uptake} = \frac{R\ N_0}{k\ \ln 2}\ (e^{kt\ \ln 2} - 1) \quad \text{[Equation 4-1]}$$

R is the rate constant per cell (DPM/cell/hour) and N_0 is the number of cells/ml at t_0, the time of isotope addition. The semi-log plot of uptake vs. time is convex with the slope decreasing until it

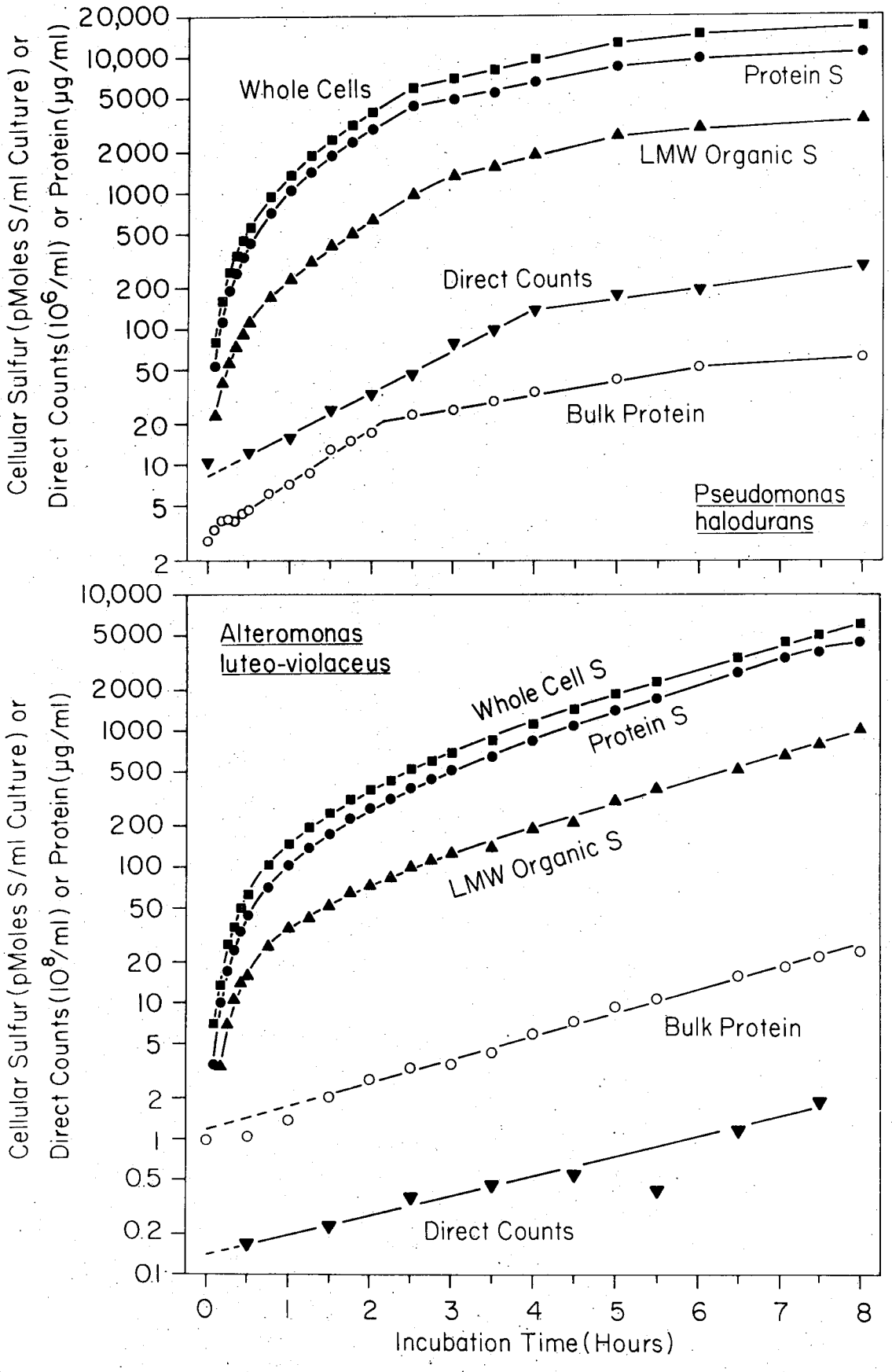
reaches the slope defined by the doubling time k. The degree of curvature is directly related to k, and can be used to determine the growth rate of exponentially-growing pure cultures (Taylor and Jannasch, 1976).

Equilibration of precursor pools with exogenous radiolabeled substrate will result in a shift of the incorporation curve towards the right (increasing incubation time) and decrease the apparent growth rate determined by the curvature until equilibrium is reached. In addition, the distribution of radiolabel in the biochemical fractions will reveal a larger proportion of metabolite in precursor pools relative to that found in equilibrium-labeled cells. An example of this phenomenon is found in Figure 4-12 and Table 4-18.

Addition of labeled sulfate to exponentially growing cultures of P. halodurans and A. luteo-violaceus results in total uptake and residue protein incorporation patterns closely resembling those modeled from Equation 4-1 (Figure 4-13). The curves for isotope uptake are fit by eye, with bulk protein and direct counts included for comparison (fit by linear regression).

Note the characteristic break in the rate of protein synthesis for P. halodurans two hours before the decrease in growth rate determined by cell counts. This does not occur in A. luteo-violaceus; bulk protein and cell counts increased exponentially during the entire experiment. The zero time values for protein are calculated from the regression line rather than the absolute values because of variable recovery at low protein concentrations. To maintain consistency, zero time cell density is determined similarly.

Figure 4-13. Direct counts, bulk protein, and $^{35}SO_4^=$ uptake and incorporation kinetics for exponentially growing cultures of Pseudomonas halodurans and Alteromonas luteo-violaceus. Complete medium containing 10mM glutamate and 1mM sulfate was inoculated with the appropriate organism at about $2x10^4$cells/ml. When the cell density was near $1x10^7$cells/ml, $^{35}SO_4^=$ was added to a final activity of $4x10^6$DPM/ml (P. halodurans) or $3x10^6$DPM/ml (A. luteo-violaceus) and aliquots sampled for radioisotope uptake at short intervals.


Cellular Sulfur (pMoles S/ml Culture) or
Direct Counts (10^6/ml) or Protein (µg/ml)
20,000
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
Whole Cells
Protein S
LMW Organic S
Direct Counts
Bulk Protein
Pseudomonas halodurans
Cellular Sulfur (pMoles S/ml Culture) or
Direct Counts (10^8/ml) or Protein (µg/ml)
10,000
5000
2000
1000
500
200
100
50
20
10
5
2
1
0.5
0.2
0.1
Alteromonas luteo-violaceus
Whole Cell S
Protein S
LMW Organic S
Bulk Protein
Direct Counts
0
1
2
3
4
5
6
7
8
Incubation Time (Hours)

It is apparent that the incorporation of sulfate into residue protein is extremely rapid in both bacteria, demonstrated by the high proportion of the whole cell sulfur residing in that fraction from the outset of the experiment. Table 4-28 lists the distribution of ^{35}S in the subcellular fractions of both microorganisms for 5 representative time points. Both complete data sets are appended in Tables A-13 (P. halodurans) and A-14 (A. luteo-violaceus). In P. halodurans over 67% of the total newly-incorporated S is incorporated into residue protein in 5 minutes. After 15 minutes of incubation (about 0.25 generation), the proportion of sulfur in residue protein, 74.3%, is only 3% less than the maximum proportion obtained in this experiment and probably lies within experimental error of that value. The proportion of L.M.W. organic ^{35}S is maximal at the first point (5 minutes) as expected, rapidly decreasing during the first 15 minutes, then declining slowly to a minimum value at 105 minutes. Two hours after isotope addition, the abrupt decrease in the rate of bulk protein synthesis usually observed with P. halodurans leads to a decrease in the proportion of residue protein-^{35}S with concommittant increase in the size of the L.M.W. organic-^{35}S pool. The proportion of alcohol soluble-^{35}S increases throughout the experiment, whereas hot TCA soluble-^{35}S closely mimics the incorporation of ^{35}S into the protein residue.

A very similar incorporation pattern is observed in A. luteo-violaceus. The growth rate and protein content per cell are about half that of P. halodurans, however, and the early data are subject to more variability due to low uptake relative to the zero time blank. Nonetheless, more

Table 4-28. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans and Alteromonas luteo-violaceus: Short-Term Isotope Uptake Kinetics of Exponentially-Growing Cells[a]

Minutes of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		Pseudomonas halodurans			
5	79.2	28.8	0.9	3.2	67.2
15	258.3	21.4	1.1	3.2	74.3
30	559.5	19.7	1.2	3.2	75.8
120	3904.6	16.2	5.0	3.0	75.8
480	16,310.9	21.0	13.4	1.9	63.7
		Alteromonas luteo-violaceus			
5	6.9	0	0	49.3	50.7
15	26.8	25.4	1.5	10.1	63.1
30	62.6	25.1	1.0	4.2	69.8
120	365.5	19.9	2.7	5.6	71.8
480	6042.2	16.8	6.5	3.6	73.1

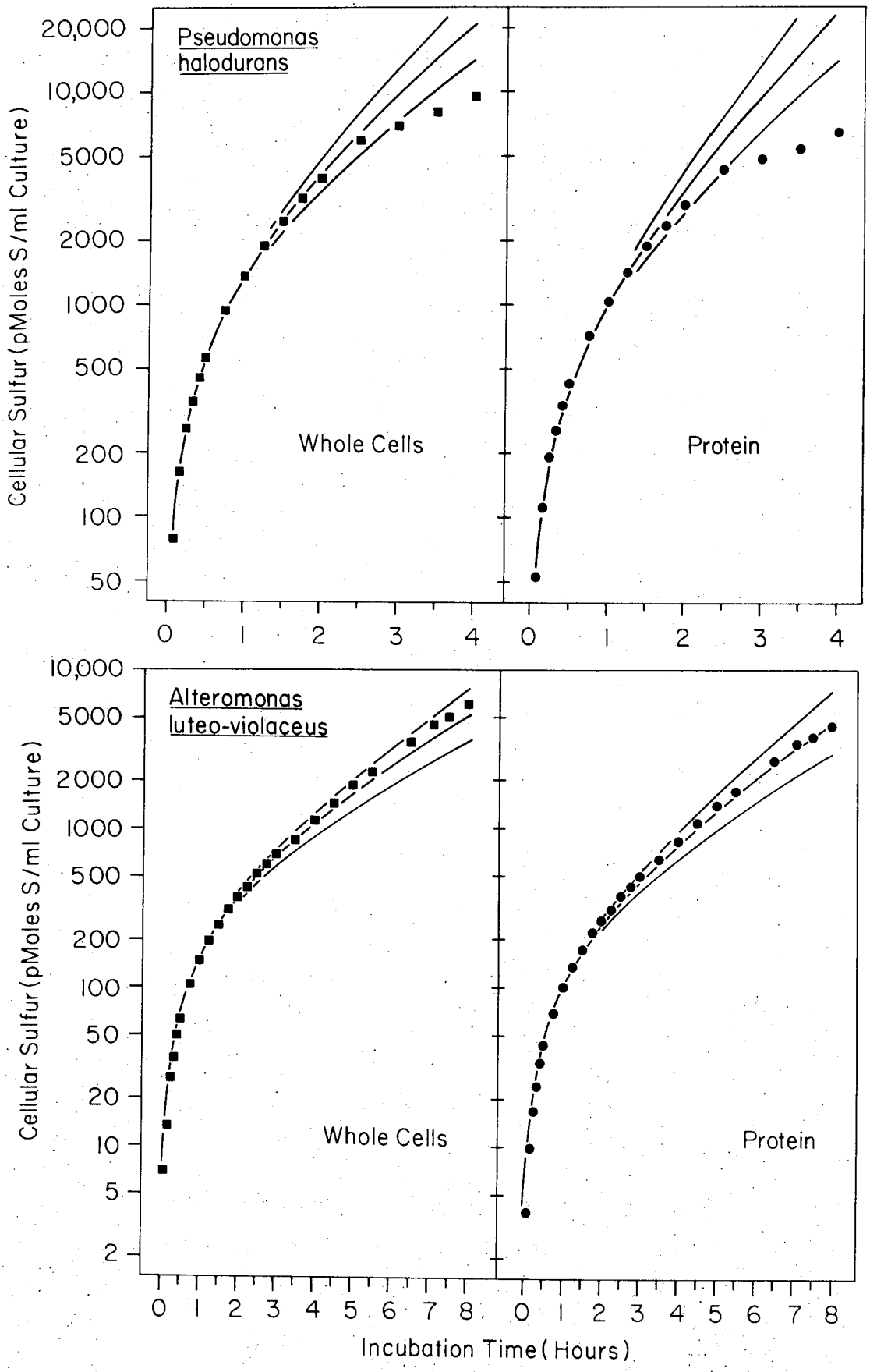
[a]Data from Figure 4-13.

than 50 % of the ^{35}S is found in residue protein in 5 minutes. An abnormally large amount of ^{35}S is contained in hot TCA soluble material at 5 and 15 minutes but is probably an artifact. After about 0.25 generations (30 minutes), the proportion of ^{35}S in residue protein is within 6% of the maximum observed in this experiment. As with P. halodurans, the L.M.W. organic-^{35}S pool of A. luteo-violaceus diminishes in relative importance with time.

The data for whole cell and residue protein-^{35}S from these experiments are modeled by Equation 4-1 in Figure 4-14. The graphs for P. halodurans and A. luteo-violaceus are fitted differently to emphasize different concepts as follows: for P. halodurans the constant $RN_0/k\ln 2$ is determined for whole cell sulfate uptake by solving Equation 4-1 for R (pMoles S/10^8cells/hour) knowing N_0 (8.25 x 10^6 cells/ml) and the k for cell division (1.03/hour) using the first data point (5 minutes; 79.2 pMoles S/ml). The resultant R (11,182 pMoles S/10^8cells/hour) is entered into the equation and expected values for total uptake calculated with increasing time (t). The expected uptake curve is drawn with a 20 % envelope of error for k, and the data are then added. The observed data are within 10 % of the calculated value for the first 150 minutes, after which the sulfate uptake rate decreases due to the change in protein synthesis rate at 2 hours.

The curve for P. halodurans residue protein-^{35}S is generated in the same way using the k determined for bulk protein, 1.29/hour. The incorporation of ^{35}S into the protein residue is slightly faster than predicted from Equation 4-1 initially, but falls under the predicted curve

Figure 4-14. Predicted and observed whole cell sulfate uptake and incorporation into protein using data from Figure 4-13. Predicted rates were calculated as described in the text; a 20 % envelope of error for the influence of growth rate is shown. The following values of k, calculated from the data in Figure 4-13, were used: P. halodurans, 1.03/hour (whole cells) and 1.29/hour (protein); A. luteo-violaceus, 0.48/hour (whole cells) and 0.57/hour (protein).


Pseudomonas halodurans
Whole Cells
Protein
Alteromonas luteo-violaceus
Whole Cells
Protein
Cellular Sulfur (pMoles S/ml Culture)
Incubation Time (Hours)

by 11% at 105 minutes, just before the break in the rate of protein synthesis.

Alteromonas luteo-violaceus provides an opportunity to test the applicability of kinetic analysis to the determination of growth rate as well as pool equilibration time. Although the growth rate determined for direct counts (0.48/hour) is less than that for bulk protein (0.57/hour), there is no deviation from exponentiality for nearly 8 hours of growth. In this case the best fit of all the data from 5 minutes to 8 hours is determined in the following manner: using the appropriate k and the zero time direct cell count determined by extrapolation (1.39×10^{7} cells/ml), the value of R is calculated for each time point. The mean R (898 ± 138 pMoles S/10^{8}cells/hour for whole cell S; 577 ± 84 pMoles S/10^{8}cells/hour for residue protein S) is then used to calculate the predicted uptake or incorporation rate.

The uptake of sulfate into whole cells of Alteromonas luteo-violaceus is modeled quite well by the equation for the first 4 hours of growth (about 2 generations), after which the effects of the faster k for bulk protein synthesis have a noticeable effect. Early uptake data for this bacterium contain a larger degree of error than for P. halodurans because of the lower growth rate and cell quota for sulfur, and the R value for whole cell uptake is influenced by the differential rates of protein synthesis and cell growth, yet the observed data are well within 20 % of the model for all points after 25 minutes. The agreement of the residue protein-^{35}S data with the expected values generated from the growth rate determined for protein is better, most data falling within 10 % of

the model for the entire experimental period. The expected curve lies above the data for the first 25 minutes, as with the whole cell uptake plot, indicating that the protein component (which dominates the whole cell uptake) does require some time to reach equilibrium with precursor pools. The distribution of ^{35}S is in agreement, both suggesting an equilibration time of less than 0.25 generations.

The results of these experiments are in marked contrast to those of a similar experiment performed with sulfur-starved cells (Figure 4-12 and Table 4-18) in which the assimilation of $^{35}SO_4^=$ into L.M.W. organic sulfur pools dominated metabolism for an extended period of time. The present evidence supports the concept of a partitioning of L.M.W. organic sulfur component such that sulfur requirements for protein synthesis are satisfied by exogenous sulfate rather than the small endogenous sulfur reservoir. The lack of a dynamic equilibrium between metabolites of exogenous sulfate and endogenous L.M.W. organic sulfur compounds is supported by the inability to model the L.M.W. organic sulfur fraction with Equation 4-1. The expected curve overestimates the amount of observed ^{35}S in the fraction at growth rate constants as low as 0.001/hour, and a linear plot of the L.M.W. organic ^{35}S vs. time shows little or no upward curvature for at least 45 minutes (not shown).

DISCUSSION

The distribution of sulfur in exponentially-growing cultures of Pseudomonas halodurans and Alteromonas luteo-violaceus is quite similar to that observed for E. coli (Roberts et al., 1963), but the distribution

of carbon is slightly different (Table 4-29). Over 90 % of the total cellular sulfur is found in L.M.W. organic and protein fractions, and generally less than 2% of the total sulfur is found in lipid components. The distribution of both sulfur and carbon remain essentially constant throughout exponential growth, but approach to the stationary phase is often foreshadowed by changes in the relative amount of L.M.W. compounds. At this time, a relative increase of L.M.W. organic sulfur is characteristic of both organisms. The effect is more pronounced in A. luteo-violaceus but this may be due to enhanced susceptibility to its protease. Protein synthesis and cell division occur at similar rates for A. luteo-violaceus throughout the growth cycle, but in P. halodurans the rates of protein synthesis and increase in cell numbers are divergent. A continuous increase in cellular protein during early exponential growth followed by an abrupt decline more than one generation before cell divison rates begin to slow results in a high degree of variability in the protein per cell. In spite of this, incorporation of carbon and sulfur into protein parallels bulk protein synthesis for both organisms.

Virtually all of the sulfur metabolism in A. luteo-violaceus and P. halodurans is concerned with the production and utilization of protein precursors. This fact is clearly demonstrated by the results of protein synthesis inhibition by chloramphenicol in P. halodurans. This bacteriostatic protein synthesis inhibitor produces an absolute cessation of both bulk protein synthesis and incorporation of sulfur into residue protein virtually instantaneously. The elimination of product formation results in a dramatic increase in the size of the L.M.W. organic sulfur pool which proceeds at a linear rate until the pool size is more than doubled.

Table 4-29. Comparison of Carbon and Sulfur Distribution in Major Biochemical Fractions of *Pseudomonas halodurans*, *Alteromonas luteo-violaceus*, and *Escherichia coli*[a]

Organism	% of Total Radioactivity				
	L.M.W.	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
		Sulfur			
P. halodurans	14.2	4.1	1.7	8.6	71.4
A. luteo-violaceus	21.1	3.0	0.9	4.9	70.1
E. coli	22.3	14.2	0.4	2.8	62.7
		Carbon			
P. halodurans	30.5	0.4	7.7	8.8	52.7
A. luteo-violaceus	18.9	1.5	9.8	15.1	54.8
E. coli	8.2	8.5	15.6	17.8	49.9

[a]Sulfur data: *E. coli*, Roberts et al. (1963); *P. halodurans*, Table 4-16; *A. luteo-violaceus*, Table 4-14. Carbon data: *E. coli*, as sulfur; *P. halodurans*, Table 4-1; *A. luteo-violaceus*, Table 4-4.

The same pattern of events occurs in A. luteo-violaceus during the first few minutes of CAP treatment, before autolysis begins.

When the production of precursor is prevented by sulfur starvation, a rapid drainage of L.M.W. organic sulfur pools is accompanied by a corresponding increase in bulk protein. When the pool size reaches a minimum value, the rate of protein synthesis declines sharply. In A. luteo-violaceus, bulk protein synthesis stops completely when the L.M.W. organic sulfur pool is drained. A threshhold concentration exists below which the organism cannot further deplete the pool: the remaining material may be primarily vitamins (thiamine and biotin) and co-enzymes (lipoic acid) which are not readily accessible for use in protein synthesis. Protein synthesis continues at a slower rate after the initial rapid pool drainage in P. halodurans. The L.M.W. organic sulfur pool does not reach a minimum but decreases linearly at a low rate. The result is the production of protein deficient in sulfur during sulfur starvation.

An investigation of the effects of sulfate concentration on the growth and sulfur distribution of Lemna reveal that changes in the external sulfate concentration are reflected in the amount of intracellular sulfate and glutathione, with no effect on the sulfur content of protein (Datko et al., 1978b). In contrast, the concentration of sulfate in the medium exerts a strong effect on the sulfur content of residue protein in both marine bacteria, and additionally influences the total cellular sulfur and protein content of both P. halodurans and A. luteo-violaceus. The increased cellular sulfur is mainly due to the change in the sulfur content of protein, however, with a similar proportion of sulfur in the major biochemical fractions at all but the lowest sulfate concentration.

Fluctuations in the relative proportion and absolute amount of L.M.W. organic sulfur can be as large as 2-fold, even in unperturbed batch growth. Most changes are due to swelling of the L.M.W. organic sulfur pool during stationary phases. In terms of the total amino acid complement of the L.M.W. pool, however, the influence of sulfurcontaining compounds is insignificant. The small contribution of L.M.W. organic sulfur compounds is qualitatively demonstrated in Figures 4-2 and 4-4; the rate of carbon incorporation into L.M.W. material decreases susbtantially at least one generation before reduction in the synthesis of L.M.W. organic sulfur compounds. Carbon-containing compounds of many kinds contribute to the L.M.W. pool, however, so quantitative amino acid analysis is necessary. Tempest et al. (1970) and Tindall et al. (1977) found that sulfur-containing amino acids constitute less than 1 mole % of the total free amino acid pool in microorganisms. Amino acid analysis of boiling water soluble extracts of P. halodurans and A. luteo-violaceus demonstrates that the organisms in this study also have very small S-amino acid pools (Chapter 3, S. Henrichs Ph. D. Thesis, Woods Hole Oceanographic Institution, 1980). No cysteine or its degradation products can be found by gas chromatography of derivatized extracts from 10^{11} cells of either bacterium, and traces of methionine (<1.5 moles % of the total amino acids) are observed only in A. luteo-violaceus.

Inorganic sulfate, an important component of L.M.W. pools in terrestrial microorganisms (Segel and Johnson, 1961) and plants (Hart and Filner, 1969), is not found in quantities >2% of the total L.M.W. sulfur in the marine bacteria. The one exception is during the recovery of A. luteo-violaceus from sulfur starvation (Table 4-18), when inorganic sul-

fate initially attains levels of over 6% of the total sulfur. As the precursor pools depleted by sulfur starvation are replenished, the proportion of sulfur in both inorganic sulfate and L.M.W. organic-S compounds declines rapidly.

The small size of the L.M.W. organic sulfur pool and the negligible contribution of inorganic sulfate to the total cellular sulfur permit extremely rapid incorporation of $^{35}SO_4^=$ into residue protein. The apparently slow equilibration of the L.M.W. organic sulfur pool with exogenous $^{35}SO_4^=$ suggests that much of the sulfur amino acid requirement for protein synthesis can be satisfied by direct reduction of sulfate rather than utilization of pre-existing precursors. Studies of the molecular organization of the cysteine synthetase system (Kredich et al., 1969) support this concept. They found that a single, multimeric complex in Salmonella typhimurium performed both O-acetylation of serine and sulfurylation of the product O-acetylserine with $S^=$ to yield cysteine. Likewise, the reduction of APS to the level of sulfide occurs in a single 6 electron step while bound to a L.M.W. protein carrier (Abrams and Schiff, 1973; Schmidt et al., 1974); 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 co-ordinated, since the intermediates homocysteine and cystathionine are also found at very low concentration (Giovanelli et al., 1973). The slowly equilibrating L.M.W. component may be primarily glutathione, which is well known as a sulfur

reserve in bacteria (Apontoweil and Berends, 1975; Datko et al., 1978a; Fahey et al., 1978). 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 (Kredich, 1971).

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 L.M.W. pool. This is especially valuable because it eliminates problems arising from dilution of label by large endogenous pools of protein precursors 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 (Figure 4-14). Although the rate of protein synthesis is often up to 25% higher than the increase in direct counts, the incorporation of sulfur into the protein residue is very accurately predicted by the model when the growth rate constant determined by protein assay (rather than by direct cell counts) is used in Equation 4-1. Surprisingly, the whole cell sulfate uptake data is nearly as well fit to the model when the lower growth rate determined by direct cell counts is used. The data for Alteromonas luteo-violaceus further demonstrate that the growth rate can be determined accurately (after incubation with ^{35}S for >1.5 generations) for an exponentially growing culture without knowledge of the initial cell density or the substrate concentration. The degree of curva-

ture of the semi-logarithmic plot of uptake or incorporation vs. time is strictly dependent on the growth rate. The initial cell density and substrate concentration only affect the rate constant; therefore the plot can be compared with a series of curves generated with Equation 4-1 using a range of growth rates, and the best fit among them gives a good approximation to the growth rate.

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 (~1μM). Methionine is especially effective at replacing sulfate-sulfur in protein in P. halodurans, with characteristics identical to those observed for E. coli (Roberts et al., 1963). The replacement is manifest by redistribution of the assimilated sulfate in favor of the L.M.W. organic sulfur pool, with a concommittant reduction in residue protein-S. Cystine is much less effective in replacing sulfate, and the response is in the opposite sense to that for methionine; the redistribution of sulfate is in favor of the protein fraction, decreasing the contribution of sulfate to the L.M.W. pool. The failure to detect sulfur-containing amino acids in seawater (Schell, 1974; Williams et al., 1976), even in coastal waters, is reassuring, but bioassay of seawater samples using sulfate incorporation into residue protein with a bacterium of known protein sulfur content and high sensitivity to replacement of sulfate is warranted.

The results of the investigation of the flow of sulfur through major biochemical fractions relative to cell growth and protein synthesis strongly support the applicability of sulfur incorporation studies to marine bacteria in natural habitats. The only factor found to cause a

significant alteration in the sulfur content of the bulk protein in Pseudomonas halodurans or Alteromonas luteo-violaceus is variation in the external sulfate concentration (Tables 4-15, 17, 22, and 24). This variable is not encountered in seawater except in zones of high dissimilatory sulfate reduction such as marshes and anoxic basins; the sulfate concentration of aerobic seawater, 25mM, is over 40-fold in excess of concentrations found to affect the sulfur content of protein in marine bacteria. Figure 4-15 shows the relationship between sulfate incorporation into residue protein and bulk protein in P. halodurans for all experiments with sulfate concentrations >500 μM. Although nitrogen-limited cultures have values above the regression line, the controls for the experiment also have unusually high residue protein:bulk protein ratios, reflecting a small degree of variability in the measurement. The residue protein: bulk protein ratio does not describe the true sulfur content of protein because some protein and protein-S are solubilized by alcohol and hot TCA. This ratio is an operational one, i.e. the measurement which can be made with natural populations. The relationship between residue protein-S and bulk protein is similar for Alteromonas luteo-violaceus (Figure 4-16), but problems encountered with protease activity in stationary phases prevented measurement of the ratio over as wide a set of conditions as those used with P. halodurans.

The work in this chapter emphasizes the relative constancy of the residue protein-S:total protein ratio because of its potential application to the measurement of protein synthesis in natural marine bacterial populations. During the course of the study, other parameters indicative of nutritional status were measured as well to determine the variability

Figure 4-15. Residue protein sulfur:bulk protein relationship for Pseudomonas halodurans. Data are shown for all cultures with sulfate concentrations >500 μM. Symbols: Unperturbed batch growth, ● ; nitrogen limited cultures, △ ; chloramphenicol inhibited culture, □ .

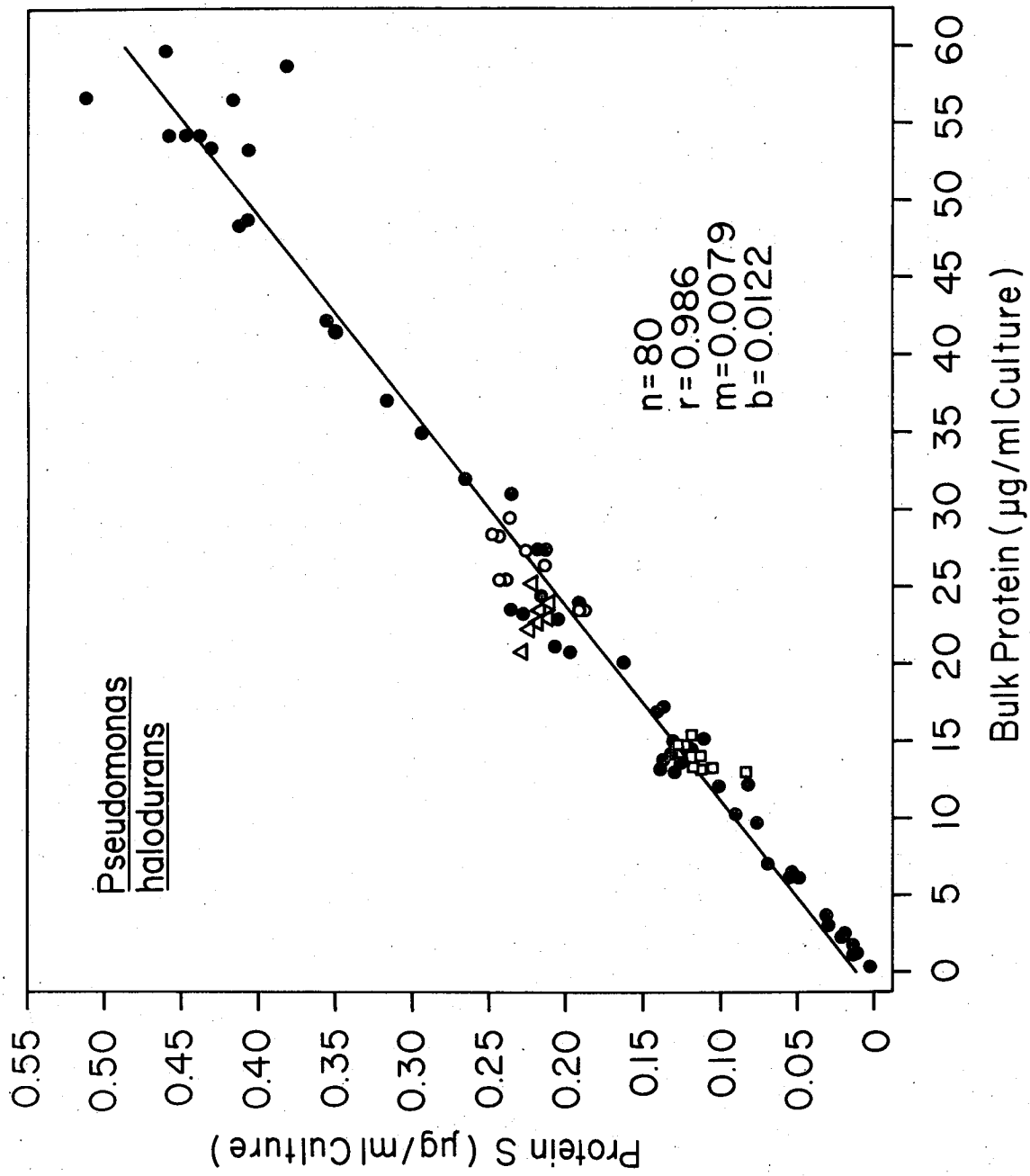
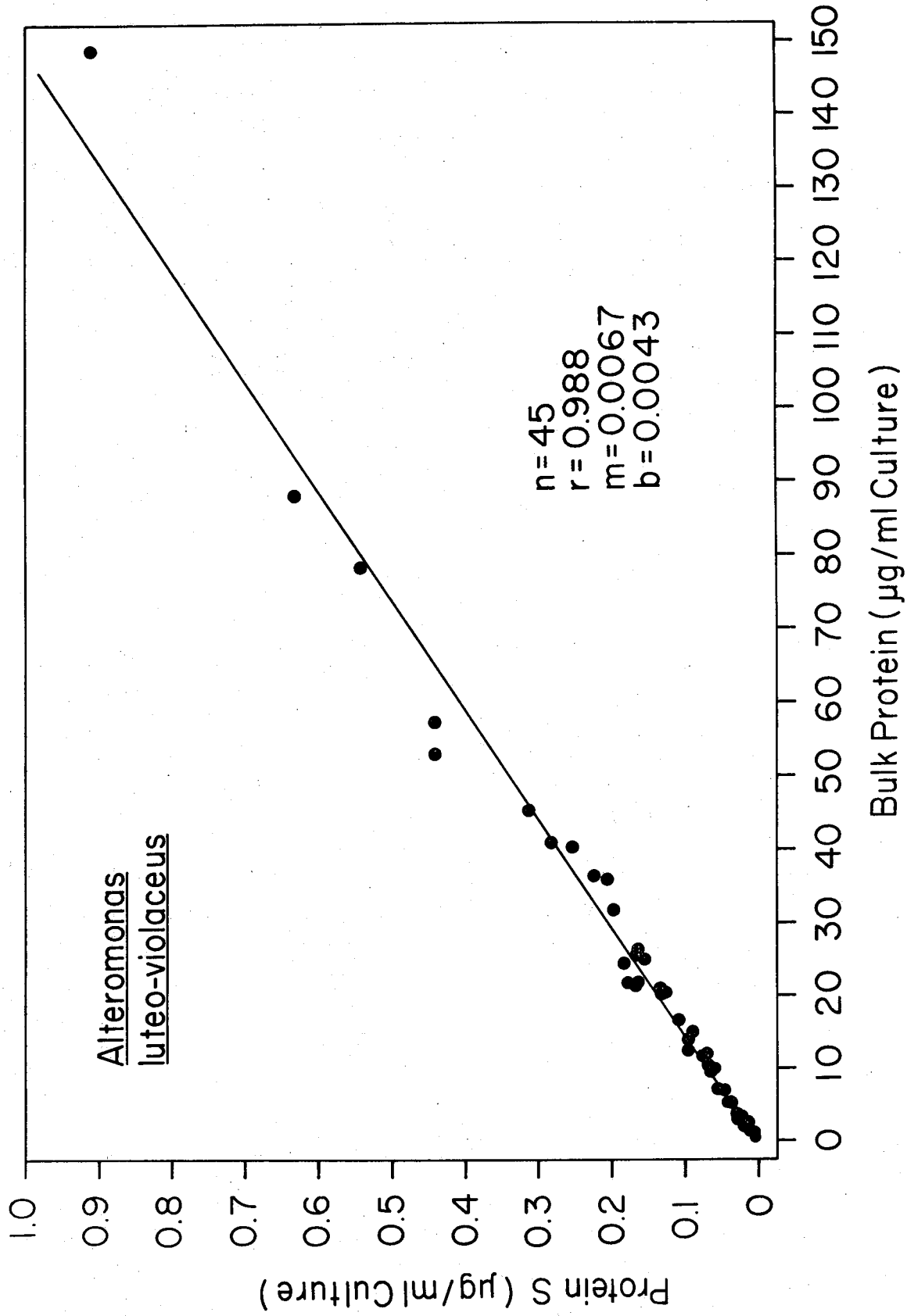

Pseudomonas halodurans
n=80
r=0.986
m=0.0079
b=0.0122
Protein S (µg/ml Culture)
Bulk Protein (µg/ml Culture)
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
0 5 10 15 20 25 30 35 40 45 50 55 60

Figure 4-16. Residue protein sulfur:bulk protein relationship for Alteromonas luteo-violaceus. Data are shown for all cultures with sulfate concentrations >100 μM.


