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Origin and Evolution of Osmoregulatory Mechanisms in Blue-Green Algae (Cyanobacteria) as a Function of Metabolic and Structural Complexity: Reflections of Precambrian Paleobiology?

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Principal Investigator: John H. Yopp, Professor of Botany
Botany Department
Southern Illinois University
Carbondale, Illinois 62901

Co-Principal Investigator:
(June 15, 1982-
December 30, 1984) Donald R. Tindall, Professor of Botany
Botany Department
Southern Illinois University
Carbondale, Illinois 62901

Postdoctoral Fellow: Kenneth Pavlicek, Ph.D.
Botany Department
Southern Illinois University
Carbondale, Illinois 62901

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ABSTRACT

The National Aeronautics and Space Administration (NASA) has, during the past decade, funded research by our laboratory designed to characterize the physiological, biochemical and ultrastructural features of a halophilic blue-green alga that enables it to survive in a hypersaline environment of extremely limited water availability. This research has led to the cultural and physiological characterization, as well as the research availability, of one of the most halophilic of the earth's oxygenic photosynthesizers, Aphanothece halophytica. In addition, this research has led to the realization that blue-green algae ('cyanobacteria') are uniquely suited to research designed to answer two of the most fundamental questions concerning the physiology of water relations in photosynthetic organisms. The first is the question of how photosynthetic organisms of diverse structural and metabolic natures adjust their intracellular water potential to that of a changing external (i.e. environmental) water potential. In other words, how do oxygenic photosynthesizers osmoregulate?

The second question concerns the time during which metabolically diverse mechanisms of osmoregulation in oxygenic photosynthetic organisms had their origin and, subsequently evolved. This question would necessarily relate to Precambrian paleobiology because oxygenic photosynthesis arose during this eon.

The unique suitability of the blue-green algae as objects of research on the second question is easily justified. The first organisms to evolve the oxygenic photosynthesis characteristic of the higher plants were the blue-green algae. Paleobiologists concerned with the phylogeny of these organisms generally believe that prokaryotes were structurally and metabolically complete by the end of the Precambrian. Indeed, the Precambrian eon has been termed "the age of the blue-green algae." We will for reasons given within the text refer to these organisms henceforth as cyanobacteria. The cyanobacteria are also unique in that they have not significantly changed morphologically and metabolically since the late Proterozoic or early Paleozoic eras. Therefore, it is assumed that modern representatives of the five major subgroups of cyanobacteria, which range from unicellular through filamentous to branched filamentous forms, possess the same metabolic (nutritional) capabilities of their Precambrian counterparts, now a part of the fossil record. These nutritional types include available strains that function as obligate photoautotrophs, facultative photoheterotrophs and facultative chemoheterotrophs. These types may in some cases function anaerobically as well as aerobically, and, even in limited numbers, as anoxygenic, hydrogen sulfide-utilizing photoautotrophs.

Recent research by prominent paleobiologists clearly indicates that ecosystems changed during the Precambrian from anaerobic-reducting to aerobic-oxidizing types.

Furthermore, the geochemical evidence suggests that the major shifts in tectonic mode during the early Proterozoic led to a great increase in favorable ecosystems for cyanobacterial productivity and adequate environmental stresses for evolution in this group. The investigators believe that the evolution of the early biosphere must have included the dominant cyanobacteria responding to changes in the water potential of their diverse, Precambrian

niches. This response could involve evolution of osmoregulatory mechanisms possible within the constraints of structural complexity and the nutritional parameters inherent in anaerobic and aerobic photoautotrophy, photoheterotrophy and chemoheterotrophy. The nature of osmoregulatory mechanisms in modern eukaryotic, unicellular and multicellular photoautotrophs is exceedingly complicated by structural complexity and nature of metabolism involved in the response. The investigators believe that such use of the structural and nutritional strains of cyanobacteria will not only provide important information concerning the nature of higher plant osmoregulation but will help decipher a major evolutionary event in Precambrian paleobiology. There exists today a considerable body of evidence that the cyanobacteria were the ancestors of the chloroplasts of higher plants.