Alteromonas
luteo-violaceus
n=45
r=0.988
m=0.0067
b=0.0043
Protein S (µg/ml Culture)
Bulk Protein (µg/ml Culture)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150

of cellular composition as a function of environmental conditions. The concurrent measurement of cell counts, protein, ^{14}C and ^{35}S assimilation, and particulate organic carbon and nitrogen permits the calculation of a number of ratios relevant to interpretation of microbial growth studies.

Variability in cellular composition is summarized in Tables 4-30 (P. halodurans) and 4-31 (A. luteo-violaceus). Particulate organic carbon and nitrogen, a measurement commonly made in phytoplankton studies, exhibited a 20-fold range of values in both organisms, with standard deviations nearly equal to or greater than the mean. The variation is predictable: most is a result of sulfur starvation and indicates storage of carbon reserves. It is well known that nitrogen limitation leads to the accumulation of carbon reserve polymers in bacteria (Dawes and Senior, 1973; Herbert, 1961; Slepecky and Law, 1961) and algae (Lehman and Wober, 1976). Starvation for sulfur results in even greater glycogen storage in bacteria (Antoine and Tepper, 1969). Accumulation of carbon and nitrogen is also observed for both P. halodurans and A. luteo-violaceus during the stationary phase if carbon and energy sources are abundant.

Increasing cellular carbon is frequently accompanied by more slowly rising particulate nitrogen. During unperturbed and sulfurlimited stationary phases the C:N ratio therefore increases, but to a smaller extent than cellular carbon. Much of the nitrogen assimilation can be ascribed to requirements for protein synthesis in sulfurlimited cultures, but amino acid pooling may be important in other stationary phases.

The protein content of P. halodurans fluctuates considerably, ranging over 2-fold during unperturbed exponential growth. This characteristic is

Table 4-30. Variability of Bulk Parameters in P. halodurans Normalized to Cell Numbers[a]

µg/10^8cells	n	$\overline{X}$	S.D.	C.V.(%)	Range
POC[b]	28	18.8	15.9	84.7	3.6-62.4
PON[b]	28	5.4	5.7	104.1	0.9-23.2
Protein	162	23.8	11.6	48.7	3.6-66.0
Total ^{14}C	23	31.5	16.1	51.3	12.3-63.7
Total ^{35}S	110	0.293	0.129	44.2	0.104-0.606
C:N (wt:wt)[b]	28	3.8	0.8	19.9	2.7-5.1
C:S (wt:wt)[c]	79	97	56	57.7	45-429

[a]Combined data from all experiments.

[b]Particulate organic carbon (POC) and particulate organic nitrogen (PON) determined by Perkin-Elmer CHN analysis.

[c]Radioisotope uptake data.

Table 4-31. Variability of Bulk Parameters in A. luteo-violaceus Normalized to Cell Numbers[a]

µg/10^8cells	n	$\bar{X}$	S.D.	C.V.(%)	Range
POC[b]	21	19.3	20.2	104.7	3.4-71.5
PON[b]	21	4.9	4.4	91.4	1.1-15.4
Protein	79	12.9	4.9	37.8	4.2-28.9
Total ^{14}C	23	8.1	2.4	29.1	3.6-14.3
Total ^{35}S	80	0.106	0.032	29.6	0.048-0.209
C:N (wt:wt)[b]	21	3.8	0.8	21.0	2.8-6.0
C:S (wt:wt)[c]	46	89	8	9.2	73-112

[a]Combined data from all experiments.

[b]Particulate organic carbon (POC) and particulate organic nitrogen (PON) determined by Perkin-Elmer CHN analysis.

[c]Radioisotope uptake data.

not shared by Alteromonas luteo-violaceus. The highly variable protein content of P. halodurans is emphasized by the >18-fold range of values, compared to <7-fold in A. luteo-violaceus, but the coefficient of variation (C.V.) is much less than for either particulate carbon or nitrogen.

Total ^{14}C data were only obtained during unperturbed batch growth experiments and should not be compared with the other data; they delineate the relatively high degree of variability during normal growth. In a chemostat, of course, this indication of great sensitivity to small environmental changes would not be observed.

The variation in total cellular sulfur is very similar to that for total protein. The slightly lower C.V. is a result of sulfur limitation experiments in which synthesis of sulfur-deficient protein occurred. Excluding these data, the agreement in the C.V. for total protein and sulfur confirm the close relationship between protein synthesis and whole cell sulfate uptake. However, there is still substantial variability due to the variations in L.M.W. pool size and divergent rates of cell division and protein synthesis. Therefore whole cell sulfate uptake is not a god measurement of growth in terms of cell numbers.

Whole cell sulfate uptake has been used in freshwater ecosystems in the hope of finding a method for estimating bacterial carbon production for inclusion in carbon budgets (Campbell and Baker, 1978; Jassby, 1975; Jordan and Peterson, 1978; Monheimer, 1974b). However, laboratory studies demonstrated a 65-fold range in C:S uptake ratios (Monheimer, 1978b), preventing unambiguous interpretation of carbon assimilation values derived from sulfate uptake studies (Monheimer, 1978a). Indeed, the C:S ratio for P. halodurans (Table 4-30) spans the range of purportedly con-

stant C:S ratios of 50:1 (Jassby, 1975) to 500:1 (Monheimer, 1974b) during exponential growth. A much smaller range is observed for A. luteo-violaceus because cell growth and protein synthesis are more tightly coupled. The mean C:S ratios are similar, and agree with the mean of 107 determined by Jordan and Peterson (1978) for 5 freshwater bacteria grown in chemostats. It is clear that non-ideal growth must be taken into account before applying a mean value derived from steady state growth to a bacterial assemblage in a variable environment.

The physiology of bacteria growing in variable environments precludes the use of a simple relationship between whole cell carbon and sulfur metabolism. If protein synthesis can be accepted as a useful index of bacterial growth, a more readily interpreted relationship becomes apparent. The parameters from Tables 4-30 and 31 are normalized to protein in Tables 4-32 (P. halodurans) and 33 (A. luteo-violaceus). The variability of total cellular sulfur per unit protein is the smallest of the three major elements of biomass studied (^{14}C data are again for exponential growth only). A large array of nonprotein carbon and nitrogen compounds in bacteria is indicated by the great variability of these elements with respect to protein.

Sulfur incorporation into protein is an excellent indicator of protein synthesis under all the conditions described in this chapter. Several useful relationships exist, depending on the desired application. For laboratory studies of protein synthesis in pure cultures, a simple and reliable method is the cold TCA-insoluble ^{35}S:total protein ratio. Determination of the ratio for each bacterium would provide a sensitive method for the routine measurement of protein synthesis, providing a

Table 4-32. Variability of Bulk Parameters in P. halodurans Normalized to Protein[a]

Weight %[b]	n	$\bar{X}$	S.D.	C.V.(%)	Range
POC	24	96	87	91.1	22-466
PON	26	37	66	178.5	6-324
Total ^{14}C	26	124	46	36.9	79-280
Total ^{35}S	95	1.20	0.20	16.2	0.78-1.73
TCA-insoluble ^{35}S	95	1.00	0.12	12.4	0.74-1.26
Protein ^{35}S	99	0.83	0.13	15.6	0.55-1.13
Protein ^{35}S[d]	80	0.86	0.12	14.3	0.64-1.13
Protein ^{14}C	18	58.0	9.2	15.8	49.2-79.9
Protein ^{35}S/ Residue Protein[e]	60	1.07	0.17	15.6	0.80-1.45
Protein ^{14}C/ Residue Protein[e]	15	61.2	13.7	22.5	48.9-88.3

[a] Combined data from all experiments.

[b] (μg/μg total protein) x 100.

[c] Perkin-Elmer CHN analysis data.

[d] Data for cultures grown with <500 μM sulfate removed.

[e] Normalized to the amount of protein found in the residue from the fractionation procedure for cultures with ≥500 μM sulfate.

Table 4-32. Variability of Bulk Parameters in A. luteo-violaceus Normalized to Protein[a]

Weight %[b]	n	$\bar{X}$	S.D.	C.V.(%)	Range
POC	20	83	40	47.6	46-226
PON	20	23	13	59.8	13-75
Total ^{14}C	14	82	14	17.2	62-110
Total ^{35}S	59	0.94	0.24	25.4	0.53-1.65
TCA-insoluble ^{35}S	59	0.78	0.14	18.1	0.48-1.20
Protein ^{35}S	59	0.69	0.13	18.5	0.43-1.09
Protein ^{35}S[d]	45	0.72	0.11	15.9	0.57-1.09
Protein ^{14}C	14	46.4	3.3	7.1	40.6-53.5
Protein ^{35}S/ Residue Protein[e]	10	0.92	0.15	15.9	0.75-1.20
Protein ^{14}C/ Residue Protein[e]	6	68.6	2.6	3.8	65.6-72.1

[a] Combined data from all experiments.

[b] (μg/μg total protein) x 100.

[c] Perkin-Elmer CHN analysis data.

[d] Data for cultures grown with <100 μM sulfate removed.

[e] Normalized to the amount of protein found in the residue from the fractionation procedure for cultures grown with ≥100 μM sulfate.

higher degree of resolution than is available using bulk protein assays yet retaining quantitative rather than qualitative features. Studies of field populations require further separation into residue protein ^{35}S because of high blanks associated with the amount of isotope necessary to detect bacterial activity in seawater (Chapter 5) but little or no added variation is introduced by the procedure. There is, therefore, a working measurement amenable to use in seawater which is quantitative.

Finally, the data obtained in this study are in excellent agreement with the model protein of Jukes et al. (1975), which indicates a sulfur content of 1.1% in protein. The true weight % S in protein, i.e. the residue protein-S:residue protein ratio, is 1.07 for P. halodurans and 0.92 for A. luteo-violaceus. Since the model protein composition is derived from numerous sources from all kingdoms, the agreement of the sulfur content of protein for the two bacteria examined strongly suggests that the relationship described above will be valid for natural assemblages of marine bacteria.

CHAPTER 5

APPLICATION OF SUBCELLULAR FRACTIONATION TECHNIQUES TO THE STUDY OF SULFUR, PHOSPHORUS, NITROGEN, AND ORGANIC CARBON METABOLISM BY NATURAL MARINE BACTERIAL POPULATIONS

INTRODUCTION

Studies of marine bacterial activity have concentrated on mineralization rates and turnover times of organic compounds (Azam and Holm-Hansen, 1973; Dietz et al., 1977; Hodson and Azam, 1977; Jannasch and Wirsen, 1973; LaRock et al., 1979; Sieburth, 1976; Wright and Hobbie, 1966). Unfortunately, the results of heterotrophic activity measurements are usually qualitative and not comparable among sampling regions or times. The concentration of some substrates, especially amino acids and glucose, can be measured in conjunction with uptake experiments (Vaccaro et al., 1968; Williams et al., 1976) and demonstrate that the bacterial assimilation of organic compounds can provide a significant source of particulate carbon for higher organisms in coastal waters. A method for the estimation of bacterial growth rates in unsupplemented seawater by a chemostat dilution technique (Jannasch, 1967) and the measurement of microbial ribonucleic acid (RNA) synthesis (Karl, 1979) have been successfully applied to natural populations. Co-ordinated efforts to quantitatively ascertain the bacterial contribution to marine food webs are still too few to provide a comprehensive understanding of bacterial growth in the oceans.

Recent observations suggest that bacteria may provide an adequate nutritional source for the reproductive growth of marine animals (Karl et al., 1980). In the open ocean, animals capable of filter-feeding on particles in the bacterial size range (0.2-2 μm) have been repeatedly observed, often in large numbers (Harbison and McAlister, 1979; Wiebe et al., 1979), and knowledge of the potential transfer of microbial biomass

to these animals would be a useful contribution to the understanding of marine food web dynamics.

Estimation of total bacterial population growth using organic tracers of metabolic activity is impractical because of the large variety of metabolizable organic compounds present in seawater (see Chapter 1). It is virtually impossible to determine the concentrations and uptake rates of more than a select few of the carbon and energy sources available to marine bacteria. The absence of a "universal organic substrate" which is taken up by all bacteria at a rate proportional to biomass production precludes the quantitative use of organic compounds as tracers of microbiological production.

Surprisingly little attention has been directed towards the use of inorganic compounds for bacterial growth measurement. Bacteria require sulfur, nitrogen, and phosphorus in proportions similar to phytoplankton, and the same sources of these elements are available to them. In addition, each of the major inorganic nutrient elements S, N, and P are contained in restricted classes of biological molecules relative to carbon. Total uptake of these elements and their patterns of incorporation into major biochemical components of microbial cells (i.e. protein, RNA, DNA, and lipid) may therefore provide information on the rates of biosynthetic processes central to reproductive growth .

No attempts have been made to measure sulfate metabolism by marine bacteria, in part due to the large isotope dilution barrier posed by the nearly 30mM sulfate concentration. A sulfate uptake method used in freshwater (Jassby, 1975; Jordan and Peterson, 1978; Monheimer, 1974a) met with frustration due to the lack of correlation between sulfate uptake

and bacterial carbon production (Monheimer, 1978b). Consideration of the physiology of unbalanced bacterial growth with respect to carbon and sulfur metabolism argues against a close correlation between whole cell carbon and sulfur assimilation (Chapters 1 and 4). Indeed, the range of C:S ratios obtained with a single marine bacterium, P. halodurans, spans the entire range of purportedly constant values for this ratio (Table 4-30). However, sulfate incorporation into protein has been shown to be a very good measure of protein synthesis, an important component of reproductive microbial growth. Sulfur incorporation into protein may therefore provide a specific measurement of total microbial protein synthesis, a valuable index for both biomass production and food web trophodynamics studies.

Results obtained by the measurement of sulfate incorporation into protein may be supported by simultaneous determination of nitrogen and phosphorus assimilation. The substantial proportion of nitrogen in nucleic acids, proteins, and precursors to these macromolecules suggests that nitrogen metabolism is also closely related to reproductive growth. Phosphorus is an essential component of nucleic acids and structural lipids and can be stored as polyphosphates, but is virtually absent in protein. The specific incoporation pattern of phosphate into RNA, DNA, phospholipids, and polyphosphates can therefore complement sulfur and nitrogen assimilation measurements, providing further data on specific aspects of microbial growth and metabolism.

Most of the world ocean inhabited by bacteria lies below the euphotic zone where mineralization and mixing with deep water provide an adequate supply of inorganic nutrients. In such an environment the uptake and incorporation of inorganic compounds is likely to be directly proportional

to growth, and often fulfill the requirements of a quantitative rate measurement of microbial growth in the field, i.e.:

(1) the substrate concentration must be measurable;

(2) the addition of labeled tracer must not alter the ambient concentration of substrate significantly nor affect the chemistry of the sample;

(3) there must be no competing compounds which interfere with the uptake of the tracer or replace it in metabolic processes;

(4) the substrate of choice must not be taken up or metabolised to a significant extent by non-microbial components of the sample;

(5) the substrate of choice must be taken up and metabolised by all actively growing members of the microbial community;

(6) the uptake or incorporation of the substrate must be interpretable in terms of microbial growth under the range of conditions likely to be found in the natural environment.

The measurement of sulfate incorporation into protein satisfies each of the above-mentioned requirements. As previously discussed (Chapter 1), 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, indicating that sulfate is the only significant source of sulfur for bacterial protein synthesis. While reductive assimilation of sulfate into protein is restricted to microorganisms, it appears to be universal among them, thereby functionally differentiating the entire microbial community from protozoa and higher animals. Additionally, the pool size of sulfur-containing protein precursors is extremely small in bacteria, minimizing compli-

cations arising from label dilution by large endogenous pools.

The results of studies on bacterial sulfate incorporation into protein (Chapter 4) establish a useful relationship between sulfur metabolism and bacterial growth for two marine bacteria. The application of a method found useful for pure cultures of individual organisms to natural, mixed populations requires that the generalized relationship be applicable within narrow limits of variation to all microorganisms encountered. If so, the sixth and final requirement for a quantitative measurement applicable to natural marine microbial populations will be fullfilled.

This chapter presents work relevant to the application of sulfate incorporation into protein as a measurement of bacterial protein synthesis in unenriched natural populations. Three major investigations were conducted as follows:

First, the applicability of the residue protein-S:total protein ratio to natural populations was verified using a series of enrichments of natural populations with analysis of the protein-S:total protein ratio. Additionally, isolates obtained from similar enrichments were tested in pure culture for the same parameter, as were bacteria representative of chemoautotrophic and anaerobic modes of metabolism.

Second, sulfate incorporation into protein and glucose metabolism were measured simultaneously in a highly eutrophic marine system in order to work out field methodology without pressing the limit of sensitivity.

Third, the rates of sulfate incorporation into protein by natural marine bacterial populations in the western North Atlantic Ocean were determined for stations representing waters of the Continental Shelf, Continental Slope, and Sargasso Sea. Simultaneous uptake measurements for

phosphate, ammonia, acetate, glucose, and glutamate were made in an effort to compare the rates of a number of metabolic and biosynthetic processes. Pertinent standing crop analyses were also made to allow the greatest degree of comparison among stations.

MATERIALS AND METHODS

Natural Population Enrichments: Enrichments of Sargasso Sea water were made on R/V Oceanus cruise numbers 69 (September, 1979) and 75 (November, 1979) in the immediate vicinity of 37°28.7'N, 64°03.4'W. The location is a standard station for the deployment of microbiological samplers and is located by pinger. Samples taken from below the euphotic zone (usually about 500m) with EtOH-washed Niskin bottles were prefiltered through 28 μm mesh Nitex net to remove particles and dispensed into sterile flasks. Inorganic nutrients (Chapter 2) 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. Isolation of pure cultures was accomplished by spreading dilutions of the enrichments on seawater medium containing inorganic nutrients and the growth substrate, solidified by 1.5 % agar (Difco). Colonies were picked, restreaked several times, and the isolates maintained on slants of the same medium.

The isolates obtained on R/V Oceanus cruise # 75 were screened for nutritional capability to select apparently different bacteria. Seawater plates containing about 25 individual carbon compounds were streaked with suspensions of the isolates, and liquid cultures using ethanol as the growth substrate were inoculated. An additional test was made for the

utilization of nitrate as the sole source of nitrogen. Based on colony morphology, visual observation, and the ability to grow on the various carbon and energy sources, 11 of the 70 isolates were determined to be different.

Determination of the Residue Protein-S:Bulk Protein Ratio for Natural Bacterial Populations: The enrichment series made on R/V Oceanus cruise # 69 was labeled with $^{35}SO_4^=$ for measurement of sulfate incoporation into protein. The experimental details are found in the text. Samples fixed with 10% TCA were centrifuged and the pellet rinsed twice with 10% TCA, then one of each pair was fractionated as described in Chapter 2. The entire protein residue was counted. The second pellet was dissolved in 0.1N NaOH and assayed for protein (Bradford, 1976). The average error for duplicate assays was 2.6%. The sulfate concentration 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 (Rosenbauer et al., 1979), giving a value of 22.64 ± 0.10mM (n=3).

Time-Course Measurement of Uptake by Natural Populations During R/V Oceanus Cruise #84: Uptake rates of isotopically-labeled sulfate, phosphate, ammonia, glucose, acetate, and glutamate were determined at three stations on a transect from Woods Hole to the Sargasso Sea. Details of date, position, water column depth, etc. are found in the text. The following general procedures were adopted: 45-50 liter samples were collected from below the euphotic zone with EtOH-washed Niskin bottles and prefiltered through a 28 μm mesh Nitex net into a large carboy which had

been rinsed three times with the sample. Samples for uptake rate measurement of organic carbon compounds (3 liters), sulfate (3 liters), and phosphate (1.5 liters) were dispensed into sterilized aspirator bottles with magnetic stirring bars and plugged with sterile cotton stoppers. A 10 liter sample was dispensed into a carboy for ammonia uptake. The samples were inoculated with 0.25ml UL-^{14}C-labeled acetate, glutamate, or glucose (50 μCi/ml; final activity about 10,000 DPM/ml); 0.3ml carrier-free $^{35}SO_4^=$ (200mCi/ml; final specific activity about 0.6 DPM/pMole); 1ml carrier-free $^{32}PO_4^=$ (final activity about 25,000 DPM/ml); or 1.5ml $^{15}NH_4^+$ (95 atom %; 10 μM final enrichment) as appropriate. Acetate and phosphate uptake were not measured at the Continental Shelf Station. Specific sampling methods are described below. All filtrations involving radioisotopes made use of the punch funnel described in Chapter 2 and were rinsed three times with 10ml unlabeled sample. The filtered samples were fumed for 10 seconds over 12N HCl to stop metabolic activity and frozen at -20°C until processing. Continental shelf and slope samples were incubated at 15°C, Sargasso Sea samples at 18°C.

Sulfate: Isotope was added with stirring and two 125ml samples were immediately withdrawn and filtered through Whatman GF/F filters for 0 time isotope adsorption blanks. Two one-ml samples were diluted 1:10 with distilled water for determination of radioactivity. Ten 125ml samples were drawn into sterile prescription bottles for long term incubation and nutrient enrichment experiments. One bottle each received (with respective final concentrations): NH_4^+ (50 μM), $PO_4^=$ (4 μM), complete inorganic nutrient mix (one-tenth strength; Eppley et al., 1967), or chloramphenicol (20 μg/ml), and all were sealed with serum stoppers. After brief

stirring, samples were filtered from the aspirator bottle (hereafter called "Flask") in duplicate until the 30 hour point (24 hours at the shelf station). At this time, one or two samples each were filtered from the flask and prescription bottles as well as the bottles containing the supplements described above. The flasks were washed and prepared for the next station. All later samples were taken from bottles.

Phosphate: The procedures for phosphate are the same as those for sulfate except that samples were taken at shorter intervals and no bottles were filled. Duplicate samples were not taken because of the limited amount of sample and the necessity of more closely spaced sampling points. All samples were incubated in the flask. One-ml samples were added to 10ml Aquasol for radioactivity determination.

Ammonia: One-liter samples were filtered immediately after isotope addition and at the same intervals as phosphate. Duplicate and enrichment samples were not taken. Filters were rinsed with unlabeled seawater, the funnel removed, and the edge rinsed again to remove label trapped in the filter matrix.

Organic Compounds: Inoculation of the flask with radio-isotope and distribution into bottles for long term incubation and enrichment experiments was performed as described for sulfate. In addition to the 105-115ml filtered samples, samples were taken for acid-volatile radioactivity (1 ml) and respiration by the wick method (10ml) as described in Chapter 2. The retention of labeled cells by the GF/F filters was moni-

tored by filtration of a 10ml aliquot through a 0.2 μm Nucleopore nitrocellulose membrane filter, which was placed directly in Aquasol for counting. Isotope addition inceased the ambient substrate concentrations as follows: acetate, <10nM; glucose and glutamate, <2nM.

Other Samples: While dispensing the sample into flasks for the isotope uptake studies, an aliquot was filtered through a sterile 0.2 μm membrane filter for nutrient analysis using an all-glass filtering flask. Portions were dispensed into sterile prescription bottles and frozen at -20°C. From the bulk seawater sample, one prescription bottle was filled and brought to 0.5 glutaraldehyde for direct counts. The remainder of the sample was filtered through combusted GF/F filters in portions up to 5 liters for analysis of particulate organic carbon and nitrogen, protein, and carbohydrate by methods described in Chapter 2. Particulate samples were frozen in combusted glass tubes with foil caps.

Biochemical Fractionation: Samples for sulfate, phosphate, and organic carbon uptake were fractionated according to the procedure described in Chapter 2. For phosphate and organic carbon labels, the additional step for extraction of RNA was incorporated. Duplicate samples for sulfate were fractionated on different days, as were several pairs of the organic carbon filters. All phosphate fractionations were done together. Data are expressed as the difference between the sample radioacivity in each fraction and the corresponding 0 time blank value for the fraction. Samples in Aquasol were counted in a Beckman LS-100C liquid scintillation counter to a statistical error of 1 % or 50 minutes, whichever came first. Samples for sulfate incorporation into protein were counted for up to 100 minutes.

^{15}N Analysis: Samples labeled with ^{15}N were analyzed by atomic emmission spectrometry using a Jasco Model NIA-1 ^{15}N analyzer (Japan Spectroscopic Ltd., Tokyo) through the courtesy of David Lean. Zero time enrichments were subtracted from the data for each station.

Other Methods: CHN analysis was performed by Phil Clarner on a Perkin-Elmer Model 240 CHN analyzer. Nat Corwin determined NH_4^+, $NO_3^-+NO_2^-$, and $PO_4^=$ on the frozen samples by the methods of Strickland and Parsons (1965). Sulfate was determined as described previously. Direct counts by epifluorescence microscopy (Daley and Hobbie, 1975) were determined on samples fixed with 0.5% glutaraldehyde.

Isotopes: Carrier-free $^{32}PO_4^=$ (as orthophosphoric acid), carrier-free $^{35}SO_4^=$ (as the sodium salt), and UL-^{14}C-glutamate (>225mCi/mMole) were obtained from Amersham (Chicago, IL). UL-^{14}C-glucose (>230mCi/mMole) and acetate (as the sodium salt, >45mCi/mMole) were purshased from New England Nuclear (Boston, MA). $(^{15}NH_4)_2SO_4$ (filter-sterilized in distilled water at a concentration of 1mg N/ml) was obtained from BioRad Laboratories (Richmond, CA). Solutions containing ethanol were evaporated to dryness and reconstituted in distilled water. The addition of the mildly acid solutions for phosphate and glutamate was without effect on the sample pH.

Cultures: Nitrosococcus oceanus was provided by Stan Watson and grown in RLC-water containing 20mM NH_4Cl. The pH was maintained near 7.5-8.0 by

addition of sterile K_2CO_3. Desulfovibrio salexigens was grown by Craig Taylor in a modified RLC-water medium supplemented with Ca-lactate (10mM) as described in Taylor et al. (MS in preparation). Mixed cultures of fermentative bacteria were grown in liquid enrichment cultures of RLC-water as described (Chapter 3, Susan Henrichs Ph. D. Thesis, Woods Hole Oceanographic Institution, 1980).

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 in order to ascertain the protein-sulfur:total protein relationship for organisms responding to different growth substrates. Subsamples of the mixed cultures to which radiosulfate had been added were analyzed for total protein and fractionated for the analysis of residue protein sulfur; a summary of the results is presented in Table 5-1. The average weight per cent sulfur in protein, 0.94 ± 0.13, is similar to those obtained for P. halodurans (0.86 ± 0.12) and A. luteo-violaceus (0.72 ± 0.11), as is the coefficient of variation for the 22 samples of mixed populations. Streaking of the cultures on solid medium gave rise to numerous colony morphologies in most flasks, indicating that the residue protein-S:bulk protein ratio was not dominated by protein from single species.

To further verify the applicability of the protein-S analysis to natural populations and increase the accuracy of the mean value to be used

Table 5-1. Sulfur Content of Protein in Marine Bacteria: Results of Enrichments of Sargasso Sea Water with Organic Carbon Compounds During R/V Oceanus Cruise # 69[a]

	µg/ml Culture					
	Low Density		High Density		Weight % S[c]	
Substrate[b]	Protein Sulfur	Bulk Protein	Protein Sulfur	Bulk Protein	Low	High
Mannitol	0.11	13.6	0.18	21.0	0.82	0.85
Glucose	0.11	13.7	0.18	21.8	0.82	0.84
Sucrose	-	-	0.17	21.0	-	0.82
Mannose	0.04	5.1	0.19	21.9	0.87	0.85
δ-Gluconolactone	0.10	11.4	0.19	20.3	0.85	0.92
Lactate	0.16	18.7	0.19	19.7	0.86	0.97
Glutamate	0.17	19.3	0.60	64.7	0.85	0.92
Glycerol	0.20	18.6	0.22	18.4	1.07	1.21
Succinate	0.14	14.4	0.23	19.0	1.00	1.20
Pyruvate	0.19	19.6	0.20	17.6	0.95	1.15
Citrate	0.17	18.6	0.18	16.3	0.91	1.14
Glucosamine	-	-	0.17	20.4	-	0.83
				Overall Mean (n=22)		0.94
				Standard Deviation		0.13
				Coefficient of Variation (%)		13.1

[a]Seawater from 500m was dispensed into sterile 125ml flasks in 100ml aliquots and supplemented with 1ml sterile substrate solution, inorganic nutrients, and 0.1ml $^{35}SO_4^{=}$ (final activity, 10 µCi/ml; 0.8 DPM/pMole). The flasks were shaken at 250 RPM at 21-25°C. Shortly after the appearance of turbidity, duplicate 25ml samples were brought to 10% TCA (final concentration) and refrigerated until processing. A similarly fixed pair of 20ml samples was taken when the culture had ceased further visible growth. The determination of bulk protein and protein-^{35}S was carried out by the methods described in Chapter 2. Samples fixed at zero time were used for isotope blanks and were subtracted from the sample data.

[b]All substrates present at 10mM final concentration except for mannitol, present at 5mM. Substrates are listed in the order of turbidity appearance.

[c](µg protein-S/ µg bulk protein) x 100.

for conversion of protein-S measurement to protein synthesis rates, isolates from a later enrichment series (R/V Oceanus cruise # 75) were grown in pure culture and the cell number, sulfur, and protein relationships determined under completely defined conditions (Table 5-2). These parameters were also measured for an anaerobic sulfate-reducing bacterium, Desulfovibrio salexigens; a marine nitrifying bacterium, Nitrosococcus oceanus; and four mixed populations of fermenters enriched from a mud sample from the Pettaquamscutt River estuary (Kingston, RI) during cooperative experiments with other laboratories at Woods Hole. The results of these analyses are presented in Table 5-3. The great degree of variability in the subcellular distribution of sulfur among the isolates (Table 5-4) is notable in view of the similarity of the sulfur content of the isolated protein.

The mean, standard deviation, and coefficient of variation for all determinations (using the mean value for pure cultures with more than one determination; e.g. Pseudomonas halodurans) of the actual weight% S in protein ([residue protein-S/residue protein] x 100) and the operational weight % S ([residue protein-S/bulk protein] x 100) are:

Actual weight % S (n=13): 1.09 ± 0.14 (13.1%)

Operational weight % S (n=41): 0.93 ± 0.14 (15.1%)

The averages contain data from autotrophic, heterotrophic, fementative, and sulfate-reducing bacteria. In spite of the wide variety of nutritional characteristics represented, the relationship of residue protein-S to total protein is similar enough that the mean value can be used in estimating the total protein synthesis of natural marine microbial communities.

Table 5-2. Cell Number, Protein, and Sulfur Relationships for Isolates from Natural Population Enrichments on R/V Oceanus Cruise #40 and 75[a]

Isolate Number	Total Amount Analyzed					per 10^8 cells		Weight % S	
	Cell Number	µg Protein		ng Sulfur		Protein	Total S	Protein S	Protein S
		Bulk	Residue	Total	Residue	(µg)	(ng)	Bulk	Residue
				R/V Oceanus Cruise #75 Isolates					
4	1.88×10^9	610.5	578.6	9592.9	6102.8	32.5	510.3	1.00	1.06
9	Clumps	Floc	85.6	1841.7	1099.2	-	-	-	1.28
26	3.79×10^9	472.2	451.1	9244.1	4865.3	12.5	243.9	1.03	1.08
34	5.90×10^9	676.2	725.2	17,510.6	7324.3	11.5	296.8	1.08	1.01
35	4.75×10^9	706.0	488.3	8609.7	4545.8	14.9	181.3	0.64	0.93
44	1.07×10^9	37.8	-	497.2	309.8	3.5	46.5	0.82	-
46	Clumps	25.4	-	362.8	247.0	-	-	0.97	-
50	5.45×10^9	755.2	543.8	16,717.2	6055.7	13.9	306.7	0.80	1.11

Table 5-2. (Continued)

Isolate Number	Total Amount Analyzed: Cell Number	µg Protein: Bulk	µg Protein: Residue	ng Sulfur: Total	ng Sulfur: Residue	per 10^8 cells: Protein (µg)	per 10^8 cells: Total S (ng)	Weight % S: Protein S / Bulk	Weight % S: Protein S / Residue
				R/V Oceanus Cruise # 75 Isolates (Continued)					
54	Clumps	543.6	522.2	10,542.8	5840.8	-	-	1.07	1.12
63	Clumps	117.4	65.4	1528.6	873.0	-	-	0.74	1.34
65	2.60×10^9	846.0	693.3	10,439.6	6421.3	32.5	401.5	0.76	0.93
				R/V Oceanus Cruise # 40 Isolates					
30	Flakes	545.8	-	9617.8	5967.2	-	-	1.09	-
31	3.69×10^9	460.2	-	7452.2	4304.2	12.5	202.0	0.94	-
					Mean	16.7	273.6	0.91	1.10
					S.D.	10.3	141.5	0.15	0.14
					C.V. (%)	61.8	51.7	16.8	12.8

[a]Cultures were recovered from minimal medium slants and inoculated into complete RLC-water medium containing 1mM sulfate and 10mM of the substrate used for their original isolation. When turbid, 0.1ml was inoculated into 40ml complete medium containing 1mM $^{35}SO_4^{=}$ (specific activity 2 DPM/pMole) and 10mM of either glutamate (# 4, 9, 34, 35, 44, 46, 54, 63, 65), glucose (# 30, 31), or succinate (# 26, 50). The cultures were shaken at 250 RPM, 20°C until visually dense, at which time they were harvested by centrifugation, rinsed twice in RLC-water, and resuspended in a small amount of RLC-water. Duplicate aliquots were prepared for bulk protein analysis and biochemical fractionation as described. Direct cell counts were made on dilutions of the remaining suspension for cultures with uniform cell distribution.

Table 5-3. Sulfur Content of Protein in Nitrosococcus oceanus, Desufovibrio salexigens, and Four Mixed Populations of Fermenters Enriched from a Mud Sample from the Pettaquamscutt River Estuary[a]

Organism	Protein Weight % S	
	Protein-S	Protein-S
	Bulk	Residue
Nitrosococcus oceanus (n=1)	0.78	1.31
Desulfovibrio salexigens (n=15)	1.10[b]	1.00
Fermentative Bacteria		
Glucose	0.83	-
Galactose	0.83	-
Fructose	1.14	-
Mannitol	0.86	-

[a] N. oceanus was grown in RLC-water medium containing 20mM NH_4Cl, 1mM $^{35}SO_4^=$ (1mM final concentration; 2 DPM/pMole), and 0.1% phenol red. The pH was adjusted as necessary with 0.1M K_2CO_3. Cells were harvested by centrifugation, washed twice with RLC-water, and resuspended in RLC-water. Duplicate aliquots were fractionated or assayed for bulk protein as described. D. salexigens was grown in an RLC-water medium modified for use with sulfate-reducing bacteria. Experimental details are found in Taylor et al. (MS in preparation). Fermenters were enriched from frozen sediments as described in Chapter 3 of S. Henrichs' Ph.D. Thesis (Woods Hole Oceanographic Institution, 1980). $^{35}SO_4^=$ was present at a specific activity of 2 DPM/pMole.

[b] Based on interpolated values for bulk protein.

Table 5-4. Distribution of $^{35}SO_4^=$ in Biochemical Fractions of Marine Bacteria in Pure Culture[a]

Organism	% of Total Radioactivity					
	$SO_4^=$	L.M.W. Organic	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
N. oceanus	2.0	19.4	8.3	4.3	13.4	52.6
D. salexigens	ND[b]	5.1	0.9	3.4	5.4	85.2
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
Unidentified Isolates: R/V Oceanus Cruise # 75						
4	0.8	15.7	13.7	1.3	4.9	63.6
9	3.4	15.7	7.5	1.9	11.8	59.7
26	3.0	19.5	16.1	1.6	7.2	52.6
34	6.4	31.0	10.8	1.4	8.5	62.3
35[c]	1.4	29.8	10.8	1.0	4.2	52.8
44	1.8	19.0	6.3	1.9	8.5	62.3
46	2.3	14.8	2.8	2.0	10.0	68.1
50	5.6	44.8	6.7	0.8	5.9	36.2
54	0.2	22.0	14.3	2.3	5.7	55.4
63	9.0	13.3	9.5	1.8	9.4	57.1
65	1.5	16.8	14.5	2.2	3.5	61.5
R/V Oceanus Cruise # 40						
30	0.6	7.9	19.3	2.3	7.9	62.0
31	2.1	16.5	15.3	1.2	7.2	57.8
Mean	2.6	19.0	9.6	1.9	7.5	60.6
Standard Deviation	2.4	9.2	5.3	0.9	2.7	10.4
C.V. (%)	92.3	48.8	55.4	47.3	36.2	17.1

[a]Experimental details in Table 5-2.

[b]None detected.

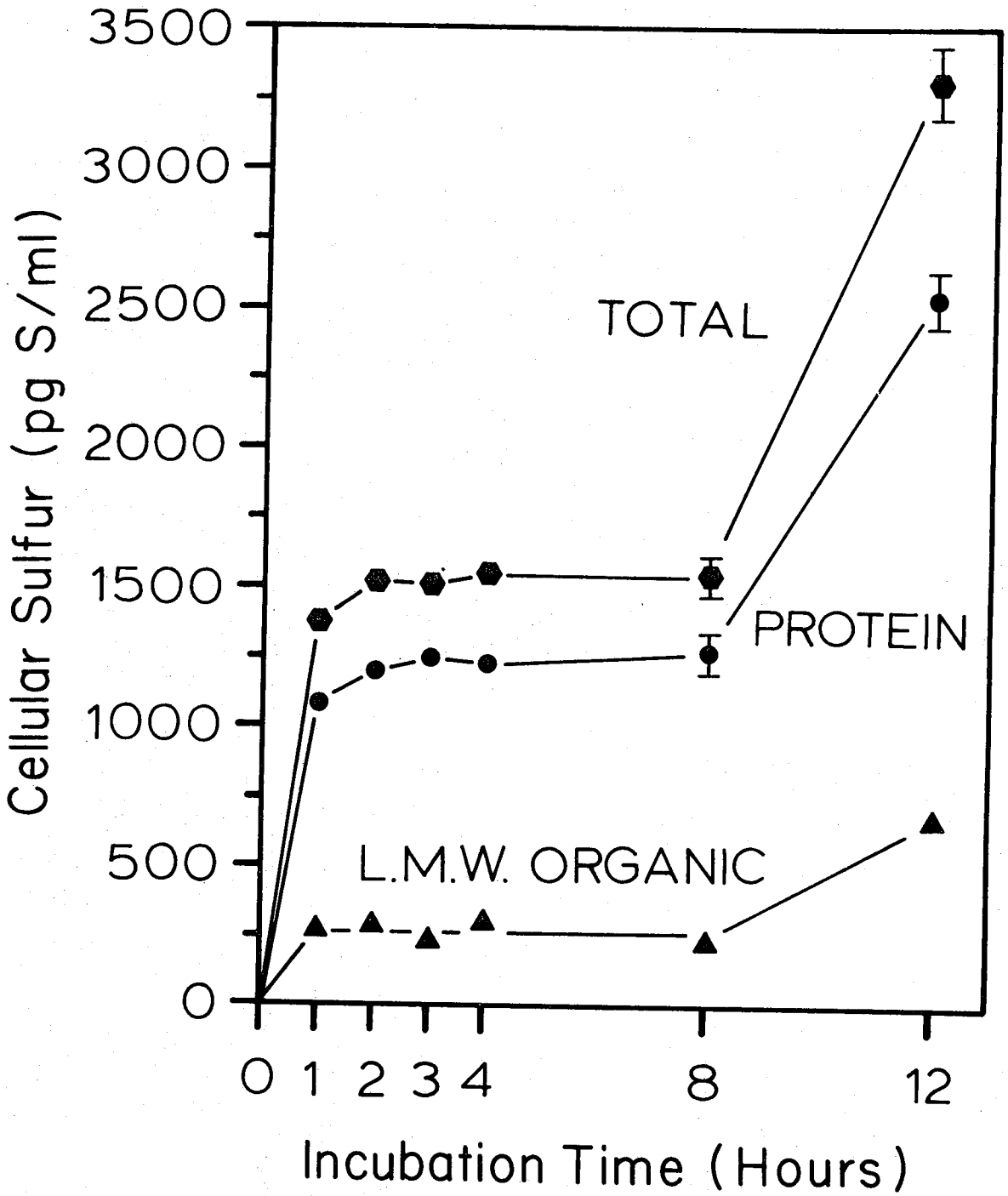
[c]Closely resembles A. luteo-violaceus.

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 (14mM) is almost 100 times the concentration at which sulfate limitation occurs in marine algae (Lewin and Busby, 1967) but gives a two-fold 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 results in an environment highly conducive to healthy bacterial growth. This was verified by the observation of a high cell density (4.4×10^6 cells/ml) of large, curved rod-shaped bacteria about 2x5 μm. Incoming seawater flushed the tank about three times per day, providing a semi-continuous culture regime; it was hoped that the sporadic input of nutrients from feeding and fish excretion would allow continued bacterial growth after removal of a sample from the system before the crash usually associated with sample withdrawl from a chemostat (Herbert, 1961; Jordan and Peterson, 1978; Karl, 1979).

The time course of sulfate incorporation into protein by the bacterial population in the fish tank is characterized by a rapid incorporation rate during the first hour followed by an extended plateau (Figure 5-1). The protein sulfur remains constant from 2-8 hours, but a doubling of

Figure 5-1. Time course of sulfur assimilation by natural bacterial populations in a brackish water fish tank. A two liter sample from a well-aerated half-strength seawater (chlorinity=9.97 o/oo) holding tank for Fundulus was rapidly filtered through 28 μm Nitex net to remove coarse particles. A one liter portion was poured into a large syringe made from a chromatography column (6x38cm; Bellco Glass Co.) fitted with a polycarbonate end cap and piston with rubber O-rings. Carrier-free $^{35}SO_4^=$ (final specific activity, 2 DPM/pMole) was added and mixed by inversion of the syringe. Subsamples were removed through a Luer-lock fitting in the end cap after flushing about 5ml of the sample through the port. Duplicate 50ml samples were filtered through Whatman GF/F filters at 0, 1, 2, 3, 4, 8, and 12 hours and processed as described in Chapter 2. The initial cell density was 4.4×10^6/ml, and the sample and incubation temperature was 22°C.


3500
3000
2500
2000
1500
1000
500
0
Cellular Sulfur (pg S/ml)
TOTAL
PROTEIN
L.M.W. ORGANIC
0 1 2 3 4 8 12
Incubation Time (Hours)

protein sulfur occurs during the next 4 hours. The assimilation of sulfate into L.M.W. organic material reaches a plateau during the first hour at about 20% of the total sulfur taken up, and increases between 8-12 hours as well, but to a more limited extent.

The total uptake of glucose and its transformation to CO_2 and acid-volatile compounds do not give any indication of the second growth phase between 8-12 hours of incubation (Figure 5-2). Glucose assimilation increases during the first three hours to a plateau which remains unchanged for the duration of the experiment. CO_2 production and transformation of glucose into acid-volatile material continue to increase for one hour after the cessation of incorporation of glucose into cell material. Acid-volatile radioactivity reaches a maximum at 4 hours, but CO_2 is continuously produced at a slow rate.

There is a great discrepancy between the respiration data obtained by the two methods described in Chapter 2, although the two methods agree well for respiration by a pure culture of an obligately aerobic bacterium. The traditional wick method indicates a maximum utilization (incorporation + respiration) of 63.5% of the total glucose (Table 5-5), compared to 83.4-93.4% for acid-volatile radioactivity. It is not clear at this time what acid-volatile materials other than CO_2 are being produced. It is likely that glucose is being transformed into some compounds which are not readily assimilable, since whole cell glucose uptake terminates at 4 hours, at which time twice as much ^{14}C is acid-volatile (total metabolism, 93.4% of the added label) relative to the amount recovered by the wick method (total metabolism, 59.5%). The M/I ratio for acid-volatile radioactivity (average 2.39) is similar to that for low

Figure 5-2. Total assimilation of ^{14}C-glucose by natural bacterial populations in a brackish water fish tank, with respiration determined by two different methods. Sampling and incubation procedures are described in Figure 5-1. UL-^{14}C-glucose (250mCi/mMole) was added at a final activity of 3.7 μCi/liter, and 10ml samples were filtered for total uptake (0.2 μm membrane filters) and fractionation into biochemical components (GF/F filters). Additional samples were taken for respiration by the wick method (5ml) and the acid-volatile method (2x0.5ml) as described in Chapter 2. Open hexagons are the sum of the fractions; closed hexagons are the activty of 0.2 μm filters counted unprocessed.

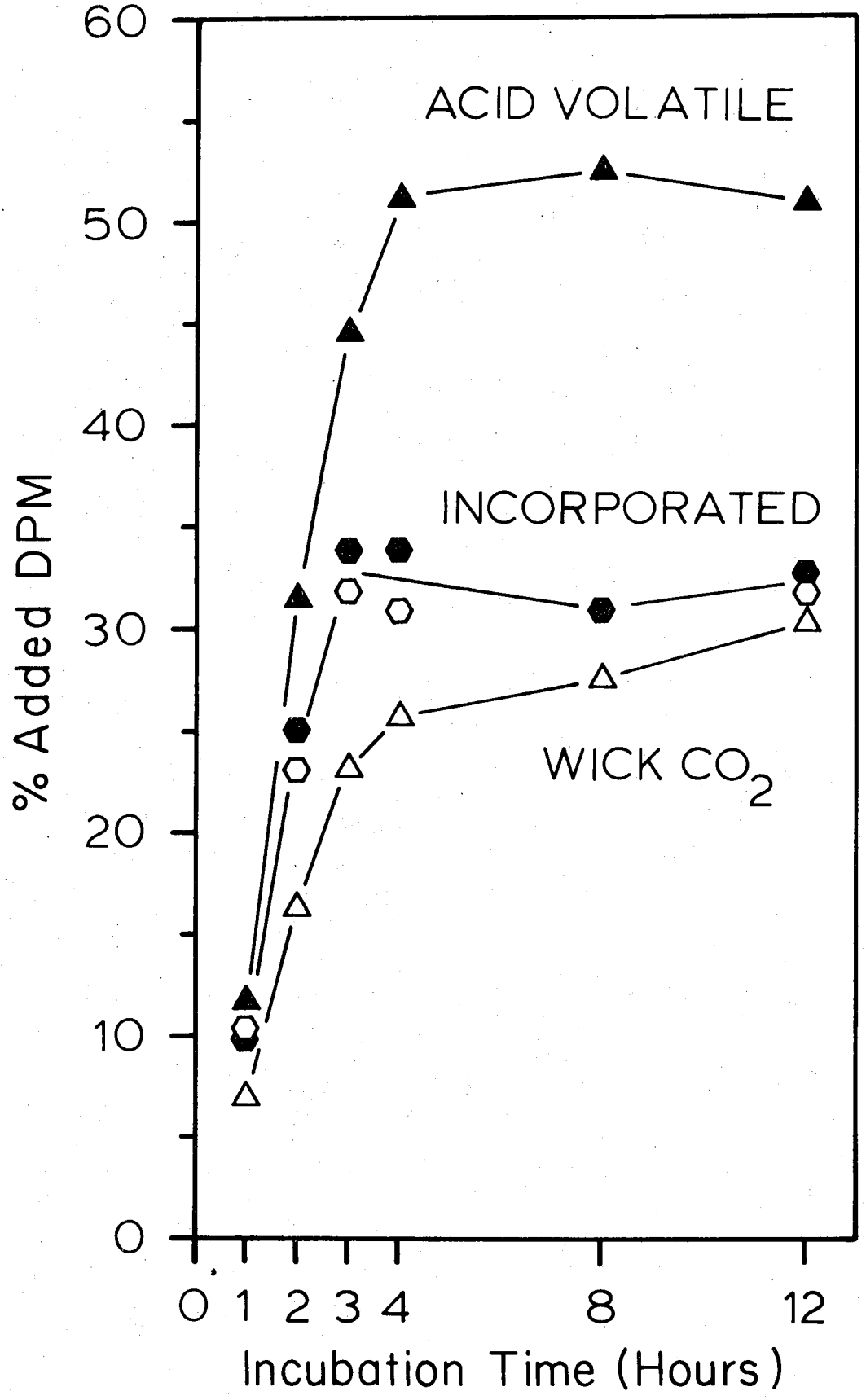

60
50
40
30
20
10
0
% Added DPM
ACID VOLATILE
INCORPORATED
WICK CO_2
0 1 2 3 4 8 12
Incubation Time (Hours)

Table 5-5. Respiration of ^{14}C-Glucose by Natural Bacterial Populations in a Fish Holding Tank Measured by Two Different Techniques[a]

Hours of Incubation	DPM ^{14}C-Glucose/ml								
	Assimilated	Respired		Total Metabolized		% Total Label Metabolized		M/I[b]	
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
1	805	568	965	1373	1770	16.7	21.5	1.71	2.20
2	2042	1338	2583	3380	4625	41.1	56.3	1.66	2.26
3	2779	1910	3664	4689	6443	57.1	78.4	1.69	2.32
4	2780	2112	4203	4892	7672	59.5	93.4	1.76	2.76
8	2535	2259	4315	4794	6850	58.3	83.4	1.89	2.70
12	2733	2485	4180	5218	6913	63.5	84.1	1.91	2.53

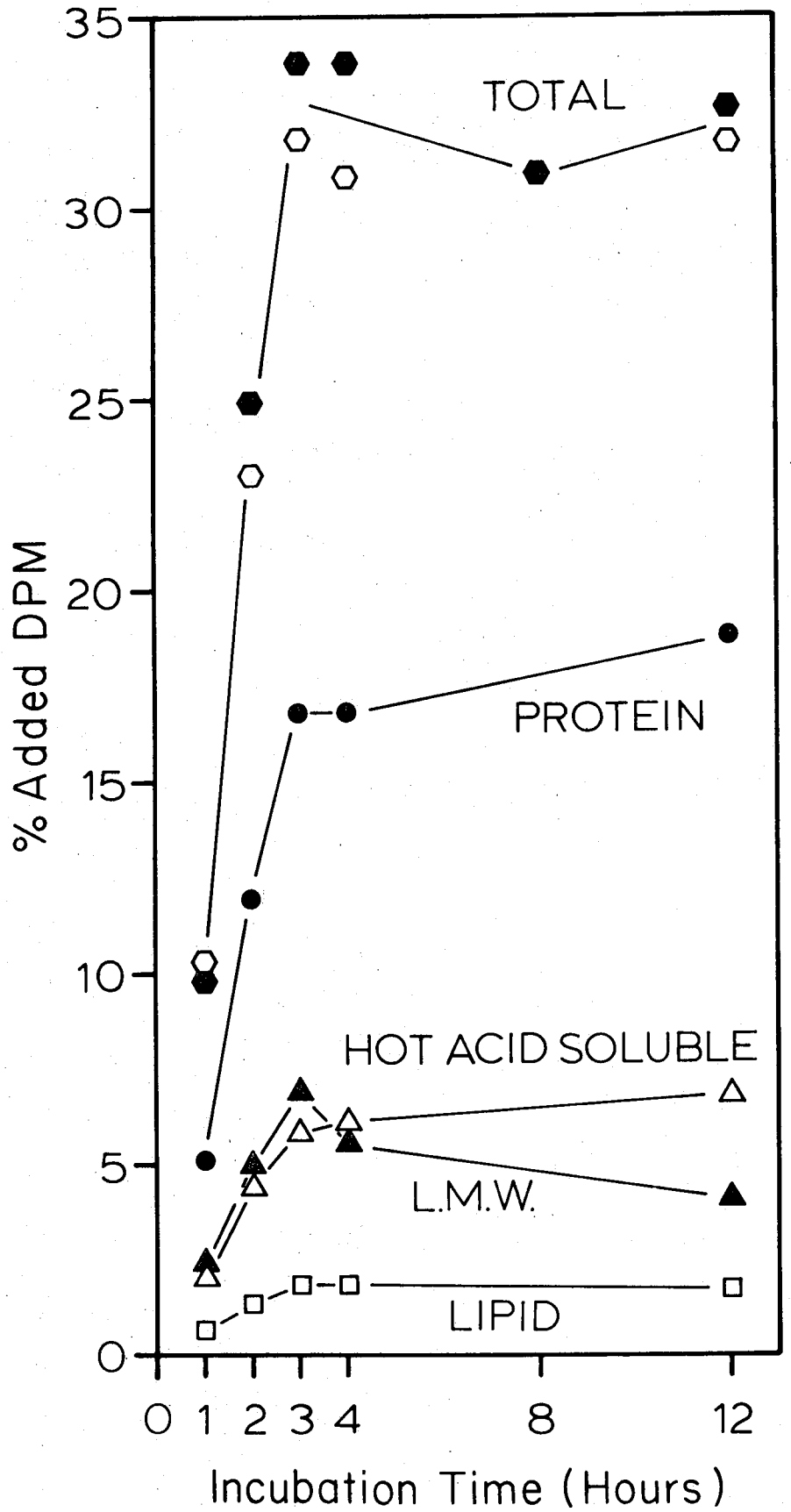
[a]Data from Figure 5-2. The methods used for the wick and acid-volatile radioactivity measurements of respiration are described in Chapter 2.

[b]Total metabolized/incorporated.

concentrations of bacteria in natural waters, whereas the M/I ratio for CO_2 (average 1.77) is lower than expected. If the acid volatile method is correct, the utilization of virtually all of the substrate explains the absence of further glucose incorporation during the 8-12 hour period.

Fractionation of the ^{14}C-labeled bacteria into major biochemical components provides indications of the secondary growth phase, even though total incorporation has ceased. Figure 5-3 shows a rapid increase in all fractions during the first three hours, with protein as the major end-product of glucose assimilation. The data on the distribution of ^{14}C and ^{35}S are appended in Table A-15. During the stationary period (as determined by whole cell ^{14}C), movement of label from the labile low molecular weight (L.M.W.) pool into residue protein (and to a lesser extent hot TCA soluble material) is evident. The ^{14}C content of lipid, a more structurally-related fraction, remains constant during this time. These data suggest 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. In the confined system containing a large amount of polymeric material (the fish food) it is possible that the action of extracellular hydrolytic enzymes provided substrates for the secondary growth period.

Figure 5-3. Total uptake of ^{14}C-glucose and its distribution in major biochemical fractions of bacteria from a brackish-water fish tank. Experimental details in Figure 5-2. Open hexagons: sum of the activity in each fraction. Closed hexagons: whole cell unprocessed controls.


35
30
25
20
15
10
5
0
% Added DPM
TOTAL
PROTEIN
HOT ACID SOLUBLE
L.M.W.
LIPID
0 1 2 3 4 8 12
Incubation Time (Hours)

Subcellular Fractionation Reproducibility and Influence of the RNA Extraction Procedure on Carbon, Sulfur, and Protein Distribution in Fractions of P. halodurans

Ribonucleic acid (RNA) is a major component of the total nucleic acids in bacteria, and its synthesis and degradation are excellent indicators of changing growth physiology (Herbert, 1961; Neidhardt and Magasanik, 1960). Bacteria in natural habitats may not be in a steady state of growth; hence the measurement of carbon and phosphorus assimilation into RNA may be a useful addition to the fractionation procedure used in this study. It is, however, necessary to test the procedure with a known system to aid interpretation. Cultures of P. halodurans labeled to equilibrium with either ^{14}C-glutamate or $^{35}SO_4^=$ were washed in unlabeled medium and resuspended at high density, and six samples each were prepared for fractionation. Three were processed by the normal method, and three were additionally extracted for RNA as described by Neidhardt and Magasanik (1960; see also Chapter 2). In addition, each supernatant fraction and the residue were assayed for protein. All six samples for each label were treated identically for cold TCA soluble, alcohol soluble, and lipid components as these treatments preceed the RNA extraction step. The results of the isotope distribution are shown in Table 5-6. The sum of the fractions of triplicates with and without RNA treatment agree within an error of about 1%. RNA treatment causes a significant redistribution of both ^{14}C and ^{35}S. Almost 25% of the sulfur and 40% of the carbon normally in the residue protein are shifted into the RNA and hot

Table 5-6. Reproducibilty of the Procedure for Fractionation of Isotopically-Labeled Bacteria into Major Biochemical Fractions[a]

	Per 10^8 Cells													
	L.M.W.		EtOH Sol.		Lipid		RNA		Hot TCA Sol.		Protein		Total	
	µg C	ng S	µg C	ng S	µg C	ng S	µg C	ng S	µg C	ng S	µg C	ng S	µg C	ng S
Without RNA Procedure														
1	4.56	33.98	1.37	25.38	1.36	3.64	–	–	2.47	8.16	11.26	127.11	21.02	198.27
2	4.43	34.20	1.55	27.52	1.33	3.74	–	–	3.67	12.87	9.99	121.65	20.96	198.99
3	4.33	33.20	1.50	26.71	1.33	2.89	–	–	3.00	11.91	10.67	124.18	20.82	198.89
Mean	–	–	–	–	–	–	–	–	3.05	10.98	10.64	124.31	20.93	199.05
S.D.	–	–	–	–	–	–	–	–	0.60	2.49	0.64	2.73	0.10	0.87
With RNA Procedure														
4	4.35	31.88	1.54	27.46	1.41	4.12	3.82	8.73	3.61	28.33	6.39	93.86	21.11	194.37
5	4.41	34.44	1.57	24.06	1.42	4.94	3.71	8.98	3.77	28.11	6.16	99.61	21.05	200.14
6	4.42	33.53	1.69	23.95	1.35	5.34	3.84	10.15	3.44	27.49	6.41	97.16	21.15	197.62
Mean	4.42	33.54	1.54	25.85	1.37	4.11	3.79	9.29	3.60	27.98	6.32	96.88	21.10	197.38
S.D.	0.08	0.93	0.10	1.62	0.04	0.90	0.07	0.76	0.17	0.44	0.14	2.89	0.05	2.89

[a] *P. halodurans* was grown in complete medium containing 5 mM UL-^{14}C-glutamate (44 DPM/nMole) or 1 mM $^{35}SO_4^{=}$ (2 DPM/pMole) and harvested by centrifugation in the late exponential phase. Six aliquots of washed cells were fractionated as described; three were extracted for RNA. Additional aliquots were taken for cell counts and protein. The number of cells fractionated was: ^{14}C, 3.3×10^9; ^{35}S, 3.6×10^9. The mean and standard deviation are shown for similarly treated samples.

TCA soluble fractions. There is a 2.5-fold increase in the amount of sulfur in hot TCA soluble material after RNA extraction, but the hot TCA soluble carbon increases by only 20%. There is a nearly equal distribution of carbon in the RNA and hot TCA soluble fractions, but only 4.5% of the total sulfur is extracted with RNA (one third as much as the hot TCA soluble sulfur). The amount of carbon need not be strictly proportional to DNA in the hot TCA soluble fraction, since this also contains cell walls and carbohydrates which have been hydrolyzed by hot acid.

The surprising redistribution of sulfur after RNA treatment is supported by protein analysis (Table 5-7). No protein (i.e. <0.2 μg/10^8cells) is found in cold-TCA soluble, lipid, or RNA fractions. In the absence of RNA treatment, protein is just at the limit of detection of the micro modification of the protein assay (Chapter 2), hence a large error is associated with the measurement. However, the RNA extraction step results in a shift of protein into the hot TCA soluble material from the residue protein. Some protein (6-8%) is lost, based on the sum of the protein from the various fractions with and without RNA treatment. The weight % carbon and sulfur in the residue protein indicate that a substantial amount of non-protein material in this fraction is released by warm alkaline hydrolysis. The loss of protein sulfur and protein by assay are equivalent, resulting in a constant 0.98% S by weight in the residue protein. On the other hand, carbon initially accounts for over 85% of the protein, a value far in excess of the 52% expected from either the Jukes protein (Chapter 4) or common sense. After RNA treatment, the weight % C in the residue protein drops to a more reasonable 63%.

Table 5-7. Distribution of Protein in Biochemical Fractions of Pseudomonas halodurans[a]

	µg Protein Per 10^8 Cells															
	EtOH Soluble				Hot TCA Soluble				Protein				Total			
	Carbon		Sulfur		Carbon		Sulfur		Carbon		Sulfur		Carbon		Sulfur	
	µg	Wt.%	µg	Wt.%	µg	Wt.%	µg	Wt.%	µg	Wt.%	µg	Wt.%	µg	Wt.%	µg	Wt.%
Without RNA Procedure																
1	3.5	38.7	3.8	0.67	0.8	308.8	0.1	8.16	12.8	88.3	12.9	0.98	17.1	65.8	16.9	0.75
2	3.3	47.0	3.5	0.79	0.7	524.3	0.4	3.22	12.1	82.3	12.3	0.99	16.2	61.6	16.2	0.75
3	3.9	38.3	3.7	0.72	0.3	1000.0	0.2	5.96	12.3	86.6	12.7	0.98	16.5	64.7	16.6	0.75
Mean	–	–	–	–	0.6	611.0	0.2	5.78	12.4	85.7	12.6	0.98	16.6	64.0	16.6	0.75
S.D.	–	–	–	–	0.3	353.7	0.2	2.47	0.3	3.1	0.3	0.01	0.5	2.2	0.3	0.0
With RNA Procedure																
4	3.4	44.9	4.3	0.63	1.8	200.6	1.6	1.77	10.1	63.2	9.4	0.99	15.3	41.8	15.3	0.61
5	4.0	39.4	3.8	0.63	1.6	235.6	1.2	2.34	9.5	64.6	10.1	0.99	15.1	40.8	15.2	0.66
6	4.3	39.4	4.0	0.60	1.6	215.0	1.3	2.11	10.3	62.5	10.1	0.96	16.1	39.8	15.4	0.63
Mean	3.7	41.3	3.9	0.67	1.7	217.1	1.4	2.07	10.0	63.4	9.9	0.98	15.5	40.8	15.3	0.63
S.D.	0.4	3.7	0.3	0.07	0.1	17.6	0.2	0.29	0.4	1.1	0.4	0.02	0.5	1.0	0.1	0.03

[a]Experimental details in Table 5-6. The weight % of carbon and sulfur in the protein in each fraction was calculated from the isotope data in Table 5-6. All fractions were assayed for protein with standards made in the appropriate solvents: no protein was detectable in L.M.W., lipid, or RNA fractions.

Sulfur is not a constituent of DNA or carbohydrate, but sulfur-containing RNA bases have been reported (Carbon et al., 1965; Lipsett, 1965). There is no information on the amount of the total cellular sulfur which is contained in RNA, but it must be small, since it only occurs in tranfer RNA. Without RNA extration, both RNA and DNA are solubilized by hot acid, and if it is assumed that no protein other than contaminants from the residue exists in this fraction, the amount of RNA sulfur in the cells would account for the hot TCA soluble radioactivity. This is 11ng S/10^8cells, and 9.3ng S are found in the RNA fraction after treatment. Furthermore, the absence of change in the weight % S in the residue protein after one-fourth of the protein has been redistributed argues that the sulfur-containing material found in the hot TCA soluble fraction after alkaline hydrolysis is purely protein.

The resolution of the redistribution of protein, carbon, and sulfur in the major fractions as a result of RNA extraction requires the identification of the compounds contained in each fraction, an analysis beyond the scope of this work. The data in Tables 5-6 and 5-7 and the chemistry of warm alkaline hydrolysis suggest that the RNA fraction contains only RNA, whereas the hot TCA soluble fraction contains protein in addition to DNA and carbohydrates. The acid soluble protein may be ribonucleoproteins, frequently of low molecular weight, which do not precipitate in the absence of nucleic acid polymers. Since virtually all the sulfur solubilized by RNA extraction resides in the hot TCA soluble material, it is clear that quantitative reprecipitation from the warm KOH solution takes place, further supporting the purity of the RNA fraction itself.

Phosphorus is not a significant component of protein, so the RNA extraction step should have little effect on its distribution except to separate RNA and DNA phosphorus. If the RNA fraction is pure, as suggested, the incorporation of carbon compounds into this fraction should be of value, even if some ambiguities exist in the interpretation of the hot TCA and residue protein fractions. Therefore, the carbon and phosphorus samples from R/V Oceanus cruise #84 were treated for RNA. Sulfate-labeled samples were not treated for RNA to maintain consistency with previous results, since all non-protein sulfur is apparently extracted by the normal procedure.

Aspects of Growth and Metabolism of Natural Marine Bacterial Populations in the Western North Atlantic Ocean

In July, 1980, a suite of time-course rate measurements was made on R/V Oceanus cruise #84 to determine the sensitivity of the measurement of sulfate incorporation into protein by natural, unenriched microbial populations. To ensure that only bacteria were being measured, samples were taken from well below the euphotic zone and prefiltered through 28 μm mesh Nitex net. It was felt that a comprehensive investigation of a number of related parameters at a few representative stations would be more valuable than a series of stations sampled in less detail for this initial application. In order to provide the fullest support for the sulfate incorporation measurement, simultaneous analysis of phosphate and ammonia uptake was made; as well as traditional uptake studies of three organic carbon compounds in common use. Standing crop parameters were analyzed to

provide background data on the chemical composition of the dissolved and particulate components of the sample.

Sampling Locations; Physical, Chemical, and Standing Crop Analyses

The sampling stations, dates, and other pertinent information are found in Table 5-8. For the remainder of this chapter, the stations will be referred to a Shelf (taken at the edge of the Continental Shelf), Slope (taken about half-way across the Continental Slope), and Sargasso Sea stations. These stations represent three different water masses of different chemistry and biology in an offshore transect from Woods Hole.

All nutrient and standing crop parameters were within the limits of detection of their representative assays. As expected, the highest concentrations of nutrients, bacterial cells, particulate organic carbon and nitrogen, protein, and carbohydrate were found at the Shelf station. The Slope station was intermediate in most parameters, and the Sargasso Sea station had many of the characteristics associated with highly oligotrophic regions, i.e. low nutrient concentrations, bacterial cell densities, and a high C:N weight ratio. Because of the depths sampled, nutrients were abundant at all stations relative to the cell numbers, even though the absolute values were low at the Sargasso Sea station.

Presentation of results of the time-course incubations will be made in the following manner: (1) profiles for sulfate incorporation into protein and whole cell uptake of ammonia, phosphate, and organic carbon compounds will be presented to give an overview of the relative rates of incorporation of the major elements; (2) results of fractionation of

Table 5-8. Physical, Chemical, and Standing Crop Data for R/V Oceanus Cruise# 84[a]

Parameter	Units	Continental Shelf	Continental Slope	Sargasso Sea
Date	1980	27 July	21 July	23 July
Latitude	-	40°07.9'N	37°49.4'N	37°29.3'N
Longitude	-	68°41.2'W	64°41.9'W	63°54.1'W
Water Column Depth	meters	175	2300	4900
Depth Sampled	meters	150	250	250
Sample Temperature	°C	11.5	11.3	18.4
Salinity	o/oo	35.636	35.092	37.101
Phosphate	µM	3.55	1.52	0.18
Nitrate + Nitrite	µM	14.12	23.23	2.62
Ammonia	µM	12.66	0.83	0.62
Particulates				
Organic Carbon	µg/l	35.1	31.3	23.5
Organic Nitrogen	µg/l	3.7	2.1	1.6
C : N	weight	9.5	14.9	14.7
Carbohydrate	µg/l	5.7	1.4	3.7
Protein	µg/l	4.8	3.7	4.0
Direct Counts	cells/ml	3.34×10^5	Lost	1.63×10^5

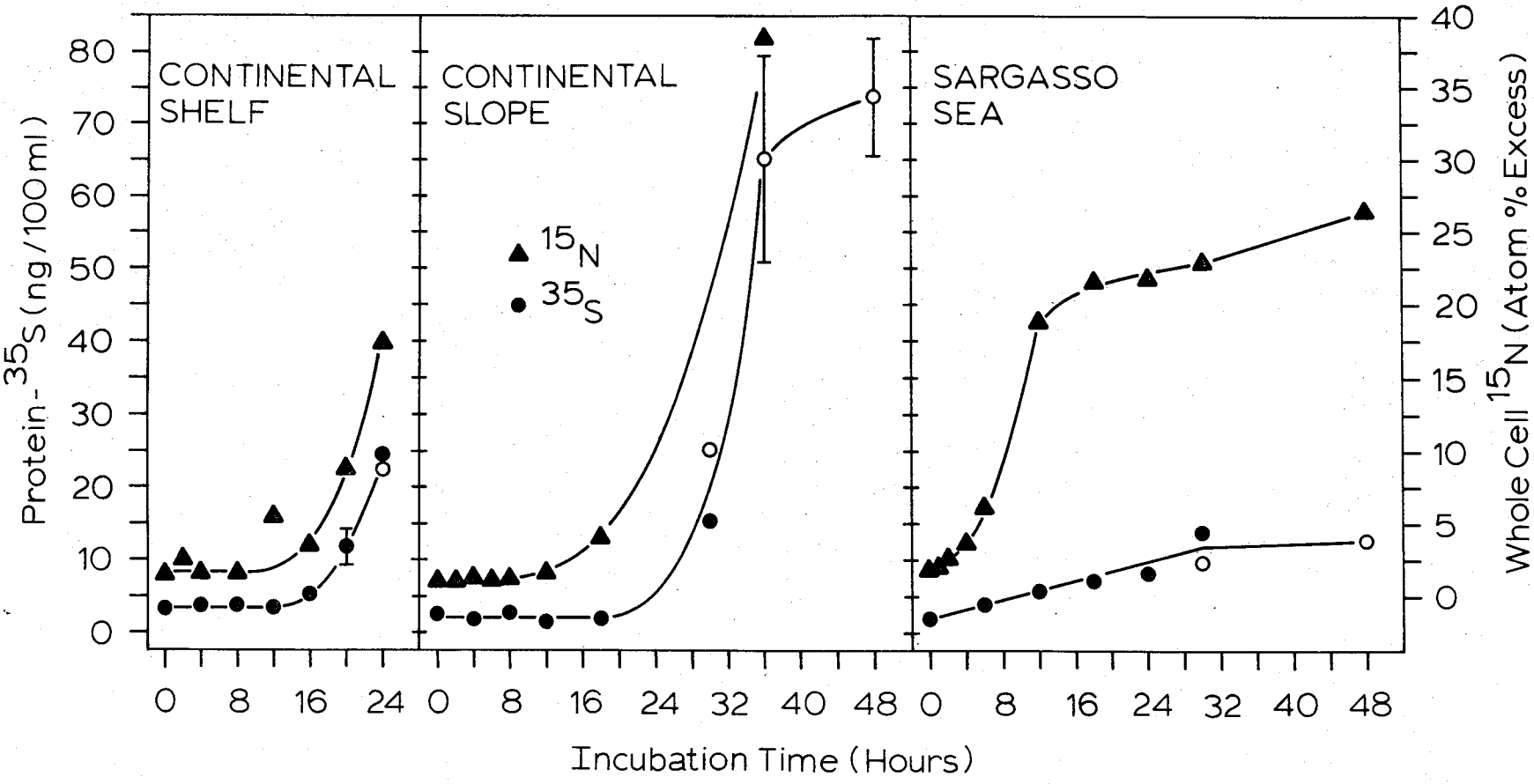
[a]Samples collected and processed as described in Materials and Methods.

$^{32}PO_4^{=}$ and ^{14}C-organic compound labeled samples will be summarized to point out specific metabolic patterns of the populations at the various stations with respect to specific nutrients; and (3) respiration of organic compounds, nutrient enrichment, chloramphenicol, and container effects will be discussed to indicate possible limiting factors and experimental artifacts. All fractionation data are found in the Appendix.

Sulfate Incorporation Into Protein and Total Uptake of Ammonia

The incorporation of sulfate into protein by bacterial populations at the three stations is shown in Figure 5-4, accompanied by total uptake of ammonia by whole cells. Detectable activity was exhibited at all stations, but some peculiarities in the uptake/incorporation profiles are apparent. A considerable lag period was observed at both continental shelf and continental slope stations for both elements, followed by nearly exponential increases in residue protein-sulfur and total ammonia uptake. In contrast, the Sargasso Sea station showed detectable uptake immediately. Although the initial rates of assimilation were not in keeping with expected decreasing activity on an off-shore transect, the absolute magnitude of uptake did fit the pattern. The continental slope and Sargasso Sea stations, for which extended incubations were made, give indications of growth cessation in the last (48 hour) sample, with 48 hour assimilation values substantially higher in the continental slope station. Since much of the data for the continental shelf station was taken in port, extended incubations could not be made, however, the end point (24 hour) assimilation data are higher than the same incubation time for the

Figure 5-4. Incorporation of sulfate into residue protein and assimilation of ammonia into whole cells of natural marine bacterial populations in the Northwest Atlantic Ocean. Sulfate incorporation measured for samples incubated in 3 liter aspirator flasks (closed circles) and 125ml prescription bottles (open circles). Sulfur data are the average of duplicates; error bars are shown when larger than the symbol.