The unique suitability of cyanobacteria as organisms for research on the first question concerning the nature of osmoregulation per se, is likewise justified in the structural and metabolic characteristics of the cyanobacteria. The major unresolved aspects of osmoregulation per se involve the resolution of the following: (1) relationship of metabolic capability to type of osmoregulatory solute formed; (2) role of intracellular compartmentalization in intracellular water potential adjustment; (3) existence of a singular or multiphasic sequence in the process of osmotic adjustment; and (4) the nature of "compatible solutes" in the metabolism of osmotically-adapted-organisms.

Cyanobacteria are prokaryotes and as such do not possess water vacuoles for compartmentalization of solutes. Cyanobacteria exist in all three major nutritional modes, making possible the establishment of relationship between nature of osmoregulatory solute and metabolic capability. Cyanobacteria have an extremely limited and relatively inflexible carbohydrate and organic acid metabolism that will facilitate the direct tracing of synthesized osmoregulatory solutes. Cyanobacteria generally lack simple control of gene regulation (feedback product and substrate control of enzyme synthesis). This attribute facilitates the interpretation of the sequence of events in a multiphasic osmoregulatory mechanism and the role of certain chemicals as "compatible solutes." Finally, cyanobacteria possess the necessary pathways for the synthesis or uptake of almost all classes of osmoregulatory solutes known for photosynthetic organisms.

Another major type of experimentation directed toward the resolution of the evolutionary history of the cyanobacteria has been the cataloging of oligonucleotide sequences of 16S ribosomal RNA. The use of this technique for measuring phylogenetic relationships among prokaryotes has proven valuable but has led to some disagreement with interpretation of such relationships from the fossil record. The investigators felt that, in general, both lines of inquiry support one another and that differences of interpretation may arise from differences in time of evolution of a particular genotype and time of environmentally-induced-expression of the phenotypes possible within the genotype. It is known that there is a direct relationship between genome size and structural complexity. It may be that differences in the environmental components of the evolving Precambrian biosphere elicited different expressions of an ever-increasing genome. The investigators further believe that a study of the nature proposed here (i.e. physiological-biochemical) will provide evidence for such differential expression in the case of osmoregulatory mechanisms within nutritional types of the cyanobacteria. In this sense, this

study serves to "bridge" the lines of inquiry involving molecular genetics and the fossil record.

The objectives of the proposed study were therefore:

(1) To ascertain whether there exists any relationship between mode of nutrition in the cyanobacteria (i.e. photoautotrophic, photoheterotrophic or heterotrophic) expressed under aerobic or anaerobic conditions and the nature of the solutes employed for adjustment or intracellular water potential (osmoregulation);

(2) To determine whether there exists any relationship between structural complexity in the cyanobacteria (unicellular, simple filamentous or multi-seriate branched) and the nature of the solutes employed for adjustment of intracellular water potential;

(3) To conduct the studies on osmoregulation under environmental conditions that correspond to those of the major stages in the evolution of the Precambrian biosphere;

(4) To identify and characterize the uptake and enzymatic mechanisms involved in the production and accumulation of the solutes employed in adjustment of intracellular water potential by representative nutritional and structural types of cyanobacteria, under the environmental conditions proposed in the previous objectives;

(5) To determine whether differences in metabolism underlie the intracellular adjustment to lowered environmental water potential achieved by the addition of sodium chloride or nonionic (penetrating or non-penetrating) solutes to the growth medium of cyanbacteria;

(6) To ascertain whether genome and other chemical characteristics of the genetic material influence the nature of the osmoregulatory response elicited under varying environmental conditions in the representative cyanobacteria;

(7) To ascertain whether adjustments of intracellular water potential in cyanobacteria is a multiphasic process culminating in the accumulation of a solute (or solutes) compatible to enzymatic functioning; and to determine whether the degree of complexity in any multiphasic process is a function of metabolic, genetic or structural complexity;

(8) To determine to what extent the inorganic and organic solutes accumulating during osmoregulation account for balancing the external water potential; and to ascertain whether the "sensing" of changes in environmental water is by a turgor-activated mechanism or not; and

(9) To reconstruct a probable sequence of evolution of osmoregulatory mechanisms as a function of the adaptive interaction between the metabolic capabilities of Precambrian cyanobacteria and fluctuating water potential in ecosystems of a changing Precambrian biosphere.