CONTINENTAL SHELF
CONTINENTAL SLOPE
SARGASSO SEA
15N
35S
Protein-35S (ng/100 ml)
Whole Cell 15N (Atom % Excess)
Incubation Time (Hours)
0
8
16
24
32
40
48
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40

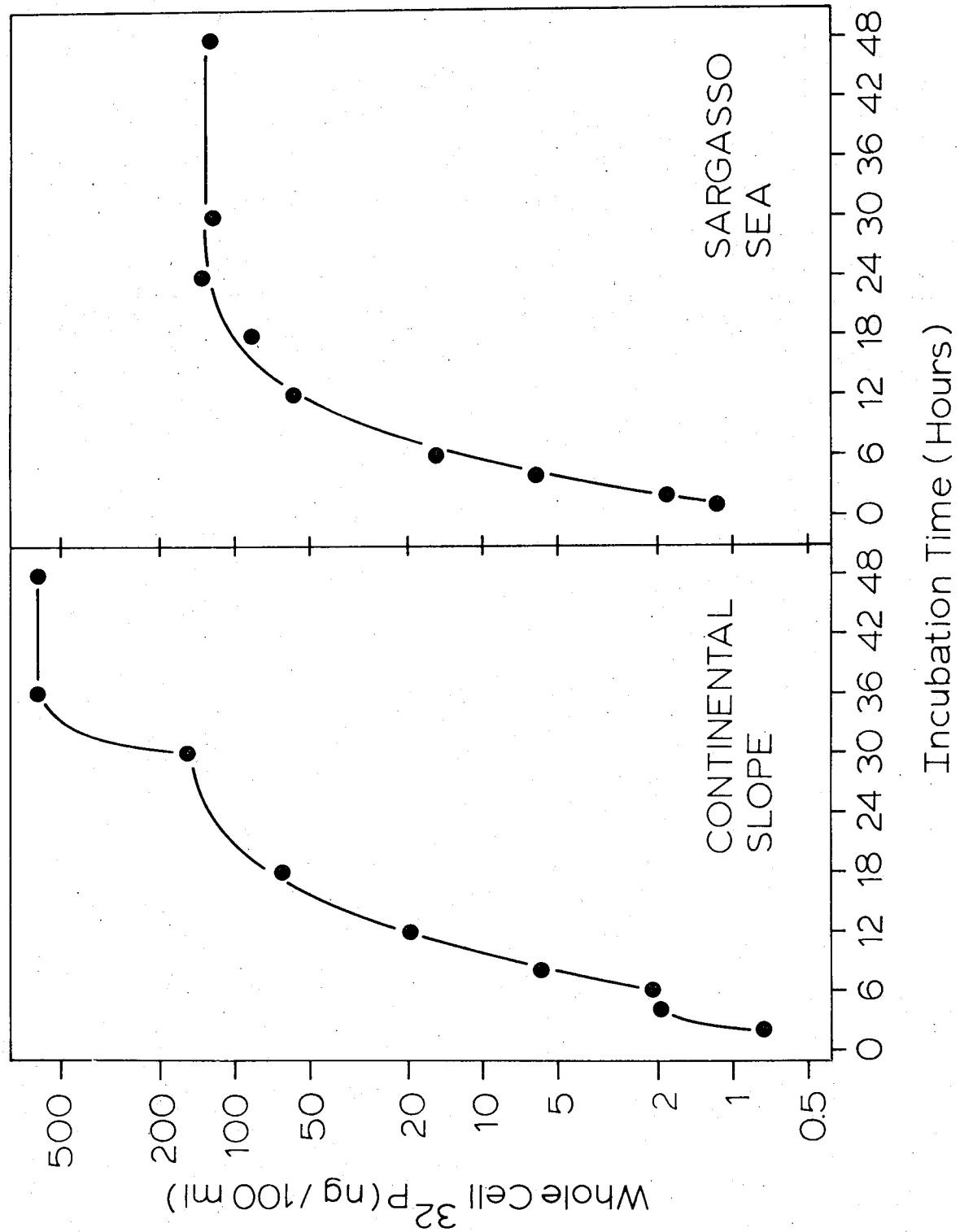
continental slope station and show no signs of decreasing uptake rate. Total utilization of the ammonia available (before enrichment with ^{15}N) was 8% at both continental shelf and continental slope stations, and 5 at the Sargasso Sea station. Quantitative results will be discussed at the end of this section, but the same trends for ammonia assimilation are obeyed since both dissolved ammonia and PON decrease on the off-shore transect.

In this figure only, the zero time blanks have not been subtracted in order to show the reproducibility of the blanks. Error bars for duplicate sulfate samples are shown when larger than the symbol. Sampling for ammonia assimilation was done at more closely spaced intervals for detection of possible luxury uptake, but no evidence for this phenomenon was observed at any station. At 30 hours of incubation (continental slope and Sargasso Sea stations) or 24 hours (continental shelf station) sulfate incorporation was determined for both flask and bottle incubated samples (closed and open symbols respectively), after which time bottle samples were used. All ammonia samples were incubated in the same 10 liter container. The blanks are similar for all stations.

Total Uptake of Phosphate

The assimilation of phosphate is depicted in Figure 5-5 for the continental slope and Sargasso Sea stations. The measurement was not made at the continental shelf station. Note the semi-logarithmic plot; all subsequent data will be shown as semi-logarithmic plots because of the great range of values during the 48 hour time-course incubations. Zero time

Figure 5-5. Total assimilation of $^{32}PO_4^{=}$ by natural bacterial populations in the Northwest Atlantic Ocean. All samples for each station were subsampled from a 1.5 liter aspirator flask. Data are the sum of activities in the major biochemical fractions of Figure 5-7 and 5-8.


Whole Cell ^{32}P (ng / 100 ml)
500
200
100
50
20
10
5
2
1
0.5
CONTINENTAL SLOPE
SARGASSO SEA
0 6 12 18 24 30 36 42 48 0 6 12 18 24 30 36 42 48
Incubation Time (Hours)

blanks have been subtracted. Phosphate uptake follows a distinctly biphasic and probably triphasic pattern at the continental slope station, with a marked increase in uptake at 30 hours. The Sargasso Sea station, on the other hand, exhibits a smooth exponential assimilation rate which decays to a plateau at 24 hours. As with sulfate incorporation and ammonia assimilation, the end points for phosphate are much higher at the continental slope station. Total utilization of the ambient phosphate was 13 % for the continental slope and 22 % for the Sargasso Sea stations.

Total Uptake of Acetate, Glucose, and Glutamate

Prior to discussion of the assimilation of organic carbon compounds, two important controls must be presented which bear on the data for all uptake and fractionation results. The first of these controls concerns the recovery of labeled cells by the glass fiber filters used for the fractionations. The nominal pore size for the GF/F filter is on the order of 0.7 μm, but recent literature indicates that a large proportion of bacteria in natural waters are <0.4 μm (Ferguson and Rublee, 1976; Hoppe, 1976; Watson et al., 1977). Therefore, at each sampling point for the organic compounds duplicate samples were filtered for fractionation and a third aliquot was filtered through a 0.2 μm membrane filter, which may be assumed to retain all the cells. A comparison of the sum of the radioactivity in each of the biochemical fractions for each sample with its associated whole cell filter is found in Appendix Table B-1 for the three stations. The overall recovery ([sum of fractions/whole cell filter]x100) for all samples (n=66) was 95.8% with 7 out of 8 time-course sets having

average recoveries between 90-106%, the only lower recovery being obtained for glutamate uptake at the continental slope station (average 85.9%). Thus the GF/F filter retains virtually all of the labeled material. It is likely that the effective pore size was reduced significantly by detritus and mucilaginous substances early in the filtration of the 105-115ml aliquots. Since all samples within a station contained the same bacterial assemblage, the recoveries for phosphate, sulfate, and ammonia-labeled cells should be similar.

The second control is the reproducibility of subcellular fractionation of isotopically-labeled cells. This was shown to be good for pure cultures (Table 5-6), but the exceedingly low amount of material fractionated with natural population samples (about $1x10^7$ cells of smaller dimension than P. halodurans) could introduce some error. Table 5-9 summarizes the error of fractionation of duplicate samples, broken down into the error for each fraction as well as the error for the sum of the fractions. Two complete time series from the Sargasso Sea station were fractionated on different days to determine the effects of new reagents, etc. The overall error for the sum of the fractions (n=37 pairs), 5.4%, was less than the error among fractions, indicating that good recovery was obtained and only redistribution of the labeled material occurred. Error associated with low molecular weight, lipid, hot TCA soluble, and protein fractions was normally under 10%. Although the % error in the alcohol soluble fraction is high, the absolute counts in this fraction are very low and a few DPM difference is hence a large % error. The largest variability among samples was inevitably associated with very low total uptake, as shown for the continental slope station where the first three

Table 5-9. Reproducibility of Subcellular Fractionation of Natural Bacterial Populations Labeled with ^{14}C-Organic Compounds[a]

		Average % Error Between Duplicates					
Substrate	Sum of Fractions	L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
			Continental Shelf Station				
Glucose	3.5(n=5)	6.2	17.9	4.3	4.3	6.2	7.9
Glutamate	3.5(n=5)	5.6	8.4	4.6	11.8	6.8	10.7
			Continental Slope Station				
Acetate	2.6(n=5)	9.0	13.5	2.5	16.4	2.3	6.8
Glucose	20.5(n=5)	20.1	10.5	0.2	21.9	3.4	7.1
Glutamate	2.2(n=6)	2.7	4.8	8.2	7.0	3.5	5.0
			Sargasso Sea Station				
Acetate	2.6(n=5)	1.9	13.8	4.0	11.2	2.8	2.1
Glucose[b]	4.4(n=5)	7.0	26.6	5.3	4.8	7.0	16.8
Glutamate[b]	0.3(n=1)	13.1	12.1	9.9	11.8	22.6	5.6
Overall Error	5.4(n=37)	7.5	13.7	4.9	10.1	5.2	8.0

[a]Data from Figures 5-9, 10, and 11 in the text. Duplicate samples filtered onto Whatman GF/F filters were fractionated as described. The error as a % of the average is listed for each fraction as well as for the sum of the fractions for each pair; the number of pairs is shown in parentheses.

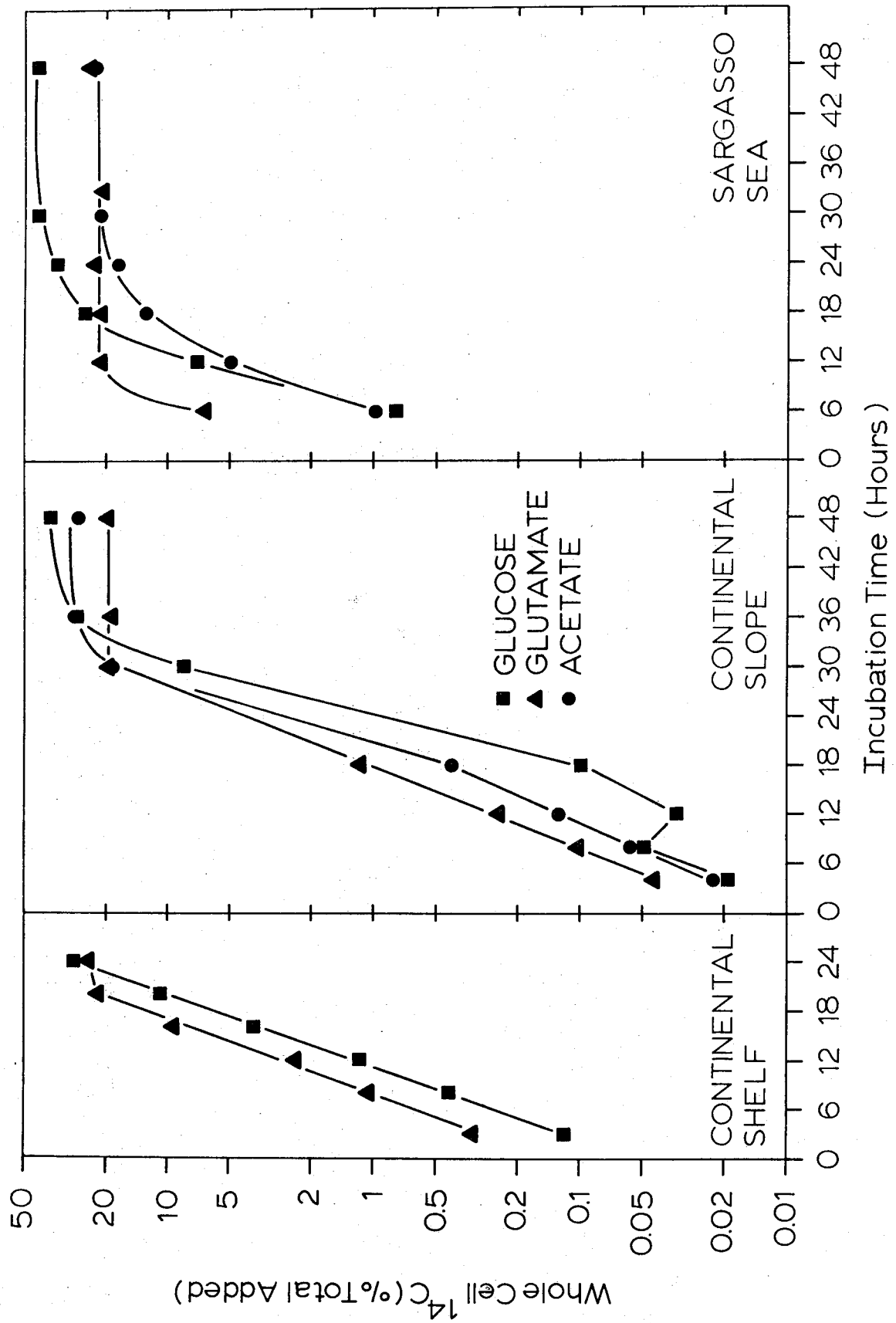
[b]Samples from each pair were fractionated on separate days.

time points for acetate and glucose were very near the blank value. It must be remembered that the error includes differences of total label incorporation between duplicates, which are substantially higher in field experiments than in pure culture experiments. Due to the great difference in total uptake between time points in most instances, an error of as much as 25% would have little effect on the interpretation of the data.

The total assimilation of acetate, glucose, and glutamate are shown in a semi-logarithmic plot for the three stations in Figure 5-6. The mean values (i.e. whole cell filters and sums of the fractions) for all samples are used. For both the continental shelf and continental slope stations, uptake is remarkably exponential rather than linear. The bacterial poplation at the Sargasso Sea station took up the substrates so rapidly that the first point (6 hours) was already near the end of the uptake curve. The relative lag time (e.g. time to reach 1% substrate utilization) obeys the same pattern as for sulfate incorporation into protein, total ammonia assimilation, and total phosphate assimilation for the three stations. The final amount of substrate metabolized depends on the respiration data as well as total uptake, and will be discussed in the next section.

The most remarkable feature of the uptake curves for the organic substrates is their apparent exponential increase. The semi-logarithmic plots are well fitted ($r>0.99$ for least-squares linear regression of t vs. ln uptake) for the continental shelf station, with doubling times for assimilation of glucose (interval 4-24 hours) and glutamate (interval 4-20 hours) both equal to 2.6 hours. Similarly well fit lines for acetate (t_d=3.3 hours, interval 4-18 hours), glucose (t_d=2.3 hours, interval

Figure 5-6. Total assimilation of UL-^{14}C-labeled acetate, glucose, and glutamate by natural bacterial populations in the Northwest Atlantic Ocean. Data are the means of the sum of activities in the major biochemical fractions (duplicate samples) and whole cell controls from Figures 5-9, 5-10, and 5-11.


Whole Cell ^{14}C (% Total Added)
5.0
2.0
1.0
0.5
0.2
0.1
0.05
0.02
0.01
0 6 12 18 24 0 6 12 18 24 30 36 42 48 0 6 12 18 24 30 36 42 48
Incubation Time (Hours)
CONTINENTAL SHELF
CONTINENTAL SLOPE
SARGASSO SEA
GLUCOSE
GLUTAMATE
ACETATE

12-36 hours), and glutamate (t_d=2.9 hours, interval 4-30 hours) are obtained at the continental slope station. In most instances, the data satisfy the requirement of measuring uptake for more than three generations to obtain logarithmically-linear plots of which the slope equals the growth rate (calculated from Equation 4-1). No such calculation may be made for the Sargasso Sea station due to the small number of pre-stationary points. It is extremely unlikely that the entire population is growing at the indicated rates, especially considering the profiles for sulfate and ammonia assimilation; however, portions of the assemblage responding to each substrate may be.

Quantitative Analysis of Uptake Data for Sulfate, Phosphate, and Ammonia Uptake

No attempt was made to determine the ambient concentrations of the organic carbon compounds used as tracers in this study. Quantitative values for sulfate, phosphate, and ammonia were obtained for all stations, however, so calculation of the absolute amount of each compound assimilated may be made. These results, along with the % total available organic compound assimilated, are presented in Tables 5-10, 5-11, and 5-12 for the continental shelf, continental slope, and Sargasso Sea stations respectively. Total assimilation of ammonia was calculated according to Equation 5-1 (Murphy, 1980)

$$\text{Total Uptake} = \frac{(\ ^{15}\text{N in Sample})\ (\text{PON})}{(\ \%\ ^{15}\text{N in } NH_4^+\ )} \qquad \text{[Equation 5-1]}$$

Table 5-10. Time-Course Incorporation Patterns for Major Nutrient Elements by Natural Bacterial Populations During R/V Oceanus Cruise # 84: Continental Shelf Station[a]

Hours of Incubation	^{14}C-Organic Carbon % Added Label Assimilated			$^{32}PO_4^=$	$^{35}SO_4^=$	$^{15}NH_4^+$	
	Acetate	Glucose	Glutamate	ng P per 100ml	ng Protein S per 100ml	Atom % Excess	ng NH_4^+-N per 100ml
2	-	-	-	-	-	1.04	8.99
4	-	0.1	0.3	-	0.55	0.11	0.95
8	-	0.4	1.0	-	0.50	0.17	1.47
12	-	1.2	2.3	-	0.29	3.97	34.31
16	-	3.8	9.4	-	2.11	2.01	17.37
20	-	10.8	22.0	-	8.65	7.32	63.27
24F	-	29.1	21.7	-	21.41	15.93	137.69
24B	-	28.7	26.4	-	19.22	-	-
24B +N	-	43.2	32.2	-	28.97	-	-
24B +P	-	22.3	26.6	-	20.23	-	-
24B +NBC	-	53.8	33.5	-	16.03	-	-
24B +CAP	-	0.2	0.7	-	0.86	-	-

[a]Data from Figures 5-4 and 5-6. Expressed as the difference between the value at the indicated time and a zero-time blank.

Table 5-11. Time-Course Incorporation Patterns for Major Nutrient Elements by Natural Bacterial Populations During R/V Oceanus Cruise #84: Continental Slope Station[a]

Hours of Incubation	^{14}C-Organic Carbon % Added Label Assimilated			$^{32}PO_4^{=}$	$^{35}SO_4^{=}$	$^{15}NH_4^{+}$	
	Acetate	Glucose	Glutamate	ng P per 100ml	ng Protein S per 100ml	Atom % Excess	ng NH_4^{+}-N per 100ml
2	–	–	–	0.75	–	0.01	0.02
4	0.0	0.0	0.0	1.93	-0.72	0.30	0.70
6	–	–	–	2.10	–	0.13	0.31
8	0.1	0.0	0.1	5.79	0.14	0.21	0.49
12	0.1	0.0	0.2	19.55	-1.00	0.63	1.48
18	0.4	0.1	0.1	64.03	-0.70	3.00	7.03
30F	12.6	9.1	16.6	153.91	15.20	–	–
30B	24.8	7.6	20.6	–	25.02	–	–
36B	28.6	28.3	18.6	617.18	64.98	37.46	87.83
48B	27.7	37.3	19.5	615.57	73.59	–	–
30B +N	26.7	23.6	20.3	–	24.38	–	–
30B +P	26.1	17.8	21.1	–	79.70	–	–
30B +NBC	28.0	45.9	19.9	–	42.13	–	–
30B +CAP	0.2	–	0.3	–	2.06	–	–

[a]Data from Figures 5-4, 5-5, and 5-6. Expressed as the difference between the value at the indicated time and a zero-time blank. Negative values indicate samples less than the blank.

Table 5-12. Time-Course Incorporation Patterns for Major Nutrient Elements by Natural Bacterial Populations During R/V Oceanus Cruise # 84: Sargasso Sea Station[a]

Hours of Incubation	^{14}C-Organic Carbon % Added Label Assimilated			$^{32}PO_4^=$	$^{35}SO_4^=$	$^{15}NH_4^+$	
	Acetate	Glucose	Glutamate	ng P per 100ml	ng Protein S per 100ml	Atom % Excess	ng NH_4^+-N per 100ml
1	-	-	-	1.16	-	0.14	0.25
2	-	-	-	1.83	-	0.75	1.32
4	-	-	-	6.09	-	1.78	3.12
6	1.0	0.8	6.6	15.23	2.02	4.36	7.64
12	5.0	7.2	21.1	57.32	3.75	16.96	29.71
18	12.9	25.3	21.1	85.38	5.29	19.73	34.56
24	17.4	34.9	22.9	133.14	6.31	19.92	34.90
30F	20.7	36.6	21.0	121.41	11.57	21.01	36.81
30B	22.6	49.0	-	-	6.63	-	-
48B	22.7	42.7	23.7	124.32	10.77	24.67	43.22
30B +N	25.8	51.5	32.1	-	9.04	-	-
30B +P	20.6	46.9	24.9	-	9.17	-	-
30B +NBC	35.5	54.7	30.7	-	10.90	-	-
30B +CAP	0.7	0.3	1.9	-	-0.20	-	-

[a]Data from Figures 5-4, 5-5, and 5-6. Expressed as the difference between the value at the indicated time and a zero-time blank. Negative values indicate samples less than the blank.

Zero time blanks have been subtracted, resulting in occassional sulfate incorporation values slightly less than 0. The trends observed for ^{15}N enrichment in samples labeled with ammonia are unaltered by the calculation.

End-point assimilation values for all three inorganic nutrient elements were in accord with the expected potential productivity of the three stations, but a substantial lag period was observed at both the continental shelf and slope stations. Inorganic nutrient limitation apparently did not occur in any of the samples, since less than 25% of the initially available nutrient was utilized. Lag times for uptake of the inorganic nutrient elements were similar in all cases and were reflected in the assimilation of the organic compounds as well.

The quantitative uptake ratios for N:P, N:S, and P:S are presented in Table 5-13. The sulfate data is for protein-S only. Several striking features are apparent, most notably the low N:P uptake ratio; a weight ratio of about 8 is expected from algal studies (Goldman et al., 1979). Based on pure protein, a 17:1 ratio would be expected for N:S uptake, but should actually be higher since nitrogen is a component of other macromolecules; the observed ratios are generally 3-4 times lower than expected. The P:S ratio for whole cells of E. coli is about 3.5:1 (Roberts et al., 1963) but 25% of the sulfur is L.M.W. organic material not included in this data. A ratio of about 4.7:1 is expected if this fraction is excluded. The observed ratios are about 4-5 times higher in the Sargasso Sea station but only 2 times higher in the continental slope station.

Table 5-13. Elemental Uptake Ratios for NH_4^+-N, $PO_4^=$-P, and $SO_4^=$-S by Natural Bacterial Populations During R/V Oceanus Cruise # 84[a]

Hours of Incubation	ng/100ml			Weight:Weight			Atom:Atom		
	N	P	S	N:P	N:S	P:S	N:P	N:S	P:S
				Continental Shelf Station					
16	17.4	-	2.1	-	8.2	-	-	18.9	-
20	63.3	-	8.7	-	7.3	-	-	16.7	-
24	137.7	-	21.4	-	6.4	-	-	14.7	-
				Continental Slope Station					
12	1.5	19.6	U	0.1	I	I	0.2	I	I
18	7.0	64.0	U	0.1	I	I	0.2	I	I
30	-	153.9	15.2	-	-	10.1	-	-	10.5
36	87.8	617.2	65.0	0.1	1.4	9.5	0.3	3.1	9.8
48	-	615.6	73.6	-	-	8.4	-	-	8.7
				Sargasso Sea Station					
1	0.3	1.2	-	0.2	-	-	0.5	-	-
2	1.3	1.8	-	0.7	-	-	1.6	-	-
4	3.1	6.1	-	0.5	-	-	1.1	-	-
6	7.6	15.2	2.0	0.5	3.8	7.5	1.1	8.7	7.8
12	29.7	57.3	3.8	0.5	7.9	15.3	1.2	18.1	15.8
18	34.6	85.4	5.3	0.4	6.5	16.1	0.9	15.0	16.7
24	34.9	133.1	6.3	0.3	5.5	21.1	0.6	12.7	21.8
30	36.8	121.4	11.6	0.3	3.2	10.5	0.7	7.3	10.9
48	43.2	124.3	10.8	0.4	4.0	11.5	0.8	9.2	12.0

[a]Data from Tables 5-10, 11, and 12: U, undetectable; I, infinity; -, no sample.

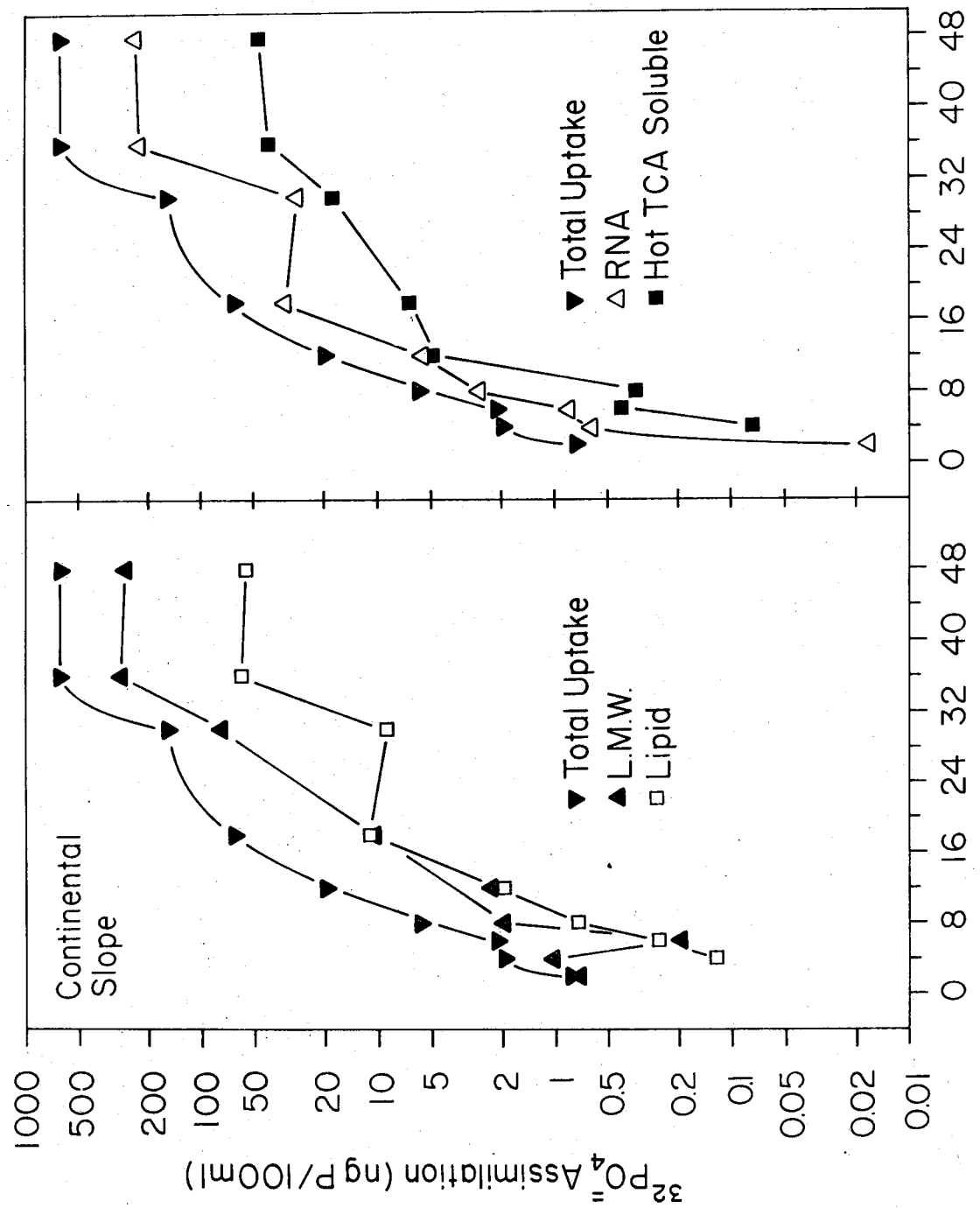
Distribution of Phosphorus and Carbon in Major Biochemical Fractions

The sensitivity of sulfate incorporation is not sufficient to permit resolution of sulfur-containg metabolites; the highest activity observed in protein was equal to only 9.1×10^{-7} of the added label. In spite of the low activity, this sample was over 30-fold greater than a very reproducible blank under 100 DPM. However, incorporation of phosphate and organic carbon compounds was high enough to permit resolution of the distribution of label in the fractions for most samples.

Phosphorus Distribution

The incorporation of phosphate into the major fractions of bacteria at the continental slope station demonstrates a great degree of variability (Figure 5-7). This variability is confined to the proportion of ^{32}P in a given fraction as a % of the total label taken up, however. The only fraction which exhibits a significant decrease in the absolute amount of ^{32}P is the L.M.W. pool, the most labile of all the fractions. Initially almost all of the phosphate is found in L.M.W. compounds, but incorporation into RNA is rapid after the first point (2 hours). It was previously pointed out that the RNA fraction is most sensitive to changes in growth physiology, whereas the lipids are structural components related more closely to increases in cell size. In this context it is significant that a plateau in the incorporation of ^{32}P into these fractions between 18 and 30 hours of incubation is followed by a large increase in both frac-

Figure 5-7. Total uptake of $^{32}PO_4^{=}$ and its distribution in major biochemical fractions of natural bacterial populations in waters of the continental slope. The phosphate concentration was 1.52 μM.


Continental Slope
Total Uptake
L.M.W.
Lipid
Total Uptake
RNA
Hot TCA Soluble
$^{32}PO_4^{=}$ Assimilation (ng P/100ml)
1000
500
200
100
50
20
10
5
2
1
0.5
0.2
0.1
0.05
0.02
0.01
0 8 16 24 32 40 48
0 8 16 24 32 40 48
Incubation Time (Hours)

tions, coincident with the first detection of sulfate incorporation into protein. The total amount of label taken up and the distribution of ^{32}P in the fractions is constant between 36 and 48 hours, suggesting that growth has ceased.

A completely different pattern of incorporation occurs at the Sargasso Sea station (Figure 5-8). The amount of ^{32}P in all fractions increases at nearly an identical rate and the relative proportions of label in the fractions are reasonable; i.e. L.M.W. compounds and RNA dominate the cellular phosphorus, lipid and hot TCA soluble (DNA + polyphosphates) each contain slightly under 10%, and the protein fraction contains less than 3% of the total. The distribution does not vary substantially during the course of incubation, indicating approximately equal incorporation rates into all fractions, a characteristic of balanced growth. There is no change in either total ^{32}P taken up or the distribution of label between 30 and 48 hours of incubation, again suggesting that growth has ceased. The proportion of label in the various fractions for several time points is found in Table 5-14; the entire data set is appended in Tables A-16 (continental slope station) and A-17 (Sargasso Sea station).

Carbon Distribution

The first portion of the results of subcellular distribution of ^{14}C derived from organic compounds will be confined to trends. Discussion of the relative amount of ^{14}C in the various fractions will be found at the end of this section.

Figure 5-7. Total uptake of $^{32}PO_4^{=}$ and its distribution in major biochemical fractions of natural bacterial populations in waters of the Sargasso Sea. The phosphate concentration was 0.18 μM and the initial cell density was 1.63×10^5/ml.

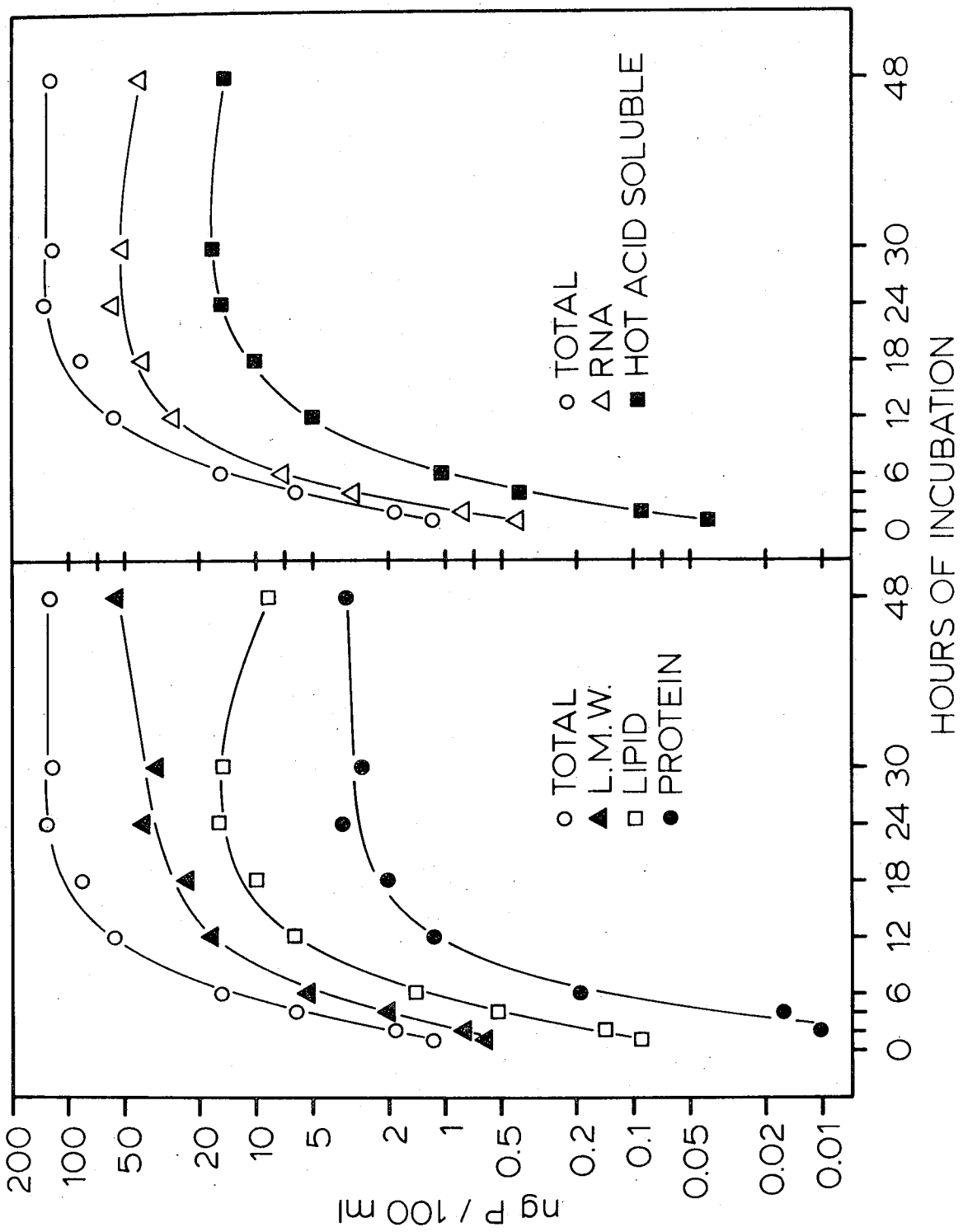

ng P / 100 ml
200
100
50
20
10
5
2
1
0.5
0.2
0.1
0.05
0.02
0.01
TOTAL
L.M.W.
LIPID
PROTEIN
TOTAL
RNA
HOT ACID SOLUBLE
0 6 12 18 24 30 48
0 6 12 18 24 30 48
HOURS OF INCUBATION

Table 5-14. Total Incorporation and Distribution of $^{32}PO_4^{=}$ in Water Samples from R/V Oceanus Cruise# 84[a]

Hours of Incubation	Total ng P Taken Up per 100ml	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
		Coninental Slope Station					
2	0.75	97.8	0.0	0.0	2.2	0.0	0.0
4	1.93	52.5	2.1	6.3	31.9	3.9	3.3
18	64.03	17.1	1.5	17.0	51.5	10.3	2.8
48	617.57	43.5	0.9	9.0	37.2	6.4	2.0
		Sargasso Sea Station					
2	1.83	42.5	0.5	7.7	43.9	5.1	0.4
4	6.09	32.9	0.9	8.6	50.0	6.6	1.0
18	85.38	27.1	0.8	11.5	46.5	11.7	2.4
48	124.32	40.7	0.7	8.2	37.1	11.3	1.9

[a]Data from Figures 5-7 and 5-8.

The incorporation patterns of glucose and glutamate at the continental shelf station are shown in Figure 5-9. Acetate uptake was not studied, and the incubations were only of 24 hour duration. Both substrates were incorporated into all fractions at similar rates, but glutamate incorporation into RNA continued at a disproportionately high rate at the end of the incubation period. Glucose assimilation shows no signs of a decrease in uptake rate, even though 30% of the total available label has been incorporated at 24 hours, and inclusion of a respiration correction will increase the total metabolized considerably. At a similar % utilization, glutamate uptake appears to be very near to a plateau. Glutamate assimilation was more rapid on a % total substrate utilized basis, but this could be due either to preference or lower ambient glutamate concentration. The total uptake and distribution of label bear a remarkable resemblance to exponential growth of a healthy population.

Acetate, glucose, and glutamate uptake and incorporation into major fractions are shown in Figure 5-10 for the continental slope station. A pronounced difference among substrates is apparent, with glucose assimilation lagging well behind the others. In addition, ^{14}C derived from glucose is almost exclusively in the L.M.W. soluble pool fraction, with label appearing in the growth related fractions (i.e. protein, hot TCA soluble, and lipid) at 30 hours of incubation, when ^{35}S incorporation into protein begins to increase. The samples labeled with acetate and glutamate contain similar amounts of label in protein and L.M.W. fractions, but acetate is clearly preferred for lipid synthesis. As in the samples from the continental shelf station, RNA and hot TCA soluble fraction contain similar proportions of label, in contrast to the results ob-

Figure 5-9. Total uptake of UL-^{14}C-labeled glucose and glutamate and their distribution in major biochemical fractions of natural bacterial populations in waters of the continental shelf. Data are the average of duplicate fractionations. The initial cell density was 3.34×10^5/ml.

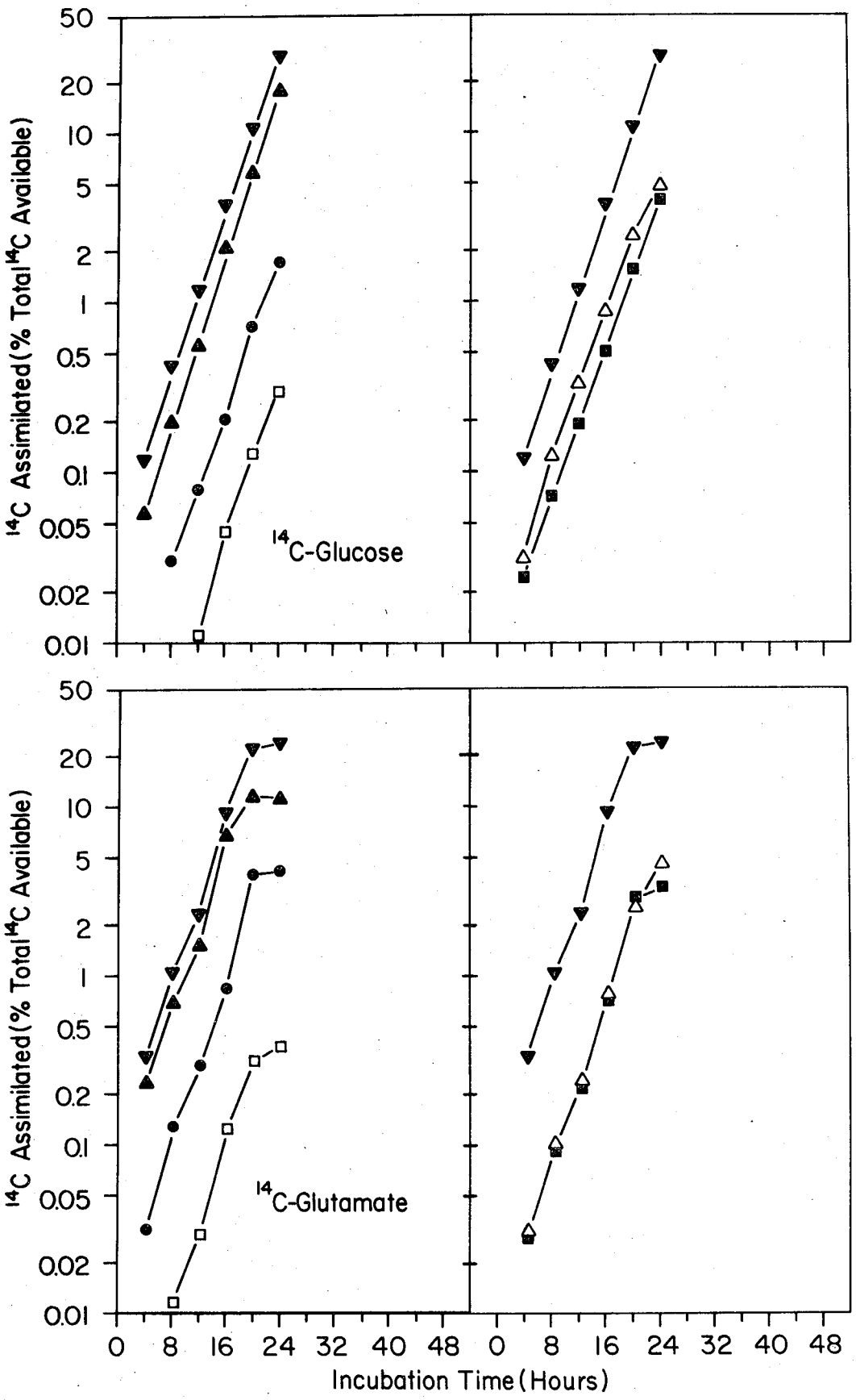
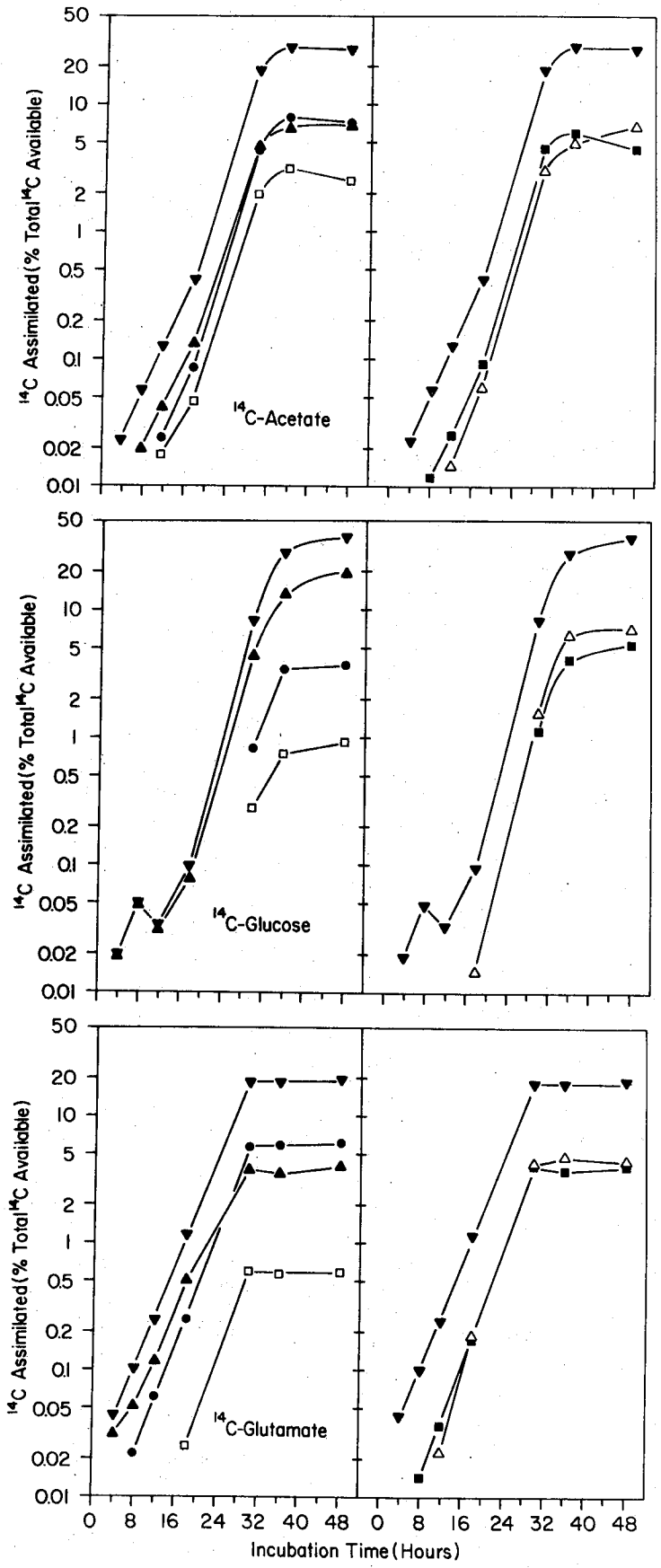

50
20
10
5
2
1
0.5
0.2
0.1
0.05
0.02
0.01
^{14}C Assimilated (% Total ^{14}C Available)
^{14}C-Glucose
^{14}C-Glutamate
0 8 16 24 32 40 48 0 8 16 24 32 40 48
Incubation Time (Hours)

Figure 5-10. Total uptake of UL-^{14}C-labeled acetate, glucose, and glutamate and their distribution in major biochemical fractions of natural bacterial populations in waters of the continental slope. Data are the average of duplicate fractionations.


^{14}C Assimilated (% Total ^{14}C Available)
^{14}C-Acetate
^{14}C-Glucose
^{14}C-Glutamate
Incubation Time (Hours)

tained with $^{32}PO_4^{=}$ at this station. Uptake of glucose was nearly terminated at the end of the incubation period, and metabolism of acetate and glutamate had virtually ceased by 36 hours.

The indications of nearly balanced growth at the Sargasso Sea station provided by the initial rates of sulfate incorporation into protein, ammonia assimilation, and total uptake and distribution of phosphate are further supported by fractionation of ^{14}C-labeled samples (Figure 5-11). Acetate and glucose were incorporated into all fractions at similar rates, but glucose contributed relatively more carbon to RNA than to the hot TCA soluble fraction, in contrast to previous stations. At the end of the incubation period, a substantial increase in L.M.W. material derived from acetate occurs at the expense of RNA and hot TCA soluble fractions. Of particular interest is the mirror-image fluctuation of L.M.W. and RNA components during what would be considered a stationary phase by whole cell uptake criteria. Although the incorporation of glutamate has ceased, its metabolism continues. At 36 hours a large increase in L.M.W. material derived from glutamate and acetate is observed, coincident with the break in protein synthesis determined by sulfate incorporation.

A more thorough understanding of the metabolism of individual carbon compounds in a complex mixture of substrates can be realized by analysis of the distribution of ^{14}C as a % of the total label taken up. Table 5-15 selects the first sample in each time series which has assimilated at least 1% of the total available substrate. As indicated in Figures 5-9, 10, and 11, the distribution of ^{14}C rarely changed substantially throughout the course of incubation unless a stationary phase had been

Figure 5-11. Total uptake of UL-^{14}C-labeled acetate, glucose, and glutamate and their distribution in major biochemical fractions of natural bacterial populations in waters of the Sargasso Sea. Data are the average of duplicate fractionations. The initial cell density was 1.64×10^5/ml.

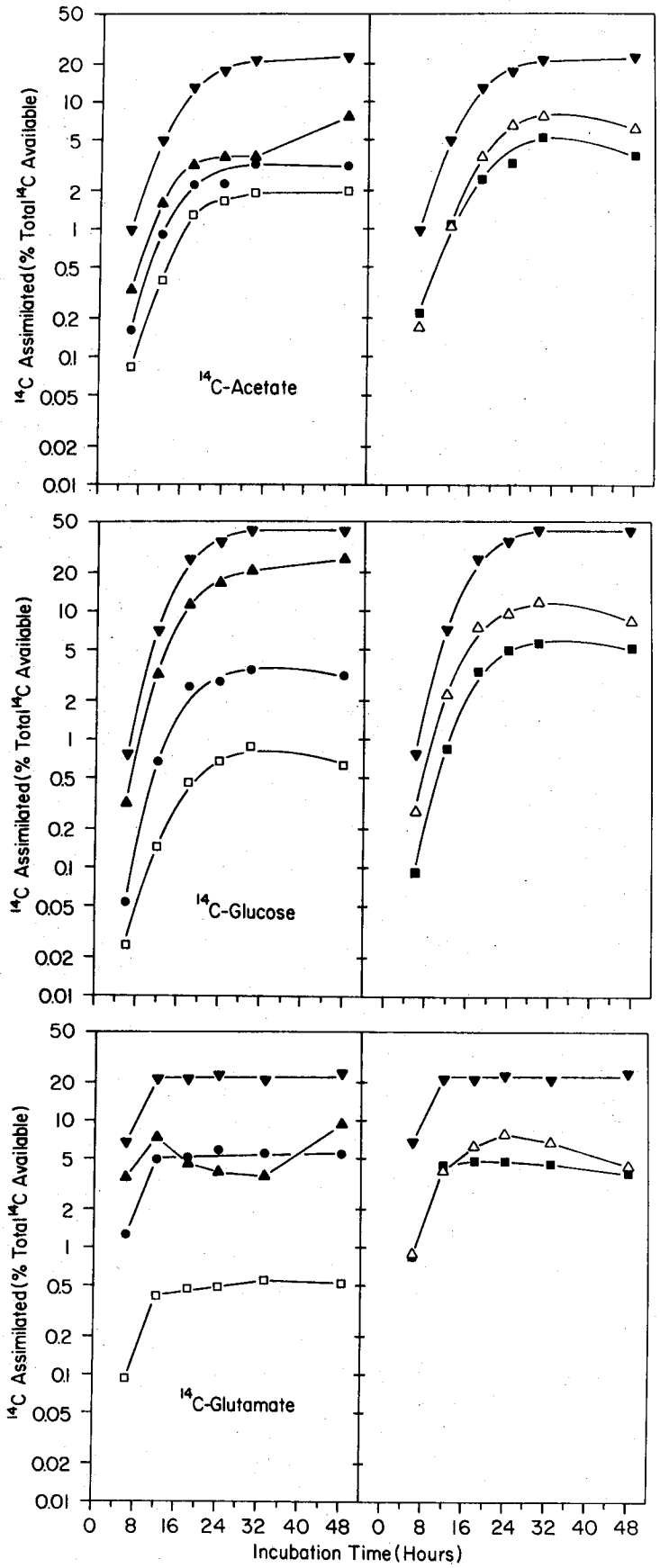

^{14}C Assimilated (% Total ^{14}C Available)
^{14}C-Acetate
^{14}C-Glucose
^{14}C-Glutamate
50
20
10
5
2
1
0.5
0.2
0.1
0.05
0.02
0.01
0 8 16 24 32 40 48
Incubation Time (Hours)

Table 5-15. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Organic Substrates in Water Samples from R/V Oceanus Cruise #84[a]

Substrate	Total DPM Taken Up	% of Total Radioactivity					
		Alcohol				Hot TCA	Residue
		L.M.W.	Soluble	Lipid	RNA	Soluble	Protein
Continental Shelf Station							
Glucose	11,759	47.5	0.9	0.9	27.8	16.2	6.7
Glutamate	10,475	67.0	1.0	1.1	9.6	9.0	12.3
Continental Slope Station							
Acetate	126,285	29.1	1.3	8.9	14.3	22.1	24.3
Glucose	90,884	53.9	1.2	3.3	18.2	13.5	9.9
Glutamate	11,427	43.7	0.9	2.2	16.5	15.1	21.7
Sargasso Sea Station							
Acetate	49,555	31.8	0.7	7.8	20.7	20.9	18.1
Glucose	71,527	45.0	0.6	2.0	31.2	11.9	9.3
Glutamate	66,497	53.1	0.4	1.4	13.3	13.0	18.8

[a]Data from Figures 5-9, 10, and 11. The first sample having assimilated greater than 1% of the total available label was chosen for comparative purposes.

reached. Therefore the distribution of ^{14}C in the selected samples is comparable; of course, the magnitude of uptake in absolute terms is unknown. Of the three compounds, acetate is the dominant contributor of carbon to the lipid fraction in both the continental slope and Sargasso Sea stations. Protein derives much of its carbon from glutamate and acetate. Glucose carbon is poorly represented in these fractions at all stations, being predominantly contained in L.M.W. intermediates and nucleic acids, especially RNA. The lag period and subsequent explosive growth revealed by sulfur and nitrogen assimilation at the continental shelf and slope stations were only reflected in the incorporation patterns of glutamate at the two stations: at the continental shelf station, the of the total glutamate in protein doubled (9.1-18.2%) between 16 and 20 hours, and increased 47 % (21.7-31.9 % of the total) between 18 and 30 hours at the continental slope station. No other evidence for the unusual growth patterns was noted, however, the total uptake (as % of the total available label) was qualitatively related to the lag time. The sulfate and ammonia assimilation methods are not as sensitive as the uptake of organic compounds, but the data from the Sargasso Sea station demonstrate what may be the lower limit of sensitivity of the methods, and support the contention that a substantial lag phase did occur at the other two stations. It must be remembered that the continental shelf and slope samples experienced a 5°C rise in temperature during the incubation period, but it is unlikely that this took more than an hour or two under the conditions of incubation. Furthermore, all samples were treated identically; thus the observed rates are strictly comparable.

Respiration of Organic Substrates and the Effects of Container Size, Nutrient Enrichment, and Chloramphenicol on Carbon and Sulfur Incorporation

Results of inorganic nutrient enrichment experiments on a previous cruise (May, 1980) to nearly identical stations indicated a substantial enhancement of uptake of ^{14}C-labeled compounds during 24 hour incubations, during which time unenriched controls had taken up less than 5% of the available label. Based on these findings, samples in prescription bottles were enriched with ammonia (50 μM), phosphate (4 μM), and inorganic nutrients (ammonia, 50 μM; phosphate, 4 μM; EDTA, 1 μM; trace metals) and sampled concurrently with unenriched flask and bottle controls after 30 hours of incubation (continental slope and Sargasso Sea stations). In this way both nutrient enrichment and container size effects could be monitored. However, on this cruise, most of the available label had been metabolized in the controls by 30 hours, obscurring possible effects of the enrichments, but several trends were apparent.

(a) Respiration

The respiration of ^{14}C-labeled substrates to CO_2 (wick method) and transformation to acid-volatile compounds were determined as described in Chapter 2. The results of the acid-volatile method were similar to those observed in the fish tank experiment (Table 5-5) and generally were twice the value obtained by the wick method. The substrates themselves were not volatilized, as evidenced by the >98 % recovery of label

at zero incubation time, and the same trends were observed for respiration measured by both methods as a function of nutrient enrichment, bottle effects, etc. Because the difference between the methods bears strongly on the total amount of label transformed, both methods were used in case later work can resolve the reason for the consistently higher acid-volatile results. The following summary of the respiration data uses only the data from the wick method, which is commonly used. The data for both methods are found in Appendix B, Tables B-2 through B-9.

Respiration accounted for 50 ± 21 % of the total label metabolized (assimilation + respiration) overall. The variation among substrates was much greater than the effects of location or incubation time. Glutamate and acetate were respired at high rates (67% and 60%, respectively), whereas glucose was respired at only 26%. Respiration of glutamate and acetate was similar at all stations. Glucose respiration was highest (35%) at the continental slope station, but was only 10 % at the continental shelf station. There was a consistent increase in the percent respired with longer incubation (36-48 hours) for all substrates, but the effect was very slight (1-5%).

(b) Container Size Effects

Samples incubated for the same period in 3 liter flasks and 125ml prescription bottles showed little effect of container size on ^{14}C assimilation. The average of all paired samples from flasks and bottles indicated minor enhancement of assimilation (22%) and respiration (24%), with most being due to the acetate-labeled sample at the continental

slope station (88%and 97% respectively). No effects were noted in the distribution of label in the major biochemical fractions.

Samples labeled with ^{35}S showed a much greater influence of container size than the ^{14}C-labeled samples. Bottle incubation enhanced sulfate incorporation into protein by 65% at the continental slope station, but inhibited incorporation by 47% at the Sargasso Sea station. These effects are far in excess of the average error of 14.5% for duplicate samples. Pairs of samples from each container type at the continental shelf station agreed within 9%. The effects of container size on bacterial metabolism do not follow any general pattern and are usually small, being always less than a factor of 2 during these long incubation periods (24-30 hours). Much shorter incubation periods should be used to test enclosure effects.

(c) Nutrient Enrichment Effects

In general, ammonia and phosphate supplements produced no substantial response in ^{14}C assimilation or respiration rates or ^{35}S incorporation into protein.. Phosphate was without effect except for a 2.4-fold stimulation of glucose uptake at the continental slope station, accompanied by a 3.4-fold increase in sulfate incorporation. At this station ammonia stimulated glucose uptake by 3.1-fold but did not influence sulfate incorporation. Total inorganic nutrients generally stimulated assimilation of all compounds to a small extent (average 26% excluding glucose assimilation at the continental slope station, where a 6-fold stimulation was noted). Since long-term incubation resulted in nearly complete util-

ization of the organic carbon compounds in unenriched samples, it can be concluded that inorganic nutrients were not limiting growth at these stations.

Small but consistent redistribution of label occurred as a result of nutrient enrichment, with similar trends among samples for all nutrient additions. Table 5-16 compares the distribution of ^{14}C in the major biochemical fractions of control and total inorganic nutrient-enriched samples. Glucose normally dominant in L.M.W. components contributes a larger proportion of label to RNA and/or protein in the enrichments. Acetate and glutamate show enhanced incorporation into protein at the expense of RNA and L.M.W. compounds, except at the continental shelf station, where glutamate carbon from RNA appears in the L.M.W. fraction, perhaps indicative of a stationary phase. Inorganic nutrient enrichment failed to increase the amount of carbon from any compound in protein to the proportion expected if it were the only carbon source, demonstrating that many compounds must be available for simultaneous assimilation.

Effects of Chloramphenicol

Much more dramatic effects on both carbon and sulfur assimilation were obtained with chloramphenicol (CAP). Only one of 11 samples was inhibited <95% by the antibiotic (glutamate uptake at the Sargasso Sea station, 83 % inhibition), and 73% of the label taken up was found in L.M.W. material. In addition to nearly complete inhibition of uptake of ^{14}C and ^{35}S, the distribution of carbon was strongly affected. In most cases over 85% of the label was found in the L.M.W. and RNA fractions.

Table 5-16. Effects of Inorganic Nutrient Enrichment on Total Uptake and Biochemical Distribution of ^{14}C-Labeled Organic Compounds in Water Samples from R/V Oceanus Cruise# 84[a]

Substrate	Total DPM Taken Up	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
		Continental Shelf Station					
Glucose							
24B	286,697	64.1	0.6	1.0	15.8	13.2	5.4
24B +NBC	537,792	57.1	1.1	1.0	21.5	12.5	6.8
Glutamate							
24B	263,739	49.2	1.3	1.7	18.3	12.6	16.9
24B +NBC	335,214	55.6	1.2	1.7	14.6	11.3	15.6
		Continental Slope Station					
Acetate							
30B	247,798	22.7	1.1	11.4	17.0	24.4	23.4
30B +NBC	279,559	20.3	1.2	13.1	11.5	24.8	29.0
Glucose							
30B	75,988	51.6	0.9	3.4	20.3	13.7	10.1
30B +NBC	458,678	39.2	1.2	1.9	25.2	17.3	15.1
Glutamate							
30B	205,549	20.3	0.9	3.0	25.3	21.3	29.3
30B +NBC	199,254	15.0	1.5	3.2	21.2	22.4	36.8

Table 5-16. (Continued)

Substrate	Total DPM Taken Up	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
		Sargasso Sea Station					
Acetate							
30B	225,681	16.2	0.6	9.1	34.8	24.5	14.8
30B +NBC	355,488	13.4	1.0	10.3	23.1	24.7	27.5
Glucose							
30B	490,243	48.0	1.1	2.0	27.7	12.4	8.7
30B +NBC	547,118	41.0	0.9	2.1	28.7	15.7	11.6

[a]Samples were incubated in 125ml prescription bottles for the time indicated (hours) with or without addition of inorganic nutrient mix, filtered, and processed as described in Materials and Methods.

Sulfate incorporation into protein was reduced to blank levels at the continental slope and Sargasso Sea stations, and was inhibited 96% at the continental shelf station. This leaves little doubt that the observed sulfate incorporation into protein in the time-course samples is due to bacterial protein synthesis.

DISCUSSION

The results of the survey of the residue protein-S:bulk protein ratio for mixed natural populations in enrichment culture and isolates from similar enrichments in pure culture confirm the validity of measuring marine bacterial protein synthesis using sulfur incorporation into protein. A wide variety of microorganisms were studied, including a chemolithotroph (Nitrosococcus oceanus), a sulfate-reducing bacterium (Desulfovibrio 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 (C.V.) of the residue protein-S:bulk protein ratio for the cruise isolates, 16.8%, is much less than the C.V. for either the protein per cell (61.8%) or the total sulfur per cell (51.7%). Further support is found in the isolation of Alteromonas luteo-violaceus from the Sargasso Sea years after its original isolation over the Puerto Rico Trench: the residue protein-S:bulk protein ratio of the new isolate, 0.64%, compares favorably with the mean from Chapter 4 for this bacterium, 0.72 ± 0.11, as does the distribution of sulfur in the major biochemical fractions.

The agreement of the true weight percent sulfur in protein determined for 13 different bacteria, 1.09%, with the 1.1% calculated from the Jukes et al. (1975) "average protein" is extremely reassuring. The variation in individual protein composition summarized by Holmquist (1978) is indeed averaged out in bulk protein. In view of the consistency of the residue protein-S:bulk 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.

Application of the sulfate incorporation method to seawater, with its large isotope dilution barrier, is greatly facilitated by the fractionation procedure. In addition to providing a more interpretable result than whole cell sulfate uptake studies, the multiple solvent extractions serve to wash adsorbed sulfate out of the residue very effectively. On R/V Oceanus cruise # 84, over 4×10^9 DPM were passed through the filters, with zero time blanks rarely over 100 DPM.

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 sulfate were very similar during the first few hours of the experiment, but the nearly complete utilization of glucose indicated the preferential utilization of L.M.W. material in the early stages of the incubation. The fish food used in the tank consists 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. Sulfate incorporation into protein was sensitive to the secondary growth period because it is never utilized to a significant extent as a % of the total label and is incorporated in direct relation to growth.

In addition to having observed the secondary growth phase in the bacterial population from the fish tank, the ratio of residue protein-S:bulk protein can be used to calculate the amount of bacterial protein synthesized during the incubation period. The operational value, 0.93 weight %, indicates the synthesis of 123 μg protein per liter during the first hour, a very healthy amount of growth. Since protein synthesis values for natural populations are not available for comparison, an assumption of 50 % carbon by weight in protein gives a little over 60 μg protein-C/liter/hour, the minimum carbon production (since carbon is contained in many non-protein cellular constituents). Values for natural bacterial population growth in a rich coastal inlet in British Columbia (Furman and Azam, 1980) determined by cell counts and thymidine incorporation into DNA range from 0.7-70 μg C/liter/day. The fish tank population was therefore more than 25 times as productive as the most rapid growth observed in their study.

The lack of agreement of wick and acid-volatile methods for the measurement of bacterial respiration of ^{14}C-labeled organic compounds is perplexing in view of the good agreement between them with P. halodurans (Chapter 2). If the higher acid-volatile results represent transformation of substrates to volatile extracellular products rather than intracellular materials, the difference will substantially alter calcula-

tions of turn-over time and the total amount of substrate metabolized in the same fashion as the original respiration correction (Hobbie and Crawford, 1969). Excretion of pyruvate has been demonstrated in aerobic culture of luminous marine bacteria grown on glucose (Ruby and Nealson, 1977), accounting for up to 50 % of the total carbon metabolized. This and other low molecular weight acids could account for the observed higher acid-volatile radioactivity relative to $^{14}CO_2$ in natural populations.

As a preface to the discussion of microbial metabolism studies in the Western North Atlantic Ocean, I must admit to my surprise at the dearth of literature concerning either inorganic nutrient assimilation or time-course studies of bacterial heterotrophic activity. I had tacitly assumed that the measurement of ammonia and phosphate assimilation would provide confirmation of the results of sulfate incorporation studies and thus be comparable to other studies of bacterial inorganic nutrient uptake. Ammonia and phosphate assimilation measurements have been made frequently with mixed assemblages of phytoplankton and bacteria both in freshwater (Lean and Nalewajko, 1976; Murphy, 1980) and marine habitats (McCarthy and Goldman, 1979; Perry, 1976). However, no studies confined to natural bacterial populations in seawater have been published in readily accessible literature. The tremendous amount of literature concerning phytoplankton nitrogen and phosphorus assimilation studies will only be alluded to for comparative purposes; a thorough coverage of this portion of the natural population experiments is not warranted for this thesis.

Samples for three stations representing continental shelf, slope, and Sargasso Sea regions were analyzed for standing crop and uptake rate

parameters. The relatively deep water used provided populations of low cell density ($<4x10^5$/ml) and adequate supplies of inorganic nitrogen and phosphorus.

The results of standing crop analyses were constistent with decreasing surface productivity in an offshore direction; POC and PON decreased offshore, with shelf and slope stations distinctly different from the Sargasso Sea station. The lower direct counts of bacteria were also observed in the Sargasso Sea.

It was surprising to note that the particulate protein did not obey the same trend as the other parameters. Because of this, the proportion of the total PON contained in protein increased going offshore. Using 17.5% N by weight in protein (from Jukes et al., 1975), the percentage of PON accounted for by protein was 22, 31, and 44% at shelf, slope, and Sargasso Sea stations respectively. For comparison, late exponential phase cells of P. halodurans contain about 65% of the PON in protein. The increasing proportion of the total PON as protein-N suggests a progressively smaller contribution of N-containing detrital material in the offshore transect and implies that bacterial biomass accounts for a greater amount of the particulate material in oligotrophic regions.

The agreement of ammonia assimilation and sulfate incorporation into protein in both lag time and subsequent rates of increase at the shelf and slope stations strongly support the hypothesis that macromolecular synthesis was not proceeding at a significant rate at the time of sample collection. The sensitivity of the ammonia measurement was 10-fold higher at the slope station due to lower ambient concentrations of ammonia, but the sulfate method was equally sensitive at all stations, since the sul-

fate concentration does not vary. Nonetheless, both measurements demonstrated very similar time-course assimilation patterns.

The probability of a true lag period is strengthened by the ability to measure low but immediate assimilation of both elements at the Sargasso Sea station. Sulfate incorporation into protein was linear for the first 30 hours, but ammonia uptake appeared to be exponential. The ammonia assimilation profile has some of the characteristics of luxury uptake, i.e. rapid uptake followed by a plateau or slower rate (c.f. Perry, 1976), but the time-course of luxury uptake events is usually measured in minutes (McCarthy and Goldman, 1979; Perry, 1976) rather than the 12 hours required to reach apparent saturation in the Sargasso Sea sample. The ammonia assimilation curve is more suggestive of exponential uptake by a growing population. Unfortunately, attempts to fractionate ^{15}N samples were unsuccessful due to the low PON concentrations (at least 1 µg N is required for analysis). The virtually complete inhibition of sulfate incorporation into protein by chloramphenicol indicates that bacterial metabolism was responsible for sulfate incorporation.

Even though the initial assimilation rates did not conform to the expected offshore-decreasing pattern, the final amount of both N and S assimilated did. Sulfate incorporation into protein reached a plateau at both continental slope and Sargasso Sea stations, suggesting that the readily assimilable organic components of the samples had been metabolized. It is conceivable that the end-point sulfate incorporation data reflect the total potential bacterial production at these stations. Neither nitrogen nor phosphorus were depleted by a significant extent as determined by isotope uptake, and the lack of substantial stimulation of ^{14}C

or ^{35}S assimilation by inorganic nutrient additions additionally implicates carbon and energy sources as the ultimate growth-limiting nutrient.

The time-course experiments at the shelf station were prematurely terminated by return to port. However, the 24 hour end-point at the shelf station was higher than the same incubation period at either of the other stations and gave no indication of decreasing rate.

Total uptake and incorporation patterns of phosphate at the continental slope and Sargasso Sea stations point out the value of the fractionation procedure as it applies to isotopes other than ^{35}S in natural populations. The initial rate of phosphate assimilation was higher at the Sargasso Sea station, as with sulfate and ammonia, but the final magnitude of uptake was greater in the slope water. Thus the third inorganic nutrient element also confirms the anticipated greater potential productivity of inshore waters. The assimilation pattern of phosphate into biochemical components in the Sargasso Sea bore a great resemblance to balanced growth. Label appeared in each fraction at nearly the same rate and in the proportions expected of healthy microorganisms (c.f. Rhee, 1973; Roberts et al., 1963). Low molecular weight material and RNA dominate the cellular phosphorus, and RNA-P was about 9 times the hot TCA soluble-P. The hot TCA soluble fraction can also include polyphosphates (poly-P), but if they were important phosphorus storage compounds in this sample the proportion of RNA-P:hot TCA soluble-P would be lower than expected. Furthermore, phosphate accumulation as poly-P in excess of growth requirements at <0.2 μM phosphate is not likely (Goldman, 1977; Rhee, 1972). Protein contained <3% of the total P, similar to findings of

Rhee (1972, 1973) and Roberts et al. (1963) verifying that the protein fraction contains little contaminating nucleic acid or other polymeric P-containing material.

The intracellular phosphorus distribution at the continental slope station was indicative of unbalanced growth. Very different rates of increase were observed in the various fractions, and L.M.W. material accounted for >95% of the total P during the first two hours. The triphasic nature of whole cell uptake was amplified by the incoporation of phosphate into RNA, a sensitive indicator of changing growth status; and lipid, a measure of structural growth. Both of these fractions increased over 5-fold from a stationary period at the same time that sulfate incorporation into protein began to increase and shortly after ammonia assimilation climbed above the limit of resolution. The great overall increase in phosphate uptake at this time and the specifically enhanced response of two fractions sensitive to changing growth status strongly support the demonstration of a substantial lag phase of growth at the continental slope station.

In contrast to the incorporation patterns of inorganic nutrient elements, the assimilation of carbon into intracellular products was qualitatively similar at all stations and with all substrates. The only indication of variability among stations was the time required for uptake of an arbitrary percent of the total available label. The term "lag time" can only be applied realistically to glucose assimilation at the slope station. In all other instances, remarkably exponential uptake wqas observed from the first sampling point. Furthermore, incorporation of carbon from glucose, glutamate, and acetate into major biochemical fractions

gave no hint of the very different growth pattern of the shelf and slope stations compared to the Sargasso Sea station. In all cases, carbon entered all biochemical fractions at similar rates. So much of the carbon labels had been metabolized by the time sulfate and ammonia assimilation rose above background levels that there was nothing left to reveal the explosive growth which occurred.