The methodology proposed to achieve the objectives involved the tracing of the pathways of formation of osmoregulatory solutes by traditional methods

involving C-14 labelled substrates; gas chromatography; amino acid analysis, X-ray analysis using scanning transmission electron microscopy, and, most importantly, C-13 labelled substrates, followed by Nuclear Magnetic Resonance (NMR) spectroscopy.

The time course proposed for achievement of the objectives was three years. An extension was approved, due to equipment failure, until May 15, 1986. Work is continuing to satisfy the objectives as originally proposed.

Major accomplishments underlying the basic understanding of cyanobacterial resistance to salt tolerance and osmotic stress were made as a result of this research. In summary, these are as follows:

(1) The cyanobacteria, once termed metabolically uniform and evolutionary, metabolically conservative, employ a diversity of organic, osmoregulatory solutes. These include glucosylglycerol in many freshwater and marine unicellular forms; sucrose and trehalose in many filamentous and branched forms of fresh and marine-water systems; and, most importantly, glycine-betaine in the extremely halotolerant forms such as our original isolate, Aphanothece halophytica.

(2) Osmoregulatory solutes were found to serve four functions in our research, i.e., (a) adjustment of water activity (Ψ_π), (b) non-inhibition of enzymes; (c) lowering K_m of enzymes to allow functioning at "normal" levels when intracellular salt accumulates (a pre-adaptation); and (d) extending the pH optimum of enzymes as intracellular pH rises due to proton-potassium ion pump action during osmoregulation. The latter two functions are original to this research.

(3) Differences in osmoregulatory solutes in cyanobacteria may, but are not always, be attributable to differences in nutritional capabilities, i.e. photoautotrophy, photoheterotrophy and chemoheterotrophy.

(4) The mechanism of osmoregulation and concomitant salt tolerance in halophilic cyanobacteria was elucidated. It involves the potassium ion (which accumulates during the initial stage of osmoregulation) activation of the enzyme S-adenosylhomocysteine (SAH) hydrolase to cause the breakdown of SAH. SAH is the key metabolic inhibitor of the essential methylation step of glycine-betaine synthesis. At the same time the reverse direction of the enzymatic reaction (i.e. SAH synthesis) is inhibited by the intracellular potassium. When betaine is synthesized to a level that protects the other salt-inhibited enzymes, including SAH hydrolase in the synthetic direction, betaine synthesis is shut-down. This is a simple, self-regulatory mechanism, originally discovered during this research.

(5) Other original discoveries include: (a) that betaine acts by lowering the Km of potassium ion-inhibited-enzymes (only), thereby "pre-adapting enzymes to inhibitory salt concentrations arising during osmotic adjustment; (b) betaine protection of an enzyme inhibited by potassium ion is inversely proportional to the degree of sensitivity of the enzyme to such salt; (c) S-Adenosylhomocysteine hydrolase, a key enzyme in control of cellular methylations was first discovered in cyanobacteria during the course of this research. This is of particular importance in view of the

current hypothesis that cyanobacteria may be the ancestors of higher plant chloroplasts.

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Description of the General Problem and Justification for and Statement of Specific Objectives

One of the fundamental principles of the biology of life forms on this planet is that their physiology is intrinsically related to the physico-chemical properties of water. Earth's life forms have evolved in a water milieu; their major constituent is water; their metabolism is primarily conducted in a water matrix; and, their communication with, and acquisition of, the essential chemicals of the abiotic environment is through a water medium. Just as earth's organisms have evolved in a diversity of environments varying with respect to water availability, so too are the physiological activities of these organisms adapted to differing degrees of water availability (i.e. water potential). Therefore, one of the most important aspects of the physiology of all life forms is the mechanism(s) whereby they regulate their intracellular water potential in response to a changing water potential of their environment. The movement of water in and out of an organism is by osmosis and the mechanisms regulating this movement are termed osmoregulatory mechanisms.