The results of the organic carbon uptake studies point out the small contribution made by any individual carbon compound to overall bacterial metabolism in natural seawater samples. However, qualitative features, shown through the use of subcellular fractionation procedures, clarify the role of individual compounds in biosynthetic processes when present as a portion of a large variety of assimilable substrates. Preferential incorporation of (1) acetate into lipids, synthesized from acetyl~SCoA; (2) acetate and glutamate into protein, through the tricarboxylic acid cycle which provides carbon skeletons for the aspartate and glutamate family of protein amino acids; and (3) glucose into RNA, drawing its ribose backbone from the hexose monophosphate shunt, all conform to preferences determined from basic biochemical principles. Thus it appears that natural populations of bacteria simultaneously presented with a number of different substrates for biosynthesis will specifically utilize the compounds most readily integrated into a given biosynthetic pathway, just as will pure cultures (c.f. Roberts et al., 1963).

Amazingly rapid generation times (<4 hours) may be calculated from the slope of semi-logarithmic plots of the organic carbon uptake data. From these calculations as many as 6 doublings of the bacteria assimilating each of the various substrates could have occurred during the incubation

period. Since the apparent rapid growth was without influence on the lag period for sulfate and ammonia assimilation, it is likely that either the populations utilizing these compounds were only a minor portion of the total bacterial assemblage or the chosen compounds were in such low concentration that their virtually complete utilization did not contribute substantially to total carbon metabolism.

Although doubling times cannot be calculated by this method for sulfur and ammonia assimilation, a quantitative amount of total metabolism can be determined. Using the ratio of 0.93 weight % S in protein, the continental shelf, slope, and Sargasso Sea stations synthesized 21 μg (24 hours), 69 μg (36 hours), and 12 μg (36 hours) of protein per liter, respectively. If protein is about 50 % C by weight (from Jukes et al., 1975) and about 50 % of the total carbon is in protein (Chapter 4), then a similar amount of total carbon metabolism occurred. These rates are only an order of magnitude less than surface primary productivity at similar stations and times of year (Peter Ortner, Ph. D. Thesis, Woods Hole Oceanographic Institution, October, 1978). Considering the greater volume of water below the euphotic zone, a substantial amount of particulate protein, and therefore carbon, are potentially available for consumption by higher organisms if the calculated rates are realized in situ. An investigation of the relative abundance of flagellated protozoa to bacteria at very similar stations two months prior to R/V Oceanus cruise #84 revealed that the population of flagellates was about 0.1% that of the bacteria (David Caron, personal communication). Previous work with flagellated protozoa demonstrated the ability of these organisms to grow rapidly on bacteria as a food source when presented at similar rela-

tive abundance (Haas and Webb, 1979). Thus both a source and an effective sink exists for large-scale particulate flux into the food web which has previously been ignored.

An extremely important outcome of the work presented herein is an emphasis on the necessity for time-course measurements rather than single end-point analyses. The fact that the uptake of organic carbon compounds yields a linear plot on semi-logarithmic paper vividly demonstrates the misconceptions which can arise from points taken at single arbitrary times. Major differences in sulfur incorporation and ammonia assimilation between the shelf and slope stations compared to the Sargasso Sea station would not have been observed.

The analysis of dissolved inorganic nutrients permitted the quantitative calculation of uptake ratios for nitrogen (as ammonia), phosphorus (as phosphate), and sulfur (as sulfate). The ratios obtained deviate significantly from those expected on the basis of algal studies, but there are no such data for mixed assemblages of bacteria for comparison. Other sources of nitrogen (e.g. urea, nitrate, and amino acids) may be utilized simultaneously with ammonia (McCarthy, 1972) and could explain the low N uptake relative to either S or P. The excess phosphate uptake is more difficult to rationalize; however, exchange of intracellular phosphate with exogenous label is well known (c.f. Lean and Nalewajko, 1976), and can cause apparently high uptake rates when net uptake is low. The uniform distribution of label among the biochemical fractions and the small population size of the samples argues against this reasoning. It is not known whether the flagellated protozoa are capable of equilibrating (and hence incorporating) intra- and extracellular phosphate.

A number of perplexing issues concerning bacterial growth and nutrient assimilation rates have been raised. The work presented in this chapter demonstrates the value of measuring sulfate incorporation into protein by natural bacterial populations and provides data suggesting that bacteria can contribute significantly to the marine food web. The method designed for use with sulfur has additionally been found effective with other tracers of microbial metabolism. Further studies of inorganic nutrient assimilation by bacteria will be a valuable contribution to the resolution of the question of bacterial productivity in marine environments.

CHAPTER 6

RELATIONSHIPS AMONG GROWTH, PROTEIN SYNTHESIS, AND SULFUR METABOLISM IN MARINE PHYTOPLANKTON: PROSPECTUS FOR FUTURE RESEARCH

INTRODUCTION

The relationship between sulfate incorporation into protein and bulk protein synthesis has been shown to provide a quantitative index of bacterial protein synthesis in the marine environment. De novo protein synthesis is also performed by marine plants, predominantly by the unicellular algae or phytoplankton. What little is known about the relative rates of production of bacteria and phytoplankton has demonstrated a good relationship between excretion of organic compounds by phytoplankton and their subsequent utilization by bacteria (Williams and Yentsch, 1976), especially near the edges of upwelling regions where senescing blooms produce copious amounts of excreted organic material (Castellvi and Ballester, 1974; Herbland, 1974). Bacteria can effectively compete with algae for nutrients (Parker et al., 1975; Rhee, 1972); the uptake of sufficient amounts of inorganic nutrient to inhibit growth means a priori that bacterial production can be substantial. Since all euphotic zone measurements of nitrogen and phosphorus assimilation include bacteria, their contribution to total community production should be known.

Uncoupling of phytoplankton growth and carbon fixation occurs under a variety of nutrient-limitation regimes, and has been shown to result in preferential synthesis of polysaccharide storage products in some cases (Morris et al., 1974). Therefore a measurement of protein synthesis, expected to be more closely related to growth than total carbon metabolism, would be a useful addition to the array of techniques available to the phytoplankton physiologist. Like bacteria, algae are capable of sulfate

reduction and assimilation. If the relationship derived from the "average protein" and demonstrated for bacteria were also valid for phytoplankton, a simple measurement for total microbial protein synthesis would be available, a big step forward in estimation of potential secondary and higher order production.

The distribution of sulfur in algae is more complex than in bacteria, since substantial amounts of sulfur are found in the plant sulfolipid (Benson, 1963; Busby and Benson, 1973) and in polysaccharide sulfate esters (Busby and Benson, 1973; Ramus and Groves, 1974). A study of sulfate uptake by marine phytoplankton (Steven Bates, Ph. D. Thesis, Dalhousie University, 1979) suggests that the relationship between sulfate assimilation into protein and de novo protein synthesis may be similar to that found for bacteria in this study, but more quantitative data must be obtained because his work did not take into account the potentially important contribution of polysaccharide sulfate esters to total sulfur metabolism (containing 20-60 % of the total sulfur).

Of special interest is the synthesis of the plant sulfolipid 6-sulfoquinovosyl diglyceride, which may provide a means for metabolically separating bacterial and algal protein synthesis in mixed populations. The plant sulfolipid is an integral component of the chloroplast thylakoid membrane, and its absence results in seriously decreased ability to perform the Hill reaction of photosynthesis (Sinensky, 1977). Because it is an essential consituent of the chloroplast, sulfolipid may be synthesized at a rate proportional to the growth rate of the chloroplast itself. The growth rate of the chloroplast, in turn, should be tightly coupled to the cellular growth rate, since changes in photosynthetic capacity are often

the result of increased pigment production rather than chloroplast number, and the chloroplast is of central importance to the biosynthesis and energy metabolism of the cell.

Although the role of the sulfolipid in chloroplast development and function is not yet clear, certain key points encourage the investigation of relationships between sulfolipid synthesis and growth. Sulfolipid is covalently bound to protein in the chloroplast membrane, but it is not related to the chlorophyll-protein complex (Gregory Schmidt, personal communication). This suggests a relationship between the sulfolipid and components of the photosynthetic electron transport chain. The absence of a physical interaction between sulfolipid and the light harvesting pigments is a positive feature, as sulfolipid is therefore less likely to be subject to the fluctuating levels of chlorophyll which often accompany light stress (c.f. Beardall and Morris, 1976).

It has therefore been proposed to carry out a study of sulfate assimilation in phytoplankton, similar to that described in Chapters 3 and 4 of this work, with a special emphasis on relationships between sulfolipid and protein synthesis. The National Science Foundation has smiled on my request, funding two years of research on this topic.

Encouraging Evidence: A Fortuitous Encounter With Trichodesmium In The Western North Atlantic

Trichodesmium is a pelagic, colonial blue-green alga occassionally found in great abundance in oligotrophic tropical and sub-tropical open ocean waters. Its blooms of incredible density in nutrient-poor regions

have been attributed to its ability to utilize elemental nitrogen aerobically as a source of nitrogen for growth (Dugdale et al., 1964; Goering et al., 1966). The adaptive significance of this capabiltity is exemplified by its higher population density in the phosphate-sufficient waters of the Caribbean (Carpenter and Price, 1977), where relief of phosphate limitation permits expression of the potential for nitrogen fixation.

Knowledge of the physiology of Trichdesmium is sparse because it has defied attempts to maintain it in culture. Its sporadic occurrence in open ocean waters renders difficult the deliberate investigation of photosynthesis and metabolism in this organism, but good use has been made of the random encounters with dense accumulations of the alga. A continually increasing body of literature on the chemical composition (Mague et al., 1974, 1977), nitrogen fixation and inorganic nitrogen assimilation rates (Carpenter and McCarthy, 1975; Carpenter and Price, 1976; Mague et al., 1977; Taylor et al., 1973), and CO_2 fixation (Carpenter and Price, 1977; Li et al., 1980) by Trichodesmium now exists.

The macroscopic (~0.5mm) nature of the colonies provides, in a sense, the algal counterpart to the fish tank experiment described in Chapter 5. Measurements of many parameters can be made without pressing the limit of sensitivity of the various methods, a benefit for initial studies of phytoplankton sulfur metabolism. In addition, the fact that it is a procaryotic organism renders more likely a correspondence between sulfate incorporation into protein and protein synthesis similar to that found for bacteria.

During R/V Oceanus cruise #84, I noticed the characteristic tufts of the alga in a relatively shallow net haul made by Ralph Evans. There

were few, and I was too busy with the Sargasso Sea station (Chapter 5) to do anything about it. The observation stimulated discussion with Steve Lohrenz, another graduate student on the cruise, who was working on CO_2 fixation studies with phytoplankton. Two days later, when much of the crush of the Sargasso Sea station was over and the tufts were more numerous in the zooplankton net hauls, Steve and I decided to combine our skills and approach a multi-parameter investigation of macromolecular synthesis in Trichodesmium.

MATERIALS AND METHODS

Sampling: Colonies of Trichodesmium were collected in an oblique tow from 30m to 5m depth at the Sargasso Sea station at noon on 26 July, 1980. A 1m net (505 μm mesh) with a gallon plastic cod end was used. Ship speed was maintained at under 0.5 knots for 25 minutes: the net was hauled up 5m every 5 minutes. When the net surfaced, the cod end was quickly removed and taken to a shaded location, where the larger zooplankton were gently removed with a 1mm mesh screen. The colonies were diluted into surface seawater which had been prefiltered through a 28 μm mesh Nitex net, and 500ml aliquots containing 80-110 colonies were gently transferred to wide mouth plastic bottles.

Inoculation: The bottles were spiked with either $^{14}CO_2$ (final specific activity 671 DPM/nMole, assuming a CO_2 concentration of 2mM), $^{32}PO_4^{=}$ (final activity 25,000 DPM/ml), $^{35}SO_4^{=}$ (final specific activity 1 DPM/pMole; sulfate concentration 29.9mM), or UL-^{14}C-glyco-

late (final activity 10,100 DPM/ml). Samples of 10 colonies were taken at zero time and at intervals thereafter by transferring with a Pasteur pipet into test tubes containing ~10ml of surface seawater. The colonies were then pipetted onto Whatman GF/F filters, rinsed with several ml of seawater, and frozen for later analysis. The bottles were incubated in a deck box cooled by flowing surface seawater with screening to reduce the light intensity to about 30% of the incident radiation (bright, clear day: ~1200 $\mu E/cm^2$). Caution was exercized to prevent disintegration of the colonies during handling.

Other Measurements: Immediately after the zero time sampling, groups of 10 colonies were placed on combusted glass filters for analysis of chlorophyll a, POC, PON, protein, and carbohydrate by the methods outlined in Chapter 2. Chlorophyll a was determined on board in 90 % acetone extracts using a Turner Model 110 fluorometer.

Radioisotopes: All radioisotopes were obtained from Amersham (Chicago, IL). $^{32}PO_4^{=}$ and $^{35}SO_4^{=}$ were carrier free. The highest specific activity glycolate and $^{14}CO_2$ available were used.

Fractionation of Radioactively-Labeled Colonies: Subcellular fractionation was carried out as described in Chapter 2. Phosphate, CO_2, and glycolate samples were extracted for RNA, the sulfate samples were not. CO_2 samples were fractionated by Steve Lohrenz.

RESULTS

General Characteristics

The colonies were dull green to green-brown. Both the linear and radial colony morphologies were found (Taylor, 1973), with linear colonies dominating by about 10:1. All descriptions refer to linear colonies unless otherwise noted. During the course of the incubation, colonies left over from the biochemical analysis samples rapidly turned brown and coagulated into nearly amorphous masses at the bottom of the bottle. This tendency was less pronounced in the labeled samples incubated at surface temperature, but colony disintegration became a factor in the incubations after about 4 hours, a problem which has been previously encountered (Mague et al., 1974).

Biochemical Composition

The amount of carbon, nitrogen, carbohydrate, protein, and chlorophyll *a* per 10 colonies are shown in Table 6-1, with results obtained by other investigators for comparison. The C:N ratio is indicative of healthy phytoplankton. Only 10% of the N can be accounted for in protein, but the cell walls of blue-green algae contain glucosamine and other nitrogenous sugars.

Table 6-1. Biochemical composition of *Trichodesmium* spp. in the Sargasso Sea[a]

Parameter	Colony Morphology: Linear	Colony Morphology: Spherical	Previously Reported Values
	µg/10 colonies		
Chlorophyll *a*	0.56 ± 0.02(9)	0.73	0.34(2) 0.20(3)
Particulate Carbon	153 ± 18(5)	132	97(2) 62 - 173(4)
Particulate Nitrogen	28 ± 3(5)	22	24(2)
Protein	19 ± 4(4)	29	-
Carbohydrate	14	39	-
	Weight:Weight		
POC/Chlorophyll *a*	274	180	285(2)
PON/Protein	1.4	0.8	-
POC/PON	5.5	5.9	5.5 - 6.0(1) 4.1(2)

[a]Colonies were picked from the net and placed onto combusted GF/F filters, rinsed with seawater, and frozen for later analysis as described. Chlorophyll *a* was determined on board on 90% acetone extracts using a Turner 110 fluorometer. The number of replicates, usually of 10 colonies each, is shown in parentheses; only one analysis of each type could be performed on radial colonies due to their limited abundance.

(1) Saino and Hattori, 1978. Deep-Sea Res. 25: 1259.

(2) Mague, Mague, and Holm-Hansen, 1977. Mar. Biol. 41: 213.

(3) Li, Glover, and Morris, 1980. Limnol. Oceanogr. 25: 447.

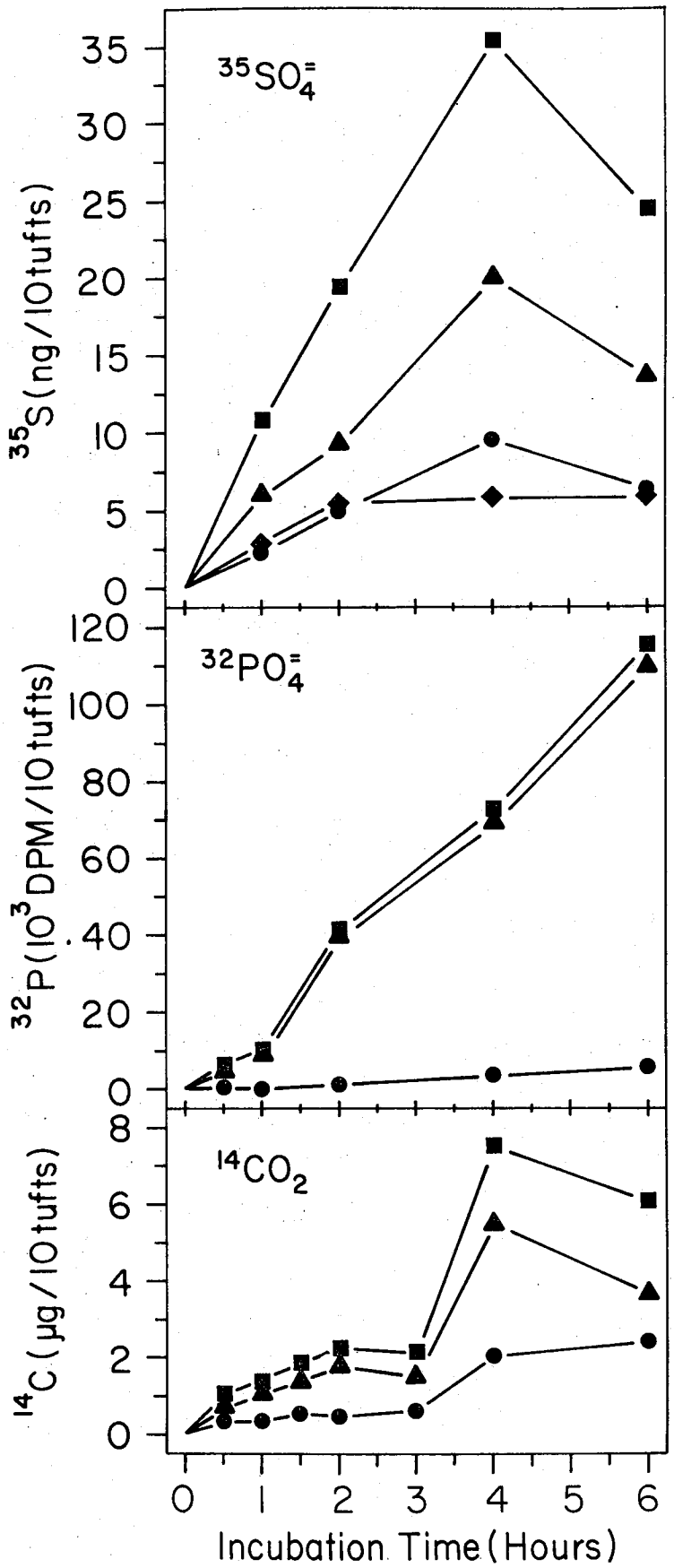
(4) Carpenter, 1973. Deep-Sea Res. 20: 285.

Isotope Incorporation

The results of isotope uptake and fractionation experiments are shown in Figure 6-1. Glycolate was not taken up to a significant extent and is not shown. Although very high rates of uptake for CO_2, $PO_4^{=}$, and $SO_4^{=}$ were noted, the subcellular distribution of all the labels was very unusual. In each case, the great majority of the label was found in L.M.W. materials. Over 95% of the phosphorus and 70 % of the carbon were contained in the cold TCA soluble fraction. In both samples, most of the acid-insoluble radioactivity was confined to RNA. Carbon was found in significant amounts in RNA from the start of the experiment, averaging about 18% of the total, but phosphate did not begin to appear in RNA until 2 hours of incubation. The extremely skewed distribution of carbon and phosphorus in L.M.W. and RNA components indicated that a small amount of protein synthesis was likely to be the only major biosynthetic event in the colonies.

The incorporation pattern of sulfate was similarly unusual, but confirmed the presence of protein synthesis activity. Protein-^{35}S increased linearly for the first four hours, then declined, probably as a result of colony disintegration. Lipid-^{35}S increased at the same rate as incorporation into protein for two hours, then leveled off. The most unexpected result was the large proportion of L.M.W. organic-^{35}S (about 50 % of the total S). If the composition of Trichodesmium with respect to sulfur distribution is similar to bacteria, the high proportion of L.M.W. organic sulfur would be indicative of extremely unbalanced growth,

Figure 6-1. Time-course incorporation of $^{35}SO_4^{=}$, $^{23}PO_4^{=}$, and $^{14}CO_2$ into major biochemical fractions of Trichodesmium spp. in the Sargasso Sea. Samples were collected and incubated as described in Materials and Methods. Symbols, all panels: ■, total uptake; ▲, low molecular weight. $SO_4^{=}$ panel: ●, residue protein; ◆, lipid. CO_2 and $PO_4^{=}$ panels: ●, TCA insoluble material.


$^{35}SO_4^=$
^{35}S(ng/10tufts)
$^{32}PO_4^=$
$^{32}P(10^3$DPM/10tufts)
$^{14}CO_2$
^{14}C(µg/10tufts)
Incubation Time (Hours)

perhaps suggesting that the colonies were senescing. This concept is supported by the high percentage of L.M.W. materials derived from the other labels.

DISCUSSION

It is not the purpose of this chapter to describe the observed patterns of incorporation of the various labels by Trichodesmium in detail. The points which are to be emphasized are: (1) sulfolipid synthesis was detectable and at a significant level with respect to total sulfur metabolism, with a good correlation between sulfolipid synthesis and protein synthesis for the first two hours of the incubation. After this time, disintegration of colonies became an important variable. As the afternoon progressed, the tufts which had previously resided at depth rose to the surface. By late afternoon one could count them in the water simply by peering over the side of the ship; by this time most were straw colored rather than green. If this indicates senescence of the population, then sulfolipid synthesis was a sensitive indicator of near-future physiology, since its synthesis halted after mid-afternoon. (2) The fractionation procedure once again revealed important physiological conditions which will strongly affect the interpretation of the growth study. The simple measurement of whole cell uptake would have lead to the conclusion that the colonies were growing at a rapid rate. The extremely high proportion of each label in L.M.W. components suggests that this is not so.

REFERENCES

Abrams, W. R. and J. A. Schiff. 1973. Studies of sulfate utilization by algae. 11. An enzyme-bound intermediate in the reduction of adenosine-5'-phosphosulfate (APS) by cell-free extracts of wild-type Chlorella and mutants blocked for sulfate reduction. Arch. Mikrobiol. 94: 1-10.

American Public Health Association: Standard methods for the analysis of water and wastewater. Washington (1975).

Anderson, G. C. and R. P. Zeutschel. 1970. Release of dissolved organic matter by marine phytoplankton in coastal and offshore areas of the northeast Pacific Ocean. Limnol. Oceanogr. 15(3): 402-407.

Anfinsen, C. B. 1973. Principles that govern the folding of protein chains. Science 181: 223-230.

Antia, N. J., C. D. McAllister, T. R. Parsons, K. Stephens, and J. D. H. Strickland. 1963. Further measurements of primary production using a large-volume plastic sphere. Limnol. Oceanogr. 8(2): 166-183.

Antoine, A. D. and B. S. Tepper. 1969. Environmental control of glycogen and lipid content of Mycobacterium phlei. J. Gen. Microbiol. 55: 217-226.

Apontoweil, P. and W. Berends. 1975. Glutathione biosynthesis in Escherichia coli K12. Properties of the enzymes and regulation. Biochim. Biophys. Acta 399(1): 1-9.

Asato, A. 1979. Macromolecular synthesis in synchronized cultures of Anacystis nidulans. J. Bacteriol. 140(1): 65-72.

Atkinson, D. E. 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry, New York 7: 4030-4034.

Atkinson, D. E. and G. M. Walton. 1967. Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J. Biol. Chem. 242: 3239-3241.

Azam, F. and O. Holm-Hansen. 1973. Use of tritiated substrates in the study of heterotrophy in seawater. Mar. Biol. 23: 191-196.

Bandurski, R. S., L. G. Wilson, and C. L. Squires. 1956. The mechanism of "active sulfate" formation. J. Amer. Chem. Soc. 78: 6408-6409.

Beardall, J. and I. Morris. 1976. The concept of light intensity adaptation in marine phytoplankton: some experiments with Phaeodactylum tricornutum. Mar. Biol. 37: 377-387.

Becker, M. A., N. M. Kredich, and G. M. Tomkins. 1969. The purification and characterization of O-acetylserine sulfhydrylase-A from Salmonella typhimurium. J. Biol. Chem 244(9): 2418-2427.

Belaich, J. P., A. Belaich, and P. Simonpietri. 1972. Uncoupling in bacterial growth: effect of pantothenate starvation on growth of Zymomonas mobilis. J. Gen. Microbiol. 70: 179-185.

Benson, A. A. 1963. The plant sulfolipid. Adv. Lipid Res. 1: 387-394.

Berman, T. 1970. Alkaline phosphatases and phosphorus availability in Lake Kinneret. Limnol. Oceanogr. 15(5): 663-674.

Berman, T. and O. Holm-Hansen. 1974. Release of photoassimilated carbon as dissolved organic matter by marine phytoplankton. Mar. Biol. 28: 305-310.

Bhovichitra, M. and E. Swift. 1977. Light and dark uptake of nitrate and ammonium by large oceanic dinoflagellates: Pyrocystis noctiluca, Pyrocystis fusiformis, and Dissodinium lunula. Limnol. Oceanogr. 22(1): 73-83.

Boffi, V. 1969. Biochemical patterns of some heterotrophic bacteria grown in defined media. J. Gen. Microbiol. 55: 227-242.

Bradfield, G., P. Somerfield, T. Meyn, M. Holby, D. Babcock, D. Bradley, and I. H. Segel. 1970. Regulation of sulfate trans- port in filamentous fungi. Plant Physiol. 46: 720-727.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quanitities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Buchanan, R. E. and N. E. Gibbons (eds.). 1974. Bergey's Manual of Determinative Bacteriology. Williams a Wilkins, Baltimore. 1245pp. 8th ed.

Busby, W. F. and A. A. Benson. 1973. Sulfonic acid metabolism in the diatom Navicula pelliculosa. Plant Cell Physiol. 14: 1123-1132.

Campbell, P. G. C. and J. H. Baker. 1978. Estimation of bacterial production in fresh waters by the simultaneous measurement of (^{35}S)sulphate and D-(^{3}H)glucose uptake in the dark. Can J. Microbiol. 24: 939-946.

Carbon, J. A., L. Hung, and D. S. Jones. 1965. A reversible oxidative inactivation of specific transfer RNA species. Proc. Natl. Acad. Sci. 53: 979-986.

Carpenter, E. J. and J. J. McCarthy. 1975. Nitrogen fixation and uptake of combined nitrogenous nutrients by Oscillatoria (Trichodesmium) thiebautii in the western Sargasso Sea. Limnol. Oceanogr. 20(3): 389-401.

Carpenter, E. J. and C. C. Price IV. 1976. Marine Oscillatoria (Trichodesmium): explanation for aerobic nitrogen fixation without heterocysts. Science 191: 1278-1280.

Carpenter, E. J. and C. C. Price IV. 1977. Nitrogen fixation, distribution, and production of Oscillatoria (Trichodesmium) spp. in the western Sargasso ans Caribbean Seas. Limnol. Oceanogr. 22(1): 60-72.

Carpenter, E. J., C. C. Remsen, and S. W. Watson. 1972. Utilization of urea by some marine phytoplankters. Limnol. Oceanogr. 17(2): 265-269.

Castellvi, J. and A. Ballester. 1974. Activite heterotrophique bacterienne en rapport avec les conditions hydrologiques des systemes marins. Tethys 6(1-2): 189-202.

Cohen, S. S. 1971. Introduction to the Polyamines. Prentice-Hall, New Jersey. pp. 64-166.

Daley, R. J. and J. E. Hobbie. 1975. Direct counts of aquatic bacteria by a modified epifluorescence technique. Limnol. Oceanogr. 20(5): 875-882.

Datko, A. H., S. H. Mudd, J. Giovanelli, and P. K. Macnicol. 1978a. Sulfur-containing compounds in Lemna perpusilla 6746 grown at a range of sulfate concentrations. Plant Physiol. 62: 629-635.

Datko, A. H., S. H. Mudd, P. K. Macnicol, and J. Giovanelli. 1978b. Phytostat for the growth of Lemna in semicontinuous culture with low sulfate. Plant Physiol. 62: 622-628.

Dawes, E. A. and P. J. Senior. 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. 10: 136-266.

Deane, E. M. and R. W. O'Brien. 1975. Sulfate uptake and metabolism in the marine chrysomonad, Monochrisis lutheri. Arch. Microbiol. 105: 295-301.

Delaney, S. F., A. Dickson, and N. G. Carr. 1973. The control of homoserine-O-transsuccinylase in a methionine-requiring mutant of the blue-green alga Anacystis nidulans. J. Gen. Microbiol. 79: 89-94.

Dietz, A. S., L. J. Albright, and T. Tuominen. 1977. Alternative model and approach for determining microbial heterotrophic activities in aquatic systems. Appl. Environ. Microbiol. 33(4): 817-823.

Dreyfuss, J. 1964. Characterization of a sulfate- and thiosulfate-transporting system in Salmonella typhimurium. J. Biol. Chem. 239(7): 2292-2297.

Dreyfuss, J. and K. J. Monty. 1963. The biochemical characterization of cysteine-requiring mutants of Salmonella typhimurium. J. Biol. Chem. 238(3): 1019-1024.

Dreyfuss, J. and A. B. Pardee. 1965. Evidence for a sulfate-binding site external to the cell membrane of Salmonella typhimurium. Biochim. Biophys. Acta 104: 308-310.

Dugdale, R. C., J. J. Goering, and J. H. Ryther. 1964. High nitrogen fixation rates in the Sargasso Sea and the Arabian Sea. Limnol. Oceanogr. 9: 507-510.

Dunstall, T. G. and C. Nalewajko. 1975. Extracellular release in planktonic bacteria. Verh. Internat. Verein. Limnol. 19: 2643-2649.

Elmore, C. D. and E. E. King. 1978. Amino acid composition of germinating cotton seeds. Plant Physiol. 62: 531-535.

Eppley, R. W., R. W. Holmes, and J. D. H. Strickland. 1967. Sinking rates of marine phytoplankton measured with a fluorometer. J. exp. mar. Biol. a Ecol. 1: 191-208.

Eppley, R. W. and J. N. Rogers. 1970. Inorganic nitrogen assimilation of Ditylum brightwellii, a marine plankton diatom. J. Phycol. 6(4): 344-351.

Fahey, R. C., W. C. Brown, W. B. Adams, and M. B. Worsham. 1978. Occurrence of glutathione in bacteria. J. Bacteriol. 133(3): 1126-1129.

Ferguson, R. L. and P. Rublee. 1976. Contribution of bacteria to the standing crop of coastal plankton. Limnol. Oceanogr. 21(1): 141-145.

Fitzgerald, J. W. 1976. Sulfate ester formation and hydrolysis: a potentially important yet often ignored aspect of the sulfur cycle of aerobic soils. Bact. Rev. 40(3): 698-721.

Fuhrman, J. A. and F. Azam. 1980. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. Environ. Microbiol. 39(6): 1085-1095.

Gauthier, M. J. 1976. Morphological, physiological, and biochemical characteristics of some violet-pigmented bacteria isolated from seawater. Can. J. Microbiol. 22: 138-149.

Gersberg, R., K. Krohn, N. Peek, and C. R. Goldman. 1976. Denitrification studies with ^{13}N-labeled nitrate. Science 192: 1229-1231.

Godchaux, W. and E. R. Leadbetter. 1980. Capnocytophaga spp. contain sulfonolipids that are novel in procaryotes. J. Bacteriol. 144(2): 592-602.

Goering, J. J. and R. C. Dugdale. 1964. Cyclic diurnal variations in the uptake of ammonia and nitrate by photosynthetic organisms in the Sargasso Sea. Limnol. Oceanogr. 9: 448-451.

Goering, J. J., R. C. Dugdale, and D. W. Menzel. 1966. Estimates of in situ rates of nitrogen uptake by Trichodesmium sp. in the tropical Atlantic Ocean. Limnol. Oceanogr. 11: 614-620.

Goldman, J. C. 1977. Steady state growth of phytoplankton in continuous culture: comparison of internal and external nutrient equations. J. Phycol. 13: 251-258.

Goldman, J. C., J. J. McCarthy, and D. G. Peavey. 1979. Growth rate influence on the chemical composition of phytoplankton in oceanic waters. Nature 279: 210-215.

Goldschmidt, E. E., M. L. S. Tsang, and J. A. Schiff. 1975. Studies of sulfate utilization by algae. 13. Adenosine-5'-phosphosulfate (APS) as an intermediate in the conversion of adenosine-3'-phosphate-5'-phosphosulfate (PAPS) to acid-volatile radioactivity. Plant Science Letters 4: 293-299.

Gunnison, D. and M. Alexander. 1975. Resistance and susceptibility of algae to decomposition by natural microbial communities. Limnol. Oceanogr. 20(1): 64-70.

Haas, L. W. and K. L. Webb. 1979. Nutritional mode of several non-pigmented microflagellates from the York River Estuary, Virginia. J. exp. mar. Biol. Ecol. 39: 125-134.

Hamilton, R. D. and K. E. Austin. 1967. Physiological and cultural characteristics of Chromobacterium marinum sp. n. Antonie van Leeuwenhoeck, J. Microbiol. Serol. 33: 257-264.

Hamilton, R. D. and J. E. Preslan. 1970. Observations on heterotrophic activity in the eastern tropical Pacific Ocean. Limnol. Oceanogr. 15: 395-401.

Harbison, G. R. and V. L. McAlister. 1979. The filter-feeding rates and particle retention efficiencies of three species of Cyclosalpa (Tunicata, Thaliacea). Limnol. Oceanogr. 24(5): 875-892.

Hart, J. W. and P. Filner. 1969. Regulation of sulfate uptake by amino acids in cultured tobacco cells. Plant Physiol. 44: 1253-1259.

Hellebust, J. A. 1965. Excretion of some organic compounds by marine phytoplankton. Limnol. Oceanogr. 10(2): 192-206.

Herbert, D. 1961. The chemical composition of micro-organisms as a function of their environment. pp. 391-416. in Microbial Reaction to Environment (11th Symposium of the Society for General Microbiology), University Press, Cambridge.

Herbland, A. 1974. Activite bacterienne dans l'upwelling Mauritanien relation avec l'oxygene et la matiere organique. Tethys 6(1-2): 203-212.

Hicks, S. E. and F. G. Carey. 1968. Glucose determination in natural waters. Limnol. Oceanogr. 13: 361-363.

Hobbie, J. E. and C. C. Crawford. 1969. Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. Limnol. Oceanogr. 14: 528-532.

Hobbie, J. E., O. Holm-Hansen, T. T. Packard, L. R. Pomery, R. W. Sheldon, J. P. Thomas, and W. J. Wiebe. 1972. A study of the distribution and activity of microorganisms in ocean water. Limnol. Oceanogr. 17(4): 544-555.

Hobson, L. A. and R. J. Pariser. 1971. The effect of inorganic nitrogen on macromolecular synthesis by Thalassiosira fluviatilis Hustedt and Cyclotella nana Hustedt grown in batch culture. J. exp. mar. Biol. Ecol. 6: 71-78.

Hodson, R. E. and F. Azam. 1977. Determination and biological significance of dissolved ATP in seawater. pp. 127-140 in 2nd Biannual ATP Methodology Symposium (G. A. Borun, ed.). SAI Technology Co., La Jolla.

Hodson, R. C., J. A. Schiff, and J. P. Mather. 1971. Studies of sulfate utilization by algae. 10. Nutritional and enzymatic characterization of Chlorella mutants impaired for sulfate utilization. Plant Physiol. 47: 306-311.

Hodson, R. C., J. A. Schiff, and A. J. Scarsella. 1968. Studies of sulfate utilization by algae. 7. In vivo metabolism of thiosulfate by Chlorella. Plant Physiol. 43(4): 570-577.

Hodson, R. C., J. A. Schiff, A. J. Scarsella, and M. Levinthal. 1968. Studies of sulfate utilization by algae. 6. Adenosine-3'-phosphate-5'-phosphosulfate (PAPS) as an intermediate in thiosulfate formation from sulfate by cell-free extracts of Chlorella. Plant Physiol. 43(4): 563-569.

Holmern, K., M. S. Vange, and P. Nissen. 1974. Multiphasic uptake of sulfate by barley roots. II. Effects of washing, divalent cations, inhibitors, and temperature. Physiol. Plant. 31: 302-310.

Holm-Hansen, O. and C. R. Booth. 1966. The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol. Oceanogr. 11: 510-519.

Holmquist, R. 1978. Evaluation of compositional nonrandomness in proteins. J. Mol. Evol. 11: 349-360.

Hoppe, H.-G. 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of autoradiography. Mar. Biol. 36(4): 291-302.

Hulanicka, M. D., S. G. Hallquist, N. M. Kredich, and T. Mojica-A. 1979. Regulation of O-acetylserine sulfhydrylase B by L-cysteine in Salmonella typhimurium. J. Bacteriol. 140(1): 141-146.

Ingraham, J. L. and G. F. Bailey. 1959. Comparative study of effect of temperature on metabolism of psychrophilic and mesophilic bacteria. J. Bacteriol. 77(5): 609-613.

Jannasch, H. W. 1967. Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. 12: 264-271.

Jannasch, H. W. 1974. Steady state and the chemostat in ecology. Limnol. Oceanogr. 19(4): 716-720.

Jannasch, H. W. and G. E. Jones. 1959. Bacterial populations in sea water as determined by different methods of enumeration. Limnol. Oceanogr. 4: 128-139.

Jannasch, H. W. and C. O. Wirsen. 1973. Deep-sea microorganisms: in situ response to nutrient enrichment. Science 180: 641-643.

Jassby, A. D. 1975. Dark sulfate uptake and bacterial productivity in a subalpine lake. Ecology 56(3): 627-636.

Jermyn, M. A. 1975. Increasing the sensitivity of the anthrone method for carbohydrate. Anal. Biochem. 68: 332-335.

Jeanjean, R. and E. Broda. 1977. Dependence of sulfate uptake by Anacystis nidulans on energy, on osmotic shock, and on sulfate starvation. Arch. Microbiol. 114: 19-23.

Jeanjean, R., A. Hourmant, and G. Ducet. 1975. Effet des inhibiteurs de groupes SH sur le transport du phosphate chez Chlorella pyrenoidosa. Biochimie 57: 383-390.

Jordan, M. J., R. J. Daley, and K. Lee. 1978. Improved filtration procedures for freshwater (^{35}S)$SO_4^{=}$ uptake studies. Limnol. Oceanogr. 23(1): 154-157.

Jordan, M. J. and B. J. Peterson. 1978. Sulfate uptake as a measure of bacterial production. Limnol. Oceanogr. 23(1): 146-150.

Jukes, T. H., R. Holmquist, and H. Moise. 1975. Amino acid composition of proteins: selection against the genetic code. Science 189: 50-51.

Kaback, H. R. and E. M. Barnes, Jr. 1971. Mechanisms of active transport in isolated membrane vesicles. II. The mechanism of energy coupling between D-lactic dehydrogenase and β-galactoside transport in membrane preparations from Escherichia coli. J. Biol. Chem. 246(17): 5523-5531.

Karl, D. M. 1978a. Distribution, abundance, and metabolic states of microorganisms in the water column and sediments of the Black Sea. Limnol. Oceanogr. 23(5): 936-949.

Karl, D. M. 1978b. Occurrence and ecological significance of GTP in the ocean and in microbial cells. Appl. Environ. Microbiol. 36(2): 349-355.

Karl, D. M. 1979. Measurement of microbial activity and growth in the ocean by rates of stable ribonucleic acid synthesis. Appl. Environ. Microbiol. 38(5): 850-860.

Karl, D. M. and D. B. Craven. 1980. Effects of alkaline phosphatase on nucleotide measurements in aquatic microbial communities. Appl. Environ. Microbiol. 40(3): 549-561.

Karl, D. M., C. O. Wirsen, and H. W. Jannasch. 1980. Deep-sea primary production at the Galapagos hydrothermal vents. Science 207: 1345-1347.

King, J. D. and D. C. White. 1977. Muramic acid as a measure of microbial biomass in estuarine and marine samples. Appl. Environ. Microbiol. 33(4): 777-783.

Kredich, N. M. 1971. Regulation of L-cysteine biosynthesis in Salmonella typhimurium. I. Effects of growth on varying sulfur sources and O-acetyl-L-serine on gene expression. J. Biol. Chem. 246(11): 3474-3484.

Kredich, N. M., M. A. Becker, and G. M. Tomkins. 1969. Purification and characterization of cysteine synthetase, a bifunctional protein complex, from Salmonella typhimurium. J. Biol. Chem. 244(9): 2428-2439.

Kredich, N. M. and G. M. Tomkins. 1966. The enzymatic synthesis of L-cysteine in Escherichia coli and Salmonella typhimurium. J. Biol. Chem. 241(21): 4955-4965.

Laishley, E. J., R. G. L. McCready, R. Bryant, and H. R. Krouse. 1976. Stable isotope fractionation by Clostridium pasteurianum. pp. 327-349 in: J. O. Nriagu (ed.). Environmental Biogeochemistry, Volume 1. Ann Arbor Science, Ann Arbor, Michigan.

LaRock, P. A., R. D. Lauer, J. R. Schwarz, K. Watanabe, and D. A. Wiesenberg. 1979. Microbial biomass and activity distribution in an anoxic, hypersaline basin. Appl. Environ. Microbiol. 37(3): 466-470.

Law, A. T. and D. K. Button. 1977. Multiple-carbon-source-limited growth kinetics of a marine coryneform bacterium. J. Bacteriol. 129(1): 115-123.

Lean, D. R. S. 1973. Phosphorus dynamics in lake water. Science 179: 678-680.

Lean, D. R. S. and C. Nalewajko. 1976. Phosphate exchange and organic phosphorus excretion by freshwater algae. J. Fish. Res. Board Can. 33(6): 1312-1323.

Lee, C. and J. L. Bada. 1977. Dissolved amino acids in the equatorial Pacific, the Sargasso Sea, and Biscayne Bay. Limnol. Oceanogr. 22(3): 502-510.

Leggett, J. E. and E. Epstein. 1956. Kinetics of sulfate absorption by barley roots. Plant Physiol. 31: 222-226.

Lehmann, M. ang G. Wober. 1976. Accumulation, mobilization, and turnover of glycogen in the blue-green bacterium Anacystis nidulans. Arch. Microbiol. 111: 93-97.

Leinweber, F.-J. and K. J. Monty. 1963. The metabolism of thiosulfate in Salmonella typhimurium. J. Biol. Chem. 238(11): 3775-3780.

Lewin, J. and W. F. Busby, Jr. 1967. The sulphate requirements of some unicellular marine algae. Phycologia 6(4): 211-217.

Li, W. K. W., H. E. Glover, and I. Morris. 1980. Physiology of carbon photoassimilation by Oscillatoria thiebautii in the Caribbean Sea. Limnol. Oceanogr. 25(3): 447-456.

Lipsett, M. N. 1965. The behavior of 4-thiouridine in the E. coli s-RNA molecule. Biochem. Biophys. Res. Comm. 20(2): 224-229.

Lyman, J. and R. H. Fleming. 1940. Composition of sea water. J. Mar. Res. 3: 134-146.

Mague, T. H., F. C. Mague, and O. Holm-Hansen. 1977. Physiology and chemical composition of nitrogen-fixing phytoplankton in the central North Pacific Ocean. Mar. Biol. 41: 213-227.

Mague, T. H., N. M. Weare, and O. Holm-Hansen. 1974. Nitrogen fixation in the North Pacific Ocean. Mar. Biol. 24: 109-119.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3: 208-218.

Marzluf, G. A. 1970a. Genetic and biochemical studies of distinct sulfate permease species in different developmental stages of Neurospora crassa. Arch. Biochem. Biophys. 138: 254-263.

Marzluf, G. A. 1970b. Genetic and metabolic controls for sulfate metabolism in Neurospora crassa: Isolation and study of chromate-resistant and sulfate transport-negative mutants. J. Bacteriol. 102(3): 716-721.

Marzluf, G. A. 1974. Uptake and efflux of sulfate in Neurospora crassa. Biochim. Biophys. Acta 339: 374-381.

McCarthy, J. J. 1972. The uptake of urea by natural populations of marine phytoplankton. Limnol. Oceanogr. 17(5): 738-748.

McCarthy, J.J. and J.C. Goldman. 1979. Nitrogenous nutrition of marine phytoplankton in nutrient-depleted waters. Science 203: 670-672.

McCready, R. G. L., E. J. Laishley, and H. R. Krouse. 1975. Stable isotope fractionation by Clostridium pasteurianum. 1. $^{34}S/^{32}S$: inverse isotope effects during SO_4^{2-} and SO_3^{2-} reduction. Can. J. Microbiol. 21: 235-244.

Monheimer, R. H. 1974. Sulfate uptake as a measure of planktonic microbial production in freshwater ecosystems. Can. J. Microbiol. 20: 825-831.

Monheimer, R. H. 1978a. Microbial heterotrophic production in two lakes of different productivity. Verh. Internat. Verein. Limnol. 20: 2222-2226.

Monheimer, R. H. 1978b. Difficulties in interpretation of microbial heterotrophy from sulfate uptake data: Laboratory studies. Limnol. Oceanogr. 23(1): 150-154.

Morris, I., H. E. Glover, and C. S. Yentsch. 1974. Products of photosynthesis by marine phytoplankton: the effect of environmental factors on the relative rates of protein synthesis. Mar. Biol. 27: 1-9.

Murphy, T. P. 1980. Ammonia and nitrate uptake in the lower Great Lakes. Can. J. Fish. Aquat. Sci. 37(9): 1365-1372.

Nalewajko, C., T. G. Dunstall, and H. Shear. 1976. Kinetics of extracellular release in axenic algae and in mixed algal-bacterial cultures: significance in estimation of total (gross) phytoplankton excretion rates. J. Phycol. 12: 1-5.

Neidhardt, F. C. and B. Magasanik. 1960. Studies on the role of ribonucleic acid in the growth of bacteria. Biochim. Biophys. Acta 42: 99-116.

Nelson, S. O., G. I. Glover, and C. W. Magill. 1975. The essentiality of sulfhydryl groups to transport in Neurospora crassa. Arch. Biochem. Biophys. 168: 483-489.

Paerl, H. W. 1973. Detritus in Lake Tahoe: structural modification by attached microflora. Science 180: 496-498.

Pardee, A. B. 1966. Purification and properties of a sulfate-binding protein from Salmonella typhimurium. J. Biol. Chem. 241(24): 5886-5892.

Pardee, A. B., L. S. Prestidge, M. B. Whipple, and J. Dreyfuss. 1966. A binding site for sulfate and its relation to sulfate transport into Salmonella typhimurium. J. Biol. Chem. 241(17): 3962-3969.

Parker, R. R., J. Sibert, and T. J. Brown. 1975. Inhibition of primary productivity through heterotrophic competition for nitrate in a stratified estuary. J. Fish. Res. Bd. Canada 32(1): 72-77.

Parsons, T. R. and J. D. H. Strickland. 1962. On the production of particulate organic carbon by heterotrophic processes in seawater. Deep-Sea Res. 8: 211-222.

Patni, N. J., S. W. Dhawale, and S. Aaronson. 1977. Extracellular phosphatases of Chlamydomonas reinhardi and their regulation. J. Bacteriol. 130(1): 205-211.

Pearce, S. M., V. A. Hildebrandt, and T. Lee. 1977. Third system for neutral amino acid transport in a marine bacterium. J. Bacteriol. 130(1): 37-47.

Perry, M. J. 1972. Alkaline phosphatase activity in subtropical central North Pacific waters using a sensitive fluorometric method. Mar. Biol. 15: 113-119.

Perry, M. J. 1976. Phosphate utilization by an oceanic diatom in phosphorus-limited chemostat culture and in oligotrophic waters of the central North Pacific. Limnol. Oceanogr. 21(1): 88-107.

Ramus, J. 1974. In vivo molybdate inhibition of sulfate transfer to Porphyridium capsular polysaccharide. Plant Physiol. 54: 945-949.

Ramus, J. and S. T. Groves. 1974. Precursor-product relationships during sulfate incorporation into Porphyridium capsular polysaccharide. Plant Physiol. 53: 434-439.

Reeck, G. R. and L. Fisher. 1973. A statistical analysis of the amino acid compositions of proteins. Int. J. Peptide Protein Res. 5: 109-117.

Reichhardt, W. 1975. Bacterial decomposition of different polysaccharides in a eutrophic lake. Verh. Internat. Verein. Limnol. 19: 2636-2642.

Renosto, F. and G. Ferrari. 1975. Mechanism of sulfate transport inhibition by cycloheximide in plant tissues. Plant Physiol. 56: 478-480.

Rhee, G.-Y. 1972. Competition between and alga and an aquatic bacterium for phosphate. Limnol. Oceanogr. 17(4): 505-514.

Rhee, G.-Y. 1973. A continuous culture study of phosphate uptake, growth rate and polyphosphate in Scenedesmus sp. J. Phycol. 9: 495-506.

Roberts, K. R. and G. A. Marzluf. 1971. The specific interaction of chromate with the dual sulfate permease systems of Neurospora crassa. Arch. Biochem. Biophys. 142: 651-659.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1963. Studies of biosynthesis in Escherichia coli. Carnegie Inst. Wash. Publ. No. 607: 521 pp.

Rosenbauer, R. J., J. L. Bischoff, and W. E. Seyfried. 1979. Determination of sulfate in seawater and natural brines by ^{133}Ba and membrane dialysis. Limnol. Oceanogr. 24(2): 393-396.

Rowbury, R. J. and D. D. Woods. 1964a. Repression by methionine of cystathionase formation in Escherichia coli. J. Gen. Microbiol. 35: 145-158.

Rowbury, R. J. and D. D. Woods. 1964b. O-Succinylhomoserine as an intermediate in the synthesis of cystathionine by Escherichia coli. J. Gen. Microbiol. 36: 341-358.

Ruby, E. G. and K. H. Nealson. 1977. Pyruvate production and excretion by the luminous marine bacteria. Appl. Environ. Microbiol. 34(2): 164-169.

Schell, D. M. 1974. Uptake and regeneration of free amino acids in marine waters of southeast Alaska. Limnol. Oceanogr. 19(2): 260-270.

Schiff, J. A. and R. C. Hodson. 1970. Pathways of sulfate reduction in algae. Ann. N.Y. Acad. Sci. 175: 555-576.

Schmidt, A., W. R. Abrams, and J. A. Schiff. 1974. Reduction of adenosine-5'-phosphosulfate to cysteine in extracts from Chlorella and mutants blocked for sulfate reduction. Eur. J. Biochem. 47: 423-434.

Schneider, J. F. and J. Westley. 1963. Direct incorporation of thiosulfate sulfur into cysteine by lysed rat liver mitochondria. J. Biol. Chem. 238(10): PC3516-3517.

Segel, I. H. and M. J. Johnson. 1961. Accumulation of intracellular inorganic sulfate by Penicillium chrysogenum. J. Bacteriol. 81(1): 91-98.

Sieburth, J. McN. 1976. Bacterial substrates and productivity in marine ecosystems. Ann. Rev. Ecol. Systematics 7: 259-285.

Sinensky, M. 1977. Specific deficit in the synthesis of 6-sulfoquinovosyl diglyceride in Chlorella pyrenoidosa. J. Bacteriol. 129(1): 516-524.

Sirevag, R. and J. G. Ormerod. 1977. Synthesis, storage, and degradation of polyglucose in Chlorobium thiosulfatophilum. Arch. Microbiol. 111: 239-244.

Slepecky, R. A. and J. H. Law. 1961. Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of Bacillus megaterium. J. Bacteriol. 82: 37-42.

Smith, I. K. 1975. Sulfate transport in cultured tobacco cells. Plant Physiol. 55: 303-307.

Smith, I. K. 1976. Characterization of sulfate transport in cultured tobacco cells. Plant Physiol. 58: 358-362.

Strickland, J. D. H., O. Holm-Hansen, R. W., Eppley, and R. J. Linn. 1969. The use of a deep tank in plankton ecology. I. Studies of the growth and composition of phytoplankton crops at low nutrient levels. Limnol. Oceanogr. 14(1): 23-34.

Strickland, J. D. H. and T. R. Parsons. 1972. A practical handbook of seawater analysis. Fish. Res. Board Can. Bull. No. 167. 310 pp.

Takahashi, M. and S. Ichimura. 1971. Glucose uptake in ocean profiles with special reference to temperature. Mar. Biol. 11: 206-213.

Taylor, B. F., C. C. Lee, and J. S. Bunt. 1973. Nitrogen-fixation associated with the marine blue-green alga, Trichodesmium, as measured by the acetylene-reduction technique. Arch. Mikrobiol. 88: 205-212.

Taylor, C. D. and H. W. Jannasch. 1976. Subsampling technique for measuring growth of bacterial cultures under high hydrostatic pressure. Appl. Environ. Microbiol. 32(3): 355-359.

Tempest, D. W., J. L. Meers, and C. M. Brown. 1970. Influence of the environment on the content and composition of microbial free amino acid pools. J. Gen. Microbiol. 64: 171-185.

Tindall, D. R., J. H. Yopp, W. E. Schmid, and D. M. Miller. 1977. Protein and amino acid composition of the obligate halophile, Aphanothece halophytica (Cyanophyta). J. Phycol. 13: 127-133.

Tsang, M. L. S. and J. A. Schiff. 1975. Studies of sulfate utilization by algae. 14. Distribution of adenosine-3'-phosphate-5'-phospho-sulfate (PAPS) and adenosine-5'-phosphosulfate (APS) sulfotransferases in assimilatory sulfate reducers. Plant Science Letters 4: 301-307.

Utkilen, H. C., M. Heldal, and G. Knutsen. 1976. Characterization of sulfate uptake in Anacystis nidulans. Physiol. Plant. 38: 217-220.

Vaccaro, R. F., S. E. Hicks, H. W. Jannasch, and F. G. Carey. 1968. The occurrence and role of glucose in seawater. Limnol. Oceanogr. 13: 356-358.

Vaccaro, R. F. and H. W. Jannasch. 1967. Variations in uptake kinetics for glucose by natural populations in seawater. Limnol. Oceanogr. 12(3): 540-542.

Vallee, M. and R. Jeanjean. 1968a. Le systeme de transport de $SO_4^{=}$ chez Chlorella pyrenoidosa et sa regulation. I. Etude cinetique de la permeation. Biochim. Biophys. Acta 150: 599-606.

Vallee, M. and R. Jeanjean. 1968b. Le systeme de transport de $SO_4^{=}$ chez Chlorella pyrenoidosa et sa regulation. II. Recherches sur la regulation de l'entree. Biochim. Biophys. Acta 150: 607-617.

Vange, M. S., K. Holmern, and P. Nissen. 1974. Multiphasic uptake of sulfate by barley roots. I. Effects of analogues, phosphate, and pH. Physiol. Plant. 31: 292-301.

Watson, S. W. 1978. Role of bacteria in an upwelling ecosystem. pp. 139-154 in: Boje, R. and M. Tomczak (eds.) Upwelling Ecosystems. Springer-Verlag, Berlin.

Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois. 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. 33(4): 940-946.

Webb, J. L. 1963. Enzyme and metabolic inhibitors. Volume 1. Academic Press New York. 949 pp.

Wiebe, P. H., L. P. Madin, L. R. Haury, G. R. Harbison, and L. M. Philbin. 1979. Diel vertical migration by Salpa aspera and its potential for large-scale particulate organic matter to the deep-sea. Mar. Biol. 53: 249-255.

Wiebe, W. J. and L. R. Pomeroy. 1972. Microorganisms and their association with aggregates and detritus in the sea: a microscopic study. pp. 325-352 in IBP-UNESCO Symposium on Detritus and its Role in Aquatic Ecosystems (Pallanza, Italy). [Mem. Ist. Ital. Idrobiol. 29 Suppl.].

Williams, P. J. LeB. 1973. The validity of the application of simple kinetic analysis to heterogeneous microbial populations. Limnol. Oceanogr. 18(1): 159-165.

Williams, P. J. LeB., T. Berman, and O. Holm-Hansen. 1976. Amino acid uptake and respiration by marine heterotrophs. Mar. Biol. 35: 41-47.

Williams, P. J. LeB. and C. S. Yentsch. 1976. An examination of photosynthetic production, excretion of photosynthetic products, and heterotrophic utilization of dissolved organic compounds with reference to results from a coastal subtropical sea. Mar. Biol. 35: 31-40.

Wirsen, C. O. and H. W. Jannasch. 1975. Activity of marine psychrophilic bacteria at elevated hydrostatic pressures and low temperatures. Mar. Biol. 31: 201-208.

Wright, R. T. and J. E. Hobbie. 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology 47(3): 447-464.

Yamamoto, L. A. and I. H. Segel. 1966. The inorganic sulfate transport system of Penicillium chrysogenum. Arch. Biochem. Biophys. 114: 523-538.

APPENDIX A

DATA USED IN PREPARING FIGURES

NOTE

All data in this appendix include a sample at zero time. This sample was taken immediately after isotope addition to determine the adsorption blank, and was fractionated with the other samples. The radioactivity in each fraction of the zero time sample was subtracted from the appropriate fraction in subsequent samples.

Table A-1. Total Uptake of UL-^{14}C-Glutamate and its Distribution in Biochemical Fractions of Pseudomonas halodurans During Batch Growth[a]

Hours of Incubation	nMoles C per ml	% of Total Radioactivity				
		L.M.W.	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
0	5.5	14.5	36.4	29.1	10.9	9.1
8	243.6	20.0	0	6.7	24.2	49.2
8.5	347.1	22.2	0	6.6	19.3	51.9
9	463.5	25.5	0	6.8	14.1	53.5
9.5	630.4	26.7	0	6.8	13.0	53.4
10	910.9	26.5	0	7.1	11.9	54.4
10.5	1306.5	27.3	0.3	7.3	11.6	53.6
11	1945.9	30.5	0.4	7.7	8.8	52.7
11.5	2265.1	29.1	0.5	8.6	7.6	54.2
12	2748.4	26.6	0.6	8.9	8.1	55.9
12.5	3294.8	26.0	0.5	9.0	7.9	56.6
13	3632.0	24.3	0.6	9.2	7.6	58.3
13.5	4026.6	24.9	0.9	8.5	8.3	57.4
14[b]	3444.7	23.4	0.5	8.6	8.3	59.1
15	4345.4	21.2	0.5	8.3	8.9	61.2
16	3997.5	19.2	0.5	8.9	8.4	63.1
18	8589.2	18.1	0.6	6.9	11.0	63.4
20	8863.8	14.9	0.7	7.3	12.5	64.6
22	5282.5	16.7	0.5	6.8	10.6	65.4
24	4839.4	18.4	0.5	7.1	10.8	63.1

[a]Data from Figure 4-2 in the text.

[b]A box of new and apparently cracked RA 984H glass fiber filters was opened and used for fractionation samples at this time. Recovery based on whole cell data using the previous lot of filters was low (about 50 %) and highly variable. Recovery of time points at 18 and 20 hours was normal. The radiocarbon distribution should not be affected, since whole cells were lost.

Table A-2. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans During Batch Growth[a]

Hours of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
0	474.4	35.0	8.3	14.5	42.2
8	478.5	16.2	1.8	6.4	75.7
8.5	794.6	14.6	1.3	5.8	78.3
9	1245.1	13.8	2.7	5.1	78.4
9.5	1913.7	13.5	2.7	4.6	79.3
10	3014.8	13.2	2.9	4.3	79.6
10.5	4741.5	13.0	3.4	4.3	79.3
11	7626.0	13.1	4.8	3.8	78.3
11.5	9497.7	15.9	4.7	3.4	76.1
12	12,238.9	16.9	5.1	3.2	74.9
12.5	14,771.7	17.9	5.0	3.3	73.8
13	17,388.8	17.9	5.2	3.0	73.9
13.5	18,793.1	19.3	6.4	2.9	71.4
14[b]	18,175.7	18.4	6.2	2.9	72.4
15	16,305.8	18.5	5.5	3.1	72.9
16	18,068.0	18.7	5.4	3.1	72.9
18	16,755.2	18.1	4.5	3.9	73.5
20	23,832.9	16.2	5.6	4.2	74.0
22	35,711.9	15.2	6.3	3.9	74.6
24	37,493.1	14.5	6.6	3.7	75.3

[a]Data from Figure 4-2 in the text.

[b]A box of new and apparently cracked RA 984H glass fiber filters was opened and used for fractionation samples at this time. Recovery based on whole cell data using the previous lot of filters was low (about 50 %) and highly variable. Recovery of time points at 22 and 24 hours was normal. The radiosulfur distribution should not be affected, since whole cells were lost.

Table A-3. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus During Batch Growth[a]

Hours of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
0	27.4	59.9	4.7	26.3	9.1
11	227.2	14.2	2.1	11.5	72.2
12	327.1	14.0	2.0	10.6	73.4
13	484.5	15.1	1.9	8.6	74.4
15	965.0	14.8	2.1	8.1	74.9
16	1442.7	14.1	2.1	7.3	76.6
17	1933.1	14.9	2.6	6.8	75.7
18	2801.2	14.2	3.0	6.8	76.0
19	3827.1	14.4	5.2	6.3	74.1
20	5347.4	16.1	4.2	6.2	73.5
21	7174.0	16.4	4.4	6.4	72.7
22	8862.7	19.1	5.8	5.6	69.5
23	10,184.3	20.7	5.4	5.2	68.6
24	11,485.6	19.6	7.0	4.8	68.6
25	13,217.7	22.5	6.9	4.3	66.2
40[b]	22,725.9	71.3	6.2	2.8	19.7
S-END[c]	22,075.8	40.5	13.7	4.1	41.8

[a] Data from Figure 4-4 in the text.

[b] 40 hour filter refrigerated two days instead of being fixed with 10 % TCA.

[c] 40 hour sample harvested by centrifugation, rinsed with RLC-water, and fixed with 10 % TCA.

Table A-4. Total Uptake of UL-^{14}C-Glutamate and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus During Batch Growth[a]

Hours of Incubation	nMoles C per ml	% of Total Radioactivity				
		L.M.W.	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
0	19.6	78.6	2.0	1.5	14.3	3.6
11	59.3	15.9	1.2	8.3	20.7	54.0
12	85.5	17.2	2.0	9.0	18.7	53.1
13	125.9	19.0	0.8	9.1	18.7	52.4
14	174.6	17.9	1.3	9.5	18.3	53.0
15	251.3	18.5	1.7	9.7	16.5	53.6
16	356.8	18.0	1.5	9.1	15.9	55.6
17	521.4	21.9	1.2	9.0	13.3	54.6
18	700.9	18.9	1.5	9.8	15.1	54.8
19	972.2	20.9	1.5	9.0	14.5	54.1
20	1291.4	19.7	1.9	9.8	14.7	54.0
21	1829.6	23.5	1.5	9.8	11.8	53.4
22	2214.5	21.3	1.9	10.5	14.2	52.2
23	2733.7	21.4	1.2	10.5	15.8	51.0
24	3181.0	19.3	2.0	11.1	19.2	48.4
25	3663.8	23.5	1.3	11.6	18.0	45.6
G-END[b]	7639.9	33.9	6.0	12.5	18.8	28.9

[a]Data from Figure 4-4 in the text.

[b]40 hour sample harvested by centrifugation, rinsed with RLC-water, and fixed with 10 % TCA. Corresponding filtered sample lost during processing.

Table A-5. Total Uptake of UL-^{14}C-Glutamate and its Distribution in Biochemical Fractions of Pseudomonas halodurans During a Carbon-Limited Stationary Phase[a]

Hours of Incubation	nMoles C per ml	% of Total Radioactivity				
		L.M.W.	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
0	7.7	49.4	13.0	2.6	10.4	24.7
2	57.8	22.1	1.4	7.4	21.8	47.2
3.5	172.9	24.3	0.7	7.2	17.2	50.5
5	597.4	26.9	0.7	7.2	12.1	53.1
6.5	1836.3	29.3	0.9	8.1	9.0	52.7
8	2071.3	22.5	0.7	8.4	7.3	61.2
9.5	2073.1	19.3	0.8	8.6	9.4	61.8
11	1856.9	19.4	0.8	8.5	8.8	62.5
12.5	1991.6	18.9	0.7	8.7	9.7	62.0
14	1467.1	20.8	0.5	8.6	8.2	61.9
15.5	1795.1	20.9	1.2	8.7	8.9	60.3
17	1502.1	20.8	1.0	8.5	9.2	60.6

[a]Data from Figure 4-5 in the text.

Table A-6. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans During a Carbon-Limited Stationary Phase[a]

Hours of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
0	51.6	32.6	13.0	21.5	32.9
2	100.8	15.5	1.3	6.9	76.3
3.5	529.2	14.8	1.5	6.3	77.5
5	2144.5	14.5	1.8	5.5	78.3
6.5	7866.7	16.2	4.8	4.1	74.8
8	10,289.8	18.3	5.6	4.3	71.8
9.5	10,717.9	19.7	3.0	4.9	72.4
11	10,718.8	20.1	3.9	4.6	71.5
12.5	9880.8	20.1	3.8	5.1	71.1
14	10,480.3	20.7	2.1	5.0	72.2
15.5	9314.4	20.5	3.1	5.0	71.4
17	10,467.4	20.8	3.1	4.5	71.6

[a]Data from Figure 4-5 in the text.

Table A-7. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans: Effects of Chloramphenicol[a]

Minutes of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		Batch Culture			
Blank	20.3	44.3	13.3	9.4	33.0
0	702.6	17.2	7.9	7.3	67.5
60	888.6	18.7	6.3	6.7	68.2
120	1366.7	18.0	8.5	5.8	67.7
180	2527.5	14.8	13.9	4.0	67.3
240	4484.2	14.8	24.0	4.3	56.9
		+ Placebo			
260	6915.6	12.5	22.1	3.7	61.7
275	7390.6	13.2	15.1	3.1	68.6
290	8386.2	14.3	13.2	2.5	70.0
305	9672.7	14.8	14.1	2.5	68.6
335	12,215.1	15.9	14.1	2.2	67.8
365	14,483.2	16.2	13.4	2.2	68.2
395	16,324.9	16.0	14.0	2.1	67.8
425	18,538.5	15.8	13.5	2.2	68.5
485	19,136.0	16.4	13.0	2.6	68.0
		+Chloramphenicol			
260	5839.3	18.4	18.0	3.7	59.8
275	6959.5	17.7	31.6	3.2	47.4
290	5330.7	26.3	20.6	4.4	48.7
305	6363.0	23.8	17.2	3.6	55.4
335	6679.2	26.1	15.4	3.3	55.2
365	7128.6	28.6	17.0	3.1	51.3
395	7097.2	30.4	13.0	2.9	53.7
425	7517.6	30.9	13.7	2.9	52.5
485	7562.6	31.9	16.5	2.9	48.7

[a] Data from Figure 4-7 in the text. At 245 minutes the culture was split into two portions. One received chloramphenicol (25 µg/ml final concentration) and the other received an equal amount of distilled water as a placebo.

Table A-8. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus: Effects of Chloramphenicol[a]

Minutes of Incubation	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		Batch Culture			
Blank	40.7	63.8	10.6	19.1	6.4
0	433.4	27.1	1.1	5.8	66.0
90	601.4	30.7	0.9	5.0	63.4
180	843.6	21.9	0.8	5.0	72.3
270	1135.3	13.0	1.5	5.4	80.2
330	1631.8	12.6	1.7	4.9	80.7
390	2052.6	13.5	2.3	4.8	79.4
450	2505.4	11.7	3.3	4.7	80.2
		+Placebo			
475	2845.6	11.5	2.7	5.3	80.5
490	2890.7	12.6	5.2	5.1	77.2
520	3827.5	12.5	5.2	4.5	77.8
580	5320.0	11.1	6.2	4.3	78.4
640	6820.7	12.2	9.3	3.7	74.8
700	8770.7	14.5	9.3	3.3	72.9
		+Chloramphenicol			
475	3055.3	17.8	3.1	5.0	74.2
490	3213.0	20.1	3.5	4.3	72.1
520	2127.1	17.2	4.3	5.3	73.3
580	903.3	7.8	7.2	8.3	76.7
640	738.3	5.6	8.3	8.5	77.6
700	751.7	5.7	9.2	8.8	76.3

[a]Data from Figure 4-9 in the text. At 460 minutes the culture was split into two portions, one receiving chloramphenicol (25 µg/ml final concentration) and the other an equal amount of distilled water as a placebo.

Table A-9. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus During Sulfur Starvation[a]

Minutes of Sulfur Starvation	pMoles S per ml	% of Total Radioactivity					
		$SO_4^{=}$	L.M.W. Organic	Alcohol Soluble	Lipid	Hot TCA Soluble	Residue Protein
Blank	174.6	79.3	6.9	3.6	2.9	1.5	5.8
Batch[b]	24,018.5	3.2	17.9	3.0	0.9	4.9	70.1
0	19,880.5	3.8	18.1	3.4	0.8	4.8	69.1
5	19,176.1	3.2	15.0	3.6	0.7	6.1	71.4
10	18,612.6	3.2	13.4	2.5	0.7	5.9	74.3
15	18,389.1	3.1	10.5	3.3	0.7	5.8	76.6
30	18,741.0	2.7	8.7	1.6	0.6	5.8	80.7
60	18,169.3	3.0	6.2	1.0	0.4	6.1	83.3
90	18,215.6	3.1	5.0	2.0	0.7	6.0	83.3
120	18,646.7	3.3	5.3	2.1	0.5	5.8	83.0
150	18,961.1	2.8	4.6	2.7	0.7	5.2	84.0
180	18,292.9	3.1	4.1	0.8	0.4	5.8	85.9
240	18,326.8	3.1	4.2	2.2	1.4	5.7	83.4
300	18,249.1	3.4	4.0	2.1	0.8	6.6	83.1
360	16,856.1	2.8	4.8	3.3	0.7	5.7	82.7
480	15,468.6	2.8	5.0	2.9	0.9	6.2	82.2

[a]Data from Figure 4-10 in the text.

[b]Sample of the culture prior to harvesting and resuspension in sulfur-free medium.

Table A-10. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans During Sulfur Starvation[a]

Minutes of Sulfur Starvation	pMoles S per ml	% of Total Radioactivity				
		$SO_4^{=}$	L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
Blank	55.7	5.0	11.1	4.8	17.4	61.6
Batch	6904.4	0.9	13.3	5.8	8.6	71.4
0	5337.7	0.9	12.6	5.8	9.1	71.5
5	5353.2	1.0	11.7	5.3	8.2	73.8
10	5198.3	1.2	11.3	4.7	8.9	73.9
15	5211.0	0.9	10.8	6.6	9.4	72.2
30	5280.8	1.2	9.2	5.6	9.8	74.3
45	5110.5	0.9	8.3	7.1	9.9	73.8
60	5035.7	0.9	7.6	7.3	10.4	73.8
75	5158.6	0.6	7.4	9.1	9.7	73.3
90	5109.3	0.6	6.9	9.2	9.1	74.1
120	4963.4	0.7	6.4	8.9	9.2	74.7
150	5305.2	0.5	5.8	11.0	8.5	74.0
180	5184.2	0.5	5.5	9.4	9.3	75.3
240	5001.9	0.7	5.3	8.1	10.3	75.7
300[b]	4997.9	0.5	5.1	4.7	19.0	70.7
360	5054.6	0.5	4.7	5.0	19.2	70.6
420	5189.9	0.5	4.4	5.3	18.2	71.6
480	5293.2	0.6	4.0	4.8	20.1	70.5
600	5039.3	0.6	3.6	5.7	19.0	71.0
720[c]	4737.9	0.7	74.0	9.9	4.5	10.9

[a]Data from Figure 4-11 in the text. A sample of the culture (Batch) was taken before harvesting and resuspension in sulfur-free medium for comparison with the 0 starvation time sample.

[b]Samples from 300-600 minutes were fractionated 12 days after the experiment, all previous samples being done the second day. During this period they were stored at 4°C in 10 % TCA.

[c]The 720 minute sample was fractionated with another experiment much later.