Water potential (Ψ) is the sum of the contribution of osmotic potential ($\Psi\pi$), which is due to solute concentration ($\Psi\pi$=-miRT); pressure potential (Ψp), which is due to the turgor pressure of cell wall resistance to volume increase; and matric potential (Ψm), that occurs as a result of reduction of water activity upon its absorption of protoplasmic surfaces. Intracellular water potential (Ψ) is commonly decreased to the level of lower external Ψ by uptake and/or synthesis of solutes. Likewise, intracellular Ψ may be increased in response to a higher external Ψ by metabolism of synthesized solutes and/or the loss of these solutes to the environment. The adjustment of the osmotic potential ($\Psi\pi$) component of a total Ψ is termed osmoregulation. Adjustment of intracellular Ψ in response to a suddenly lowered (termed upshock), or gradually lowered, external Ψ may employ the regulation of the other components of water potential (Ψm and Ψp) as well. In this sense osmoregulatory mechanisms and mechanisms of adjustment of intracellular Ψ may not be synonymous.

There have been eight recent reviews on the subject of osmoregulation in photosynthetic algae and higher plants (9, 21, 22, 36, 43, 59, 75, 82). Two general conclusions may be drawn from these extensive reviews. The first is that there are at least six fundamental aspects of osmoregulatory systems that must be yet elucidated, all of which are somehow related to the organism's mode of nutrition and structural complexity. The second conclusion is that little is known concerning the osmoregulatory mechanisms of the cyanobacteria. The photoautotrophic prokaryotes were the first organisms on earth to possess the photosynthetic machinery (i.e. photosystems I and II) of the eukaryotic algae and higher plants (62,64). The eight major books and review articles on cyanobacteria over the past decade have also added only very little to the subject of osmoregulation in cyanobacteria (14, 24, 28, 66, 70, 71, 75, 79). Walsby (75) has best reviewed the literature concerning this topic in the

group. Our recent publication on osmoregulation in this group, which was the first such study, and one other publication represent an initial view of this process in earth's earliest oxygenic photosynthesizers (85).

The investigators believe that the cyanobacteria represent organisms that possess unique attributes that would facilitate the elucidation of the six fundamental aspects of osmoregulatory systems in photosynthesizing plants. The investigators also believe that a study of the osmoregulatory mechanisms in this group will yield important information on one of the fundamental aspects of Precambrian paleobiology. As stated by Schopf (64), "the ultimate goal of Precambrian paleobiology is to decipher and document both the timing and nature of major events in the early history of life." Truly the evolution of osmoregulatory mechanisms in the dominant organisms of the Precambrian is one such major event.

The investigators postulate the following scenario based upon the excellent interpretations of the data on the evolution of the earth's earliest ecosystems by Knoll (45) and Schopt (64): (1) The cyanobacteria evolved from probable anaerobic, photosynthetic bacterial ancestors during (or possibly much prior to) the late Archean era (2900 to 2500 m.y. ago) of the Precambrian eon (4500 to 570 m.y. ago) (1, 61, 62, 63). These life forms are aerobic photoautotrophs possessing a chlorphyll a based-photosynthesis that include photosystems I and II (66). The latter photosystem functions to utilize water as a source of hydrogen for the reduction of CO_2 and the oxygen liberated as a by-product. (2) The cyanobacteria evolved to the full range of metabolic and structural complexity of present-day forms by the late Pre-Cambrian (45, 62, 64). During this period of evolution, cyanobacteria developed and retained the following modes of nutrition (metabolic capabilities): anaerobic and aerobic photoautotrophy; aerobic and anaerobic photoheterotrophy; aerobic chemoheterotrophy; and perhaps as evidenced by a few modern forms, anaerobic chemoheterotrophy (24, 64, 66). As such, modern representatives of these prokaryotes retained most of the major metabolic innovations (called "benchmarks" by Schopf et al, 64) that characterized earth's earliest ecosystems. (3) Prior to, and during the development of a stable aerobic hydrosphere and atmosphere during the early Proterozoic (2500 and 1600 m.y. ago) as a consequence of cyanobacterial photosynthetic activity (18, 45, 63), major geologic events occurred that "challenged" the metabolic machinery of these prokaryotes to develop osmoregulatory mechanisms. The interpretation of the geologic record by Knoll (45), Cloud (18) and Schopf et al (64) would mean, therefore, that the earth's earliest ecosystems included the dominant cyanobacteria responding to changes in the water potential of their diverse, Precambrian niches by osmoregulatory mechanisms possible within the constraints of anaerobic and aerobic photoautotrophy, anaerobic and aerobic photoheterotrophy and anaerobic and aerobic chemoheterotrophy.

It is important to note at this point that there has been during the past decade an enormous amount of research by molecular biologists directed toward resolving the evolution of the prokaryotes, including the cyanobacteria (24, 29). Pointing out the difficulties in utilizing solely morphological characters to assess phylogenetic, as well as taxonomic relationships, Stackebrandt and Woese (68) recently reviewed the methodology of genetic research used to establish genealogies among the prokaryotes. This research has been responsible for a new concept of cellular evolution (as presented by Woese and Fox, 78). Consequently, a new phylogenetic structure of the prokaryotic domain (77) has

likewise resulted as recently discussed by Fox et al (29). As noted by Stackebrandt and Woese (68), the cataloging of oligonucleotide sequences in the evolutionarily conservative 16S ribosomal RNA is the best method for establishing genealogical relationships among prokaryotes. This method has been used to study such relationships among the cyanobacteria by Doolittle and Bonen and their associates at Dalhousie (5, 6, 24, 29). The assessment of the extent of similarities in these 16S rRNA sequences by this group has led to some possible disagreement with the interpretation of phyletic relationships rendered from the fossil record (6, 24, 29, 77). However, there is also much agreement among interpretations from either method, especially with respect to the time and sequence of origin of the most structurally complex cyanobacteria (e.g. Fisherella and Nostoc). Stackebrandt and Woese (68) point out in this regard that these approaches represent "two measures of evolution....the phenotypic versus the genotypic. The phenotypic measure is the classical one; it is the one we use in speaking of evolutionary progression... For the most part it reflects ecological change, or the working out of some 'evolutionary potential' in a line of descent." The investigators realize that osmoregulatory strategies may reflect environmentally induced-expression of the same geneotype that changed with the evolution of the Precambrian biosphere. However, we are also aware from the recent work of Herdman et al (38, 39) and Rippka et al (56) that morphologically identical species (or strains) of cyanobacteria may have greatly differing genome size. It may well be possible, therefore, that genome doubling (and further increases) provided the genetic material for metabolic and structural advancement during the Precambrian. We propose to test these possibilities and hope to contribute to the reconciliation between the phenotypic and genetic approach by careful selection of experimental material.

Perhaps the best summary statement of justification for this aspect of our study is given in the most recent relevant publication by Schopf et al (64):

> "Bearing such limitations ('interpretation of the fossil record') in mind, it seems evident that questions regarding the earliest phases of ecologic development must be phrased biochemically and metabolically (rather than solely in terms of organismal morphology); that their answers should be based on consideration of totality of relevant chemical, biological and geological evidence now available (rather than on data from any single discipline alone); and that at present, any fruitful approach to such questions, must, of necessity, be largely model-dependent (determined to a substantial degree by theoretical and indirect consideration, rather than solely by direct observable features of the fossil record as now known.)"

It seems clear from the literature that while the paleobiological and the molecular genetic approaches are well represented, the physiological-biochemical approach is still underrepresented.

It is relatively easy then to justify a study on an undoubtedly primitive physiological response if its relevance to earth's earliest ecosystems can be established. It is somewhat more difficult to justify the use of the cyanobacteria to study the six basic problems yet unresolved for osmoregulation in photosynthetic eukaryotes. These problems involve the elucidation of the following: (1) The influence of the type (ionic versus non-ionic and