Table A-11. Total Uptake of $^{35}SO_4^=$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus During Recovery from Sulfur Starvation[a]

Minutes of Incubation	pMoles S per ml	% of Total Radioactivity				
		$SO_4^=$	L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
0	20.2	15.4	20.9	9.1	16.5	38.2
5	1409.5	6.6	70.3	1.7	4.1	17.4
10	2531.4	4.2	66.4	2.0	4.3	23.1
15	3525.9	4.1	61.3	2.6	4.7	27.3
20	4342.7	3.1	58.2	2.2	5.0	31.6
25	5491.9	2.6	58.4	1.7	5.0	32.3
30	6234.4	2.5	55.2	3.4	2.8	36.1
45	10,046.3	2.3	48.5	2.8	6.0	40.4
60	12,448.7	1.9	42.8	3.3	6.4	45.7
75	15,611.1	2.3	37.7	3.4	6.6	50.0
90	18,980.5	2.1	34.4	5.5	6.7	51.4
105	23,035.2	1.7	31.7	5.2	6.9	54.4
120	25,209.7	2.3	30.3	5.7	7.1	54.6
150	32,842.7	2.4	25.6	5.1	7.1	59.8
180	42,433.0	2.4	17.6	5.5	7.7	66.9
240	45,585.5	2.6	12.7	5.2	8.0	71.6

[a]Data from Figure 4-12 in the text.

Table A-12. Total Uptake of $^{35}SO_4^=$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans Grown on Various Carbon and Energy Sources[a]

Cells/ml	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		Acetate (0.47 hr^{-1})			
1.55×10^8	8323.3	13.4	6.8	6.0	73.8
2.49×10^8	8674.7	8.6	8.5	6.8	76.2
		Citrate (1.03 hr^{-1})			
5.81×10^7	6025.3	15.1	6.3	5.8	72.8
1.82×10^8	9364.2	13.8	7.5	5.9	72.8
		Ethanol (0.82 hr^{-1})			
1.34×10^8	8742.7	14.5	5.4	4.8	75.3
2.42×10^8	9123.3	11.1	6.0	5.5	77.4
		Fructose (0.76 hr^{-1})			
4.27×10^7	5442.7	14.5	6.1	5.6	73.7
1.86×10^8	9486.8	14.7	8.3	5.7	71.4
		Glucose (0.44 hr^{-1})			
3.49×10^7	2765.3	13.7	2.4	6.3	77.6
2.59×10^8	9203.3	11.8	7.0	5.9	75.3

Table A-12. (Continued)

Cells/ml	pMoles S per ml	% of Total Radioactivity: L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
		Glutamate (~ 1 hr^{-1})[b]			
1.08×10^8	9754.2	18.6	7.0	5.4	69.0
		Lactate (0.70 hr^{-1})			
6.67×10^7	5227.3	13.4	5.7	6.0	74.9
1.87×10^8	9536.3	14.0	7.0	5.5	73.5
		Pyruvate (0.70 hr^{-1})			
4.08×10^7	5217.9	15.6	5.3	6.4	72.7
1.13×10^8	9354.3	16.6	6.4	6.7	70.4

[a] Experimental details in Table 4-25.

[b] Growth rate not determined. The normal growth rate in this medium is about 1/hour.

Table A-13. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Pseudomonas halodurans: Short-Term Isotope Uptake Kinetics of Exponentially-Growing Cells[a]

		% of Total Radioactivity			
Minutes of Incubation	pMoles S per ml	L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
0	21.0	46.7	2.9	4.8	45.7
5	79.2	28.8	0.9	3.2	67.2
10	159.7	24.8	1.1	3.4	70.8
15	258.3	21.4	1.1	3.2	74.3
20	345.1	21.1	1.1	3.4	74.4
25	447.9	20.3	1.3	3.2	75.2
30	559.5	19.7	1.2	3.2	75.8
45	937.2	18.1	1.9	3.3	76.7
60	1351.2	17.2	2.3	3.2	77.3
75	1881.6	16.5	4.4	3.1	75.9
90	2460.6	16.6	3.4	3.0	76.9
105	3133.9	16.0	5.9	2.8	75.4
120	3904.6	16.2	5.0	3.0	75.8
150	5891.0	16.5	7.2	2.3	74.0
180	6884.8	19.1	7.7	2.6	70.6
210	7995.3	19.5	9.8	2.4	68.2
240	9545.2	19.9	9.4	2.4	68.3
300	12,548.9	20.8	10.2	2.4	66.6
360	14,598.6	20.4	10.9	2.1	66.6
480	16,310.9	21.0	13.4	1.9	63.7

[a]Data from Figure 4-13 in the text.

Table A-14. Total Uptake of $^{35}SO_4^{=}$ and its Distribution in Biochemical Fractions of Alteromonas luteo-violaceus: Short-Term Isotope Uptake Kinetics of Exponentially-Growing Cells[a]

Time (Minutes)	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
0	13.9	46.8	25.2	15.8	12.2
5	6.9	0	0	49.3	50.7
10	13.3	25.6	0	0	74.4
15	26.8	25.4	1.5	10.1	63.1
20	35.7	29.1	1.7	2.0	67.2
25	49.3	28.0	1.2	2.4	68.4
30	62.6	25.1	1.0	4.2	69.8
45	103.1	24.8	1.8	5.8	67.5
60	146.3	24.0	2.5	4.9	68.7
75	193.3	21.4	2.0	6.1	70.5
90	243.2	21.1	2.7	5.4	70.7
105	309.1	20.7	2.5	5.0	71.8
120	365.5	19.9	2.7	5.6	71.8
135	427.2	19.4	3.0	5.1	72.5
150	514.0	19.0	2.5	5.2	73.3
165	591.7	18.7	3.2	4.9	73.2
180	680.7	18.6	2.8	5.0	73.7
210	846.4	16.2	3.6	5.2	75.0
240	1115.1	16.6	3.8	5.3	74.2
270	1427.6	14.6	3.9	5.7	75.9
300	1850.8	16.4	3.4	4.6	75.5
330	2287.1	16.3	4.9	4.4	74.5
390	3457.0	14.8	5.4	3.7	76.1
425	4453.4	14.6	5.4	3.8	76.2

Table A-14. (Continued)

Time (Minutes)	pMoles S per ml	% of Total Radioactivity			
		L.M.W. Organic	Alcohol-Ether Soluble	Hot TCA Soluble	Residue Protein
450	5018.1	15.5	6.2	3.5	74.8
480	6042.2	16.8	6.5	3.6	73.1
510	7004.9	17.0	7.0	3.1	72.9
540	7741.7	18.0	7.1	3.1	71.7
570	9146.9	18.0	7.1	3.1	69.5

[a]Data from Figure 4-13 in the text.

Table A-15. Total uptake of UL-^{14}C-Glucose and $^{35}SO_4^=$ and their distribution in biochemical fractions of natural bacterial populations from a fish holding tank[a]

Hours of Incubation	Total DPM per ml	% of Total Radioactivity				
		Alcohol			Hot TCA	Residue
		L.M.W.	Soluble	Lipid	Soluble	Protein
		^{14}C-Glucose				
0	556	49.6	1.6	3.4	20.5	24.8
1	848	23.5	1.8	5.8	19.3	49.6
2	1891	21.6	2.0	5.6	18.9	51.8
3	2612	21.6	1.6	5.6	18.3	52.9
4	2530	18.2	1.9	5.8	19.7	54.4
12	2607	13.0	1.1	5.4	21.3	59.1

Hours of Incubation	pMoles S per ml	% of Total Radioactivity		
		L.M.W.	Alcohol-Ether	Residue
		Organic	Soluble	Protein
		$^{35}SO_4^=$		
0	19.7	55.8	3.0	41.1
1	42.9	19.3	2.3	78.3
2	47.5	18.7	2.7	78.5
3	47.1	15.5	2.5	82.0
4	48.5	19.0	2.5	78.6
8	48.1	14.8	3.3	81.9
12	103.3	20.0	3.6	76.4

[a]Data from Figures 5-1 and 5-2 in the text.

Table A-16. Total Incorporation and Distribution of $^{32}PO_4^{=}$ in Water Samples from R/V Oceanus Cruise #84: Continental Slope Station[a]

Hours of Incubation	Total ng P Taken Up per 100ml	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	3.70	58.4	3.7	6.0	20.0	7.4	4.4
2	0.75	97.8	0.0	0.0	2.2	0.0	0.0
4	1.93	52.5	2.1	6.3	31.9	3.9	3.3
6	2.10	9.2	3.2	1.2	39.4	19.7	16.3
8	5.79	34.0	0.5	12.9	45.6	5.9	1.1
12	19.55	11.4	4.2	9.9	27.8	24.8	21.9
18	64.03	17.1	1.5	17.0	51.5	10.3	2.8
30	153.91	49.4	2.0	5.8	18.1	11.5	13.2
36	617.18	45.6	0.8	9.4	35.9	6.5	1.9
48	617.57	43.5	0.9	9.0	37.2	6.4	2.0

[a]Data from Figure 5-7 in the text.

Table A-17. Total Incorporation and Distribution of $^{32}PO_4^{=}$ in Water Samples from R/V Oceanus Cruise #84: Sargasso Sea Station[a]

Hours of Incubation	Total ng P Taken Up per 100ml	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	0.60	35.2	4.3	12.9	32.5	10.9	4.1
1	1.16	52.0	0.8	7.5	35.9	3.4	0.4
2	1.83	42.5	0.5	7.7	43.9	5.1	0.4
4	6.09	32.9	0.9	8.6	50.0	6.6	1.0
6	15.23	34.5	1.0	9.3	47.1	6.8	1.3
12	57.32	29.9	1.0	10.8	47.7	8.6	2.0
18	85.38	27.1	0.8	11.5	46.5	11.7	2.4
24	133.14	30.0	0.9	11.8	43.5	11.4	2.6
30	121.41	27.8	1.0	12.2	42.8	13.9	2.3
48	124.32	40.7	0.7	8.2	37.1	11.3	1.9

[a]Data from Figure 5-8 in the text.

Table A-18. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Glucose in Water Samples from R/V Oceanus Cruise #84: Continental Shelf Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
			Alcohol			Hot TCA	Residue
		L.M.W.	Soluble	Lipid	RNA	Soluble	Protein
0	4,294	40.5	7.0	7.1	14.1	15.9	15.5
4	1,190	47.9	0.0	1.4	26.7	20.2	3.8
8	4,250	46.1	0.0	1.0	29.0	16.8	7.2
12	11,759	47.5	0.9	0.9	27.8	16.2	6.7
16	37,693	56.0	0.9	1.2	22.9	13.5	5.5
20	107,635	54.0	1.1	1.2	22.7	14.5	6.7
24F	290,599	59.8	0.7	1.1	17.4	14.5	6.5
24B	286,697	64.1	0.6	1.0	15.8	13.2	5.4
24B +N	432,002	56.5	0.8	1.2	21.5	13.4	6.7
24B +P	223,179	57.7	0.9	1.1	23.2	12.8	4.3
24B +NBC	537,792	57.1	1.1	1.0	21.5	12.5	6.8
24B +CAP	1,971	76.0	0	0.4	12.0	11.6	0.0

[a]Data from Figure 5-9 in the text.

Table A-19. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Glutamate in Water Samples from R/V Oceanus Cruise #84: Continental Shelf Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	882	51.4	3.7	5.0	17.0	14.7	8.2
4	3,335	70.4	0.8	1.1	9.5	8.6	9.5
8	10,475	67.0	1.0	1.1	9.6	9.0	12.3
12	23,285	64.9	1.2	1.3	10.5	9.3	12.8
16	93,713	72.3	1.2	1.3	8.4	7.7	9.1
20	220,382	52.9	1.4	1.4	11.7	13.1	18.2
24F	217,118	42.6	1.2	1.5	20.4	15.8	18.4
24B	263,739	49.2	1.3	1.7	18.3	12.6	16.9
24B +N	322,294	59.5	1.1	1.6	17.0	9.8	11.0
24B +P	266,467	55.4	1.4	1.8	16.0	11.7	13.8
24B +NBC	335,214	55.6	1.2	1.7	14.6	11.3	15.6
24B +CAP	6,826	70.0	2.0	2.9	12.6	7.8	4.8

[a]Data from Figure 5-9 in the text.

Table A-20. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Acetate in Water Samples from R/V Oceanus Cruise #84: Continental Slope Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	139	40.3	7.2	8.6	22.3	18.0	3.6
4	226	32.7	2.7	22.6	11.5	22.1	8.4
8	562	34.2	1.8	16.9	9.8	20.5	16.9
12	1,246	33.0	2.0	14.1	11.6	20.2	19.0
18	4,164	31.1	1.5	11.1	14.4	21.7	20.2
30F	126,285	29.1	1.3	8.9	14.3	22.1	24.3
30B	247,798	22.7	1.1	11.4	17.0	24.4	23.4
36	286,482	22.5	1.3	10.8	17.1	20.7	27.6
48	277,033	24.9	0.9	9.2	24.1	16.0	24.9
30B +N	266,609	33.5	1.1	9.5	12.5	20.0	23.4
30B +P	261,068	25.8	1.2	9.7	13.5	21.2	28.6
30B +NBC	279,559	20.3	1.2	13.1	11.5	24.8	29.0
30B +CAP	1,583	29.4	1.9	20.2	13.4	20.8	14.3

[a]Data from Figure 5-10 in the text.

Table A-21. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Glucose in Water Samples from R/V Oceanus Cruise #84: Continental Slope Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
		Alcohol Soluble L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	4,168	24.7	10.1	8.3	18.9	20.9	17.1
4	190	100.0	0.0	0.0	0.0	0.0	0.0
8	482	100.0	0.0	0.0	0.0	0.0	0.0
12	335	91.6	0.0	0.0	0.0	0.0	8.4
18	967	79.4	0.0	0.0	15.1	0.0	5.5
30F	90,884	53.9	1.2	3.3	18.2	13.5	9.9
30B	75,988	51.6	0.9	3.4	20.3	13.7	10.1
36	283,049	46.7	0.9	2.7	22.7	14.8	12.1
48	373,013	52.6	0.7	2.5	19.4	14.8	10.0
30B +N	236,288	38.1	1.0	3.0	30.9	15.7	11.3
30B +P	178,274	44.3	0.7	2.6	26.4	15.5	10.5
30B +NBC	458,678	39.2	1.2	1.9	25.2	17.3	15.1

[a]Data from Figure 5-10 in the text.

Table A-22. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Glutamate in Water Samples from R/V Oceanus Cruise #84: Continental Slope Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
		L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	328	45.4	5.2	5.2	35.7	4.9	3.7
4	431	70.8	2.3	1.2	0.0	10.2	15.5
8	998	51.2	1.5	2.2	9.1	14.1	21.8
12	2,438	47.7	1.3	1.9	9.2	15.1	24.9
18	11,427	43.7	0.9	2.2	16.5	15.1	21.7
30F	165,990	20.0	0.9	3.4	20.3	23.5	31.9
30B	205,549	20.3	0.9	3.0	25.3	21.3	29.3
36	186,485	18.8	0.8	3.0	25.9	20.2	31.2
48	195,078	20.7	0.8	3.0	22.9	21.1	31.4
30B +N	202,765	20.4	0.7	2.7	20.4	19.9	35.9
30B +P	210,606	21.0	0.8	3.2	20.5	21.0	33.6
30B +NBC	199,254	15.0	1.5	3.2	21.2	22.4	36.8
30B +CAP	2,923	41.8	2.1	5.0	14.3	21.9	14.9

[a]Data from Figure 5-10 in the text.

Table A-23. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Acetate in Water Samples from R/V Oceanus Cruise #84: Sargasso Sea Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
		Alcohol				Hot TCA	Residue
		L.M.W.	Soluble	Lipid	RNA	Soluble	Protein
0	4,403	3.8	0.4	1.8	90.0	2.9	1.2
6	9,968	34.1	1.1	8.5	17.3	22.5	16.5
12	49,555	31.8	0.7	7.8	20.7	20.9	18.1
18	128,690	24.7	0.7	10.0	28.3	19.2	17.2
24	174,168	21.0	1.1	9.5	37.0	18.5	13.0
30F	207,028	17.4	0.5	8.4	35.6	23.2	14.9
30B	225,681	16.2	0.6	9.1	34.8	24.5	14.8
48	226,955	33.1	1.3	8.5	27.1	16.5	13.6
30B +N	258,243	14.0	0.7	10.2	39.3	18.2	17.6
30B +P	206,220	19.3	0.7	9.6	28.9	20.9	20.7
30B +NBC	355,488	13.4	1.0	10.3	23.1	24.7	27.5
30B +CAP	7,023	11.8	0.9	5.2	74.9	5.1	2.1

[a]Data from Figure 5-11 in the text.

Table A-24. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Glucose in Water Samples from R/V Oceanus Cruise #84: Sargasso Sea Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
		Alcohol Soluble L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	5,128	31.1	7.3	7.7	20.9	14.4	18.6
6	7,729	40.8	1.2	3.2	36.0	12.0	6.9
12	71,527	45.0	0.6	2.0	31.2	11.9	9.3
18	252,739	44.6	0.4	1.8	29.5	13.5	10.2
24	348,644	48.0	0.6	1.9	27.2	14.2	8.1
30F	366,490	48.8	0.5	2.0	27.4	14.1	7.2
30B	490,243	48.0	1.1	2.0	27.7	12.4	8.7
48	426,999	58.9	0.6	1.5	19.5	12.1	7.4
30B +N	515,293	46.4	0.6	2.6	27.4	12.8	10.2
30B +P	469,084	43.6	1.1	1.9	25.9	16.3	11.3
30B +NBC	547,118	41.0	0.9	2.1	28.7	15.7	11.6
30B +CAP	3,407	52.3	0.0	0.2	38.1	7.8	1.6

[a]Data from Figure 5-11 in the text.

Table A-25. Total Uptake and Distribution of ^{14}C from Uniformly Labeled Glutamate in Water Samples from R/V Oceanus Cruise #84: Sargasso Sea Station[a]

Hours of Incubation	Total DPM Taken Up	% of Total Radioactivity					
		Alcohol Soluble L.M.W.	Alcohol Soluble	Lipid	RNA	Hot TCA Soluble	Residue Protein
0	1,459	47.4	1.6	7.3	26.4	9.5	7.8
6	66,497	53.1	0.4	1.4	13.3	13.0	18.8
12	210,896	34.5	0.8	1.9	18.9	20.5	23.3
18	211,326	21.4	0.6	2.2	29.4	22.7	23.7
24	228,852	17.1	0.4	2.1	34.2	20.8	25.4
33F	209,565	17.2	0.4	2.6	32.0	21.7	26.1
48	236,687	39.6	0.8	2.2	18.4	16.1	22.8
33B +N	320,525	44.9	1.2	1.8	15.0	12.4	24.8
33B +P	249,236	50.3	0.9	1.6	12.1	12.5	22.7
33B +NBC	307,277	49.0	1.0	1.7	13.3	14.0	21.0
33B +CAP	19,333	73.3	0.9	1.0	13.9	4.3	6.6

[a]Data from Figure 5-11 in the text.

APPENDIX B

RECOVERY OF RADIOLABELED CELLS ON WHATMAN GF/F FILTERS AND RESPIRATION DATA USING WICK AND ACID-VOLATILE METHODS: R/V OCEANUS CRUISE #84

NOTE

All data in this appendix are the difference in activity between the sample and a zero time isotope adsorption or volatilization blank.

Table B-1: Recovery of ^{14}C-Labeled Natural Bacterial Populations on GF/F Glass Fiber Filters Compared to 0.2 µm Nucleopore Filters[a]

Hours of Incubation	Acetate			Glucose			Glutamate		
	0.2 µm	GF/F	%	0.2 µm	GF/F	%	0.2 µm	GF/F	%
				Continental Shelf Station					
4	–	–	–	1946	1190	61.2	3432	3335	97.2
8	–	–	–	4743	4250	89.6	11,303	10,475	92.7
12	–	–	–	12,422	11,759	94.7	19,481	23,285	119.5
16	–	–	–	30,920	37,693	121.9	85,377	93,713	109.8
20	–	–	–	106,398	107,635	101.2	229,096	220,382	96.2
24F	–	–	–	277,919	290,599	104.6	249,440	217,118	87.0
24B	–	–	–	341,254	286,697	84.0	331,166	263,739	79.6
24B +N	–	–	–	481,253	432,002	89.8	367,931	322,294	87.6
Mean					(n=9)	92.8		(n=9)	94.3

Table B-1. (Continued)

Hours of Incubation	Acetate			Glucose			Glutamate		
	0.2 µm	GF/F	%	0.2 µm	GF/F	%	0.2 µm	GF/F	%
				Continental Slope Station					
4	254	224	88.2	0	190	-	574	431	75.1
8	649	560	86.3	0	482	-	1228	998	81.3
12	1475	1244	84.3	367	335	91.3	2696	2438	90.4
18	4789	4164	86.9	984	966	98.1	14,862	11,427	76.9
30F	137,077	126,285	92.1	99,082	90,884	91.7	177,895	165,990	93.3
36	278,450	286,480	102.9	334,961	283,048	84.5	189,143	186,485	98.6
Mean		(n=6)	90.1		(n=4)	91.4		(n=6)	85.9

Table B-1: (Continued)

Hours of Incubation	Acetate			Glucose			Glutamate		
	0.2 µm	GF/F	%	0.2 µm	GF/F	%	0.2 µm	GF/F	%
				Sargasso Sea Station					
6	10,313	9668	93.7	7997	7729	96.6	67,195	66,497	99.0
12	45,715	49,555	108.4	73,066	71,527	97.9	215,053	210,896	98.1
18	116,765	128,690	110.2	224,711	252,739	112.5	221,163	211,326	95.6
24	166,412	174,168	104.7	353,427	348,644	98.6	236,523	228,852	96.8
30F	186,335	207,028	111.1	342,726	366,490	106.9	230,012	209,565	91.1
30B	208,545	225,681	108.2	412,408	490,423	118.9	-	-	-
48	210,074	226,955	108.0	392,999	426,999	108.7	240,200	236,687	98.5
30B +P	204,172	206,220	101.0	408,042	469,084	115.0	279,356	249,236	89.2
30B +N	198,904	258,243	129.8	429,273	515,293	120.0	299,263	320,525	107.1
30B +NBC	331,983	355,488	107.1	510,555	547,118	107.2	329,700	307,277	93.2
30B +CAP	9099	7023	77.2	13,129	3407	26.0	25,235	19,333	76.6
Mean		(n=11)	105.4		(n=11)	100.8		(n=10)	94.5
Overall Mean Recovery:		(n=66)	95.8						

[a]At each sampling point, 2 100-115ml samples were filtered through Whatman GF/F filters for biochemical fractionation and 1 10ml sample was filtered through a Nucleopore 0.2 µm nitrocellulose membrane. The 10ml sample was counted in Aquasol and compared with the sum of the counts in the fractions from the GF/F filtered samples. All data have been normalized to an addition of 10,000 DPM 14C/ml.

Table B-2. Respiration of ^{14}C-Glucose by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise# 84, Continental Shelf Station[a]

Hours of Incubation	DPM ^{14}C-Glucose/100ml					% Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
4	1582	-	3324	-	4906	-	0.5	-	3.10
8	4530	-	33,602	-	38,132	-	3.8	-	8.42
12	12,088	-	54,269	-	66,357	-	6.6	-	5.49
16	34,523	-	120,737	-	155,260	-	15.5	-	4.50
20	107,776	-	211,235	-	319,011	-	31.9	-	2.96
24F	286,232	44,127	327,834	330,359	614,066	33.0	61.4	1.15	2.15
24B	316,398	20,222	83,561	336,620	399,959	33.7	40.0	1.06	1.26
24B +N	460,045	-	134,807	-	594,852	-	59.5	-	1.29
24B +P	239,326	-	114,731	-	354,057	-	35.4	-	1.48
24B +NBC	537,792	39,744	100,101	577,536	637,893	57.8	63.8	1.07	1.19
24B +CAP	1971	727	5665	2698	7636	0.3	0.8	1.37	3.87

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

Table B-3. Respiration of ^{14}C-Glutamate by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise #84, Continental Shelf Station[a]

Hours of Incubation	DPM ^{14}C-Glutamate/100ml					% Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
4	3221	-	24,832	-	28,053	-	2.8	-	8.71
8	10,352	-	79,104	-	89,456	-	8.9	-	8.64
16	85,489	-	341,254	-	426,743	-	42.7	-	4.99
20	212,349	-	622,860	-	835,209	-	83.5	-	3.93
24F	221,423	391,410	713,676	612,833	935,099	61.3	93.5	2.77	4.22
24B	281,723	414,749	654,396	696,472	936,119	69.6	93.6	2.47	3.32
24B +N	327,636	-	585,646	-	913,282	-	91.3	-	2.79
24B +P	284,854	-	649,207	-	934,061	-	93.4	-	3.28
24B +NBC	335,214	344,772	576,566	679,986	911,780	68.0	91.2	2.03	2.72
24B +CAP	6826	17,638	25,227	24,464	32,053	2.4	3.2	3.58	4.70

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

Table B-4. Respiration of ^{14}C-Acetate by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise #84, Continental Slope Station[a]

Hours of Incubation	DPM ^{14}C-Acetate/100ml					% Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
8	605	-	U[b]	-	605	-	0.1	-	1.00
18	4477	-	U	-	4477	-	0.4	-	1.00
30F	131,681	178,966	122,870	310,647	254,551	31.1	25.5	2.36	1.93
30B	247,798	352,776	282,667	600,574	530,465	60.1	53.0	2.42	2.14
36	282,465	446,961	411,619	729,426	694,084	72.9	69.4	2.58	2.46
48	277,033	455,801	420,017	732,834	697,050	73.3	69.7	2.65	2.52
30B +NBC	279,559	419,839	-	699,398	-	69.9	-	2.50	-
30B +CAP	1583	7676	-	9259	-	0.9	-	5.85	-

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

[b]Undetectable.

Table B-5. Respiration of ^{14}C-Glucose by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise #84, Continental Slope Station[a]

Hours of Incubation	DPM ^{14}C-Glucose/100ml					% Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
8	482	-	U[b]	-	482	-	0.0	-	1.00
18	975	-	U	-	975	-	0.1	-	1.00
30F	94,983	42,791	93,855	137,774	188,838	13.8	18.9	1.45	1.99
30B	75,998	73,978	117,963	149,976	193,961	15.0	19.4	1.97	2.55
36	309,005	103,766	205,793	412,771	514,798	41.3	51.5	1.34	1.67
48	373,013	188,525	345,536	561,538	718,549	56.2	71.9	1.51	1.93
30B +N	236,288	-	115,509	-	351,797	-	35.2	-	1.49
30B +P	178,274	-	95,288	-	273,562	-	27.4	-	1.53
30B +NBC	458,678	-	244,797	-	703,475	-	70.3	-	1.53

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

[b]Undetectable.

Table B-6. Respiration of ^{14}C-Glutamate by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise # 84, Continental Slope Station[a]

Hours of Incubation	DPM ^{14}C-Glutamate/100ml					% Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
8	1113	-	U[b]	-	1113	-	0.1	-	1.00
18	13,145	-	29,238	-	42,383	-	4.2	-	3.22
30F	171,943	442,671	755,328	614,614	927,721	61.5	92.7	3.57	5.39
30B	205,549	499,508	728,701	705,057	934,250	70.5	93.4	3.43	4.55
36	187,814	511,470	768,930	699,284	956,744	69.9	95.7	3.72	5.09
48	195,078	514,187	749,983	709,265	945,061	70.9	94.5	3.64	4.84
30B +N	202,765	-	734,520	-	937,285	-	93.7	-	4.62
30B +P	210,606	-	734,824	-	945,430	-	94.5	-	4.49
30B +NBC	199,254	492,980	741,035	692,234	940,289	69.2	94.0	3.47	4.72
30B +CAP	2923	8313	9563	11,236	12,486	1.1	1.2	3.84	4.27

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

[b]Undetectable.

Table B-7. Respiration of ^{14}C-Acetate by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise #84, Sargasso Sea Station[a]

Hours of Incubation	DPM ^{14}C-Acetate/100ml					% Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
6	9991	-	33,173	-	43,164	-	4.3	-	4.32
12	47,635	-	218,210	-	265,845	-	26.6	-	5.58
18	122,728	-	401,735	-	524,463	-	52.4	-	4.27
24	170,290	-	491,144	-	661,434	-	66.1	-	3.88
30F	196,682	287,451	520,228	484,133	716,910	48.4	71.7	2.46	3.65
30B	217,113	345,535	444,779	562,648	661,892	56.3	66.2	2.59	3.05
48	212,821	367,802	466,921	580,623	679,742	58.1	68.0	2.73	3.19
30B +N	237,726	-	459,629	-	697,355	-	69.7	-	2.93
30B +P	213,085	-	451,271	-	664,356	-	66.4	-	3.12
30B +NBC	332,852	404,980	534,607	737,832	867,459	73.8	86.7	2.22	2.61
30B +CAP	8061	11,454	32,270	19,515	40,331	2.0	4.0	2.42	5.00

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

Table B-8. Respiration of ^{14}C-Glucose by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise # 84, Sargasso Sea Station[a]

Hours of Incubation	DPM ^{14}C-Glucose/100ml					% Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
6	7863	-	12,658	-	20,521	-	2.1	-	2.61
12	72,297	-	100,347	-	172,644	-	17.3	-	2.39
18	238,725	-	228,723	-	467,448	-	46.7	-	1.96
24	351,036	-	358,316	-	709,352	-	70.9	-	2.02
30F	354,608	96,953	421,985	451,561	776,593	45.2	77.7	1.27	2.19
30B	451,416	116,793	268,952	568,209	720,368	56.8	72.0	1.26	1.60
48	409,999	184,853	338,576	594,852	748,575	59.5	74.9	1.45	1.83
30B +N	472,283	-	221,324	-	693,607	-	69.4	-	1.47
30B +P	438,563	-	272,050	-	710,613	-	71.1	-	1.62
30B +NBC	528,837	112,499	235,542	641,336	764,379	64.1	76.4	1.21	1.45
30B +CAP	8268	1679	1487	9947	9755	1.0	1.0	1.20	1.18

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

Table B-9. Respiration of ^{14}C-Glutamate by Natural Bacterial Populations Measured by Two Different Techniques: R/V Oceanus Cruise# 84, Sargasso Sea Station[a]

Hours of Incubation	DPM ^{14}C-Glutamate/100ml					%Total Label Metabolized		M/I[b]	
	Assimilated	Respired		Total Metabolized					
		Wick	Volatile	Wick	Volatile	Wick	Volatile	Wick	Volatile
6	66,486	-	183,737	-	250,233	-	25.0	-	3.76
12	212,975	-	608,228	-	821,203	-	82.1	-	3.86
18	216,245	-	697,068	-	913,313	-	91.3	-	4.22
24	232,688	-	703,461	-	936,149	-	93.6	-	4.02
30F	219,789	386,657	707,718	606,446	927,507	60.6	92.8	2.76	4.22
30B	256,048	423,727	662,019	679,775	918,067	68.0	91.8	2.65	3.59
48	236,562	447,626	683,113	684,188	919,675	68.4	92.0	2.89	3.89
30B +N	294,622	-	599,445	-	894,067	-	89.4	-	3.03
30B +P	389,091	-	700,360	-	1,089,451	-	108.9	-	2.80
30B +NBC	295,805	382,522	595,632	678,327	891,437	67.8	89.1	2.29	3.01
30B +CAP	44,658	38,708	71,471	83,366	116,129	8.3	11.6	1.87	2.60

[a]Data normalized to 10,000 DPM/ml added activity. Assimilation values are the average of whole cell filters and the sum of fractions from processed samples.

BIOGRAPHICAL NOTE

Although there is a Hollywood, Florida, no one has mistaken the Hollywood from which I originated. The lasting impressions from my formative years (ages 17-present) have ingrained in me a profound sense of exhibitionism and personal projection. The result gives no indication of the very straight, close cropped nature of my early years.

From February 11, 1952 (an anniversary of Thomas Edison's birthday) to some time in sixty-nine I cavorted in the City of the Stars and environs, collecting rare coins from bus drivers' coin boxes and acting pretty normal. The traumatic events associated with my parents' divorce when I was six must have skimmed over my head, but I do remember feeling a certain kinship with a basketball as my fat step-father's threats bounced me from Daddy-O's to mothers' and back.

Not yet as rowdy as I was soon to be, my years in high school were devoted to the development of "smooth hustling"; not of girls at that time, but rather towards the acquisition of goals just a hair outside of the rules. The pertinent example was a partnership with Roger Vargo; as free-lance photographers for the Crescenta Valley High School Talon, Yearbook, and other literary functions we took on the logo CV Photography. This trade requires a substantial investment in equipment which we were not able to raise. However, we made a deal: we would provide, free of charge, coverage of local school events, sports, etc. for the newspaper, and they, in turn, let us use the moth-eaten, dust covered,

fully-equipped school darkroom. The cost of the trade was well returned in many sales of action photos to pro-jock football and basketball players and their parents at 1000 %profit.

But I had to get out. My step-father was driving me mad, and time for college was at hand. San Diego seemed close enough to visit without having to live at home. The $200 per quarter tuition was within my grasp, and away I went. Of course, some scholarships were obtained, and it was my luck that the work-study program was relatively under-subscribed. Imagine walking into a job office and hearing, "What kind of job would you like ? " Either photography or something at Scripps Institution of Oceanography (as advertised on TV); even a job dishwashing would get my foot in the door....

The unlucky Angelo Carlucci scored my services for a reasonable 75¢ an hour. Dishwashing seemed safe enough, but I was able to prove my intelligence time and time again, like baking Nalgene plastic bottles in an oven overnight at 300^{o}C. However, Angelo weathered the storm. Then he had to put up with my nagging, every week; "When can I go on a cruise? Even a day trip ? Please ? " Fortuitously, events about to change my life were in the works. A cruise to Antarctica had been scheduled, but its lengthy duration (73 days port to port) left most people cold. I believe Angelo thought he would cure me for good by sending me on that one. Much to everyone's surprise, I ate it up.

The single-handed performance of the sampling program on that fateful cruise launched me into an era of blissful world travel at an early age. The years at U.C.S.D./S.I.O. combined the rigorous biochemistry/molecular

biology training at Upper Campus (it could also be taken as slang) with rewarding experience in field biological oceanography with the Food Chain Research Group, at that time in the hey-day of co-operative, multi-disciplinary research. I am indebted to an extreme extent to this group, particulary Angelo Carlucci and Ozzie Holm-Hansen, for turning me on to Oceanography. In addition, we had some great poker games at lunch.

I actually applied to the Joint Program as a joke. There was a spot on the NSF Doctoral Fellowship application for the school of your choice, and the name W.H.O.I. rang a bell. I didn't really know anything about it, but it was "the other one" (I was at Scripps at the time).

PUBLICATIONS

Brown, W. C. and R. L. Cuhel. 1975. Surface-localized cortex-lytic enzyme in spores of Bacillus cereus T. J. Gen. Microbiol. 91: 429-432.

Brown, W. C., Cuhel, R. L., and C. Greer. 1977. Isolation and properties of a surface-bound cortex-lytic enzyme from spores of Bacillus cereus T. pp. 335-349. In Barker, A. N., Wolf, J., Ellar, D. J., Dring, G. J., and G. W. Gould (eds.), Spore Research 1976. Academic Press, London.

Carlucci, A. F. and R. L. Cuhel. 1977. Vitamins in the South Polar Seas: distribution and significance of dissolved and particulate vitamin B_{12}, thiamine, and biotin in the southern Indian Ocean. pp. 115-128. In G. A. Llano (ed.), Adaptations Within Antarctic Ecosystems: Proceedings of the Third SCAR Symposium on Antarctic Biology. Smithsonian Institution, Washington, D. C.

Holm-Hansen, O., El-Sayed, S. Z., Franceschini, G. A., and R. L. Cuhel. 1977. Primary production and the factors controlling phytoplankton growth in the Southern Ocean. pp. 11-50. In G. A. Llano (ed.), Adaptations Within Antarctic Ecosystems: Proceedings of the Third SCAR Symposium on Antarctic Biology. Smithsonian Institution, Washington, D. C.

Jannasch, H. W., Cuhel, R. L., Wirsen, C. O., and C. D. Taylor. 1981. An approach for in situ studies of deep-sea amphipods and their microbial gut flora. Deep-Sea Res. (in press).