penetrating versus non-penetrating) of solute used to lower the external Ψ on the resulting internal solutes employed to lower the intracellular Ψ. Several studies have shown that an influence of this type exists (11, 12, 32, 33, 36, 47, 76). (2) The influence of metabolic capabilities of the upshocked organism on the quantity and quality of osmoregulatory solutes formed. Again the published literature indicates that, depending upon the metabolic status of an organism, different intracellular solutes may result from imposition of an external water stress (48, 85). (3) The existence of a possible sequence of different intracellular solutes accumulating as a consequence of changes in metabolism elicited by the initial ionic or non-ionic solute accumulated has also been postulated (12, 21, 43, 85). This may explain the commonly observed photosynthetic and respiratory shock just prior to deplasmolysis (2, 3, 35, 36, 58). (4) The functional role of the internal osmoregulatory solutes as compatible solutes remains to be fully resolved. The concept of compatible solute derives from the fact that the solute employed for lowering the intracellular $\Psi\pi$ must allow enzymatic function as well. Ion (K^+ and Na^+) perform this role in halophilic bacteria but are inhibitory (in vitro) to the same enzymes from eukaryotic photoautotrophs (7, 26, 40, 82). This latter group employs a diversity of organic solutes (polyols, amino acids, carbohydrates) to lower intracellular $\Psi\pi$ and still allow enzymatic activity (3, 9, 10, 85). However, recent studies (12, 13, 31) indicate that some of these eukaryotic photoautotrophs also accumulate high levels of ions (at least initially) to aid in lowering intracellular $\Psi\pi$. In this case the comparable solutes may protect enzymes from the salt inhibition as well as aid in lowering intracellular $\Psi\pi$ (7, 9, 59, 60). The mechanism by which this protection is afforded is one of much current research. (5) The specific mechanism(s) whereby the enzyme(s) responsible for production and/or uptake of the osmoregulatory solutes in the well-studied systems remain largely unknown (22, 43, 44). Only the enzymes regulating the production of the organic solutes in the flagellate Ochromonas malhamensis as a function of external $\Psi\pi$ are well studied. This is due to the excellent work of Kauss and his coworkers over the past decade (43). (6) The role of vacuolization and intracellular compartmentalization of the solutes accumulating during osmoregulatory recovery.

The advantage of using cyanobacteria to study these aspects of the mechanism of regulation of Ψ lies in their structural and metabolic characteristics. First, they are prokaryotes that possess low water content and no vacuolar system. Therefore, there should be no structural compartmentalization of penetrating solutes from the lower external Ψ into vacuolar and cytoplasmic sites. In addition, internal osmoregulatory solutes that may accumulate sequentially will not be compartmentalized in vacuolar and cytoplasmic sites, thereby complicating a time course study of deplasmolysis. Finally, the accumulating osmoregulatory solutes would be in presumed contact with lamellar and interlamellar enzymes rather than sequestered in a vacuole. In this sense a better evaluation of the degree of compatibility of the solute could be obtained.

Secondly, species of the cyanobacteria differ with respect to structural complexity; such differences constitute traditional characters for taxonomic distinction. It is then possible to study osmoregulation within a single nutritional mode (e.g. the photoheterotrophic) as a function of structural complexity. Species of blue-green algae exist as unicells, simple filaments, multi-seriate-branched filaments and as heterocyst-containing-simple and branched filaments.

Thirdly, the cyanobacteria possess metabolic characteristics that permit direct experimental testing of the relationship between metabolic capability and nature of osmoregulatory solute(s) employed for adjustment of intracellular Ψ. These characteristics are as follows:

(1) Cyanobacteria characteristically exist as aerobic or microaerophilic, oxygenic photosynthesizers (i.e. are photoautotrophs) but can exist (often the same strain or isolate) in the other nutritional modes (i.e. photoheterotrophy and chemoheterotrophy) (14, 66, 69, 70). Therefore, by manipulating the environmental conditions and/or using an inhibitor of photosystem II, such as DCMU (3'-3, 4-dichlorophenyl) 1'-1 dimethylurea) the photoheterotrophic or chemoheterotrophic (by dark maintenance) mode may be induced (55). Only a relatively few organisms, other than the cyanobacteria, may be so utilized to demonstrate the relationship between nutritional mode and nature of osmoregulatory solute employed. Furthermore, some cyanobacterial isolates are obligate photoautotrophs while other closely related isolates are facultative photo- and even chemoheterotrophs (56). This permits yet another insight to be made regarding an obligate or facultative nutritional relationship to osmoregulation.

Use of the photoheterotrophic cyanobacteria should provide evidence of the effect of a photoassimilated organic solute on the capability for formation of a potential osmoregulatory solute. Osmoregulatory solutes formed from photoassimilated substrates would, therefore, be formed in the absence of metallic involvement from photosynthesis. This will allow the assessment of a further degree of metabolic involvement in osmoregulatory mechanisms in light-treated organisms.

The use of chemoheterotrophic cyanobacteria will allow the study of osmoregulation in organisms with the principal metabolic machinery of the other two nutritional types, but in the total absence of light. Supplying these algae with various (though still very limited) organic substrates may result in further insight into osmoregulation in the absence of even photosystem I of photosynthesis.

Recent research has demonstrated that certain cyanobacteria (unicells and filamentous types) may also be induced to use a primitive, anoxygenic photosynthesis using H_2S as a hydrogen donor (18, 30). Use of a blue-green alga in this nutritional mode will allow the study of osmoregulation not only under the conditions of an extremely primitive atmosphere, but also under conditions in which only photosystem I of photosynthesis is operative (18).

(2) Cyanobacteria have an extremely limited, and relatively inflexible carbohydrate and organic acid metabolism (15, 17, 24, 54, 66, 69). Indeed it has been shown that the Pentose Phosphate Pathway is the major pathway of carbohydrate metabolism functioning as such in the photoautotrophic, photoheterotrophic, and chemoheterotrophic nutritional modes (66, 70, 79). Furthermore, the Krebs Cycle is incomplete (interrupted by a lack of alpha-ketoglutarate dehydrogenase) and serves only a biosynthetic function (53, 66). These metabolic characteristics allow a much more direct assessment of the relationship of energy-yielding, metabolic reactions to osmoregulatory ion

fluxes or synthesis of osmoregulatory solutes. In addition, a lack of respiratory breakdown of certain photoheterotrophically- or chemoheterotrophically-assimilated, organic acid substrates will permit a more direct synthesis into measureable osmoregulatory solutes.

(3) Cyanobacteria are unique among the organisms possessing oxygenic photosynthesis with respect to a general lack a control of enzyme synthesis (gene expression) by potential substrates or products of the reactions mediated by the enzymes (17, 24, 66). As N.G. Carr notes "With only one or two exceptions the rate of synthesis of enzymes of both intermediary metabolism and of biosynthesis have been shown to be unaltered by the availability of substrates or by the end product of the biosynthetic pathway respectively" (17). While Carr interprets this as a possible cause of photoautotrophy and Doolittle notes that it may in fact be a consequence of it, there is little doubt that cyanobacterial metabolism is characterized by a deficiency (though not total lack) in "simple" controls of gene regulation. Whatever the explanation, the cyanobacterial-obligate photoautotroph represents an ideal system for the study of the nature and sequence of appearance of osmoregulatory solutes. Unlike photoautotrophic eukaryotes, which do respond (transcriptional systems present) to added substrates, and which do possess end-product feedback controls, the cyanobacterial metabolic system should respond more directly to external water stress. Presumably organic solutes arising in the obligate photoautotrophs as a result of lowered environmental Ψ_π would not induce the enzymes of their further dissemination. Likewise penetrating organic solutes used to lower the environmental Ψ_π should not induce enzymes that would insure their structural rearrangement.

(4) Previous research, including a great deal of our own over the past ten years, has demonstrated that cyanobacteria possess the biosynthetic pathways needed to produce the organic osmoregulatory solutes reported for all other photosynthetic eukaryotes. Therefore, the pathways, and in many cases the individual enzymes, involved in the production of polyols, monosaccharide esters with glycerol, proline, glutamic acid, sucrose, and other carbohydrates have been studied in cyanobacteria (16, 21, 28, 66, 79).

(5) Not only are the cyanobacteria linked to the photosynthetic eukaryotes by their possession of an identical photosynthetic machinery, but a type of organic solute (monosaccharide ester of glycerol) has been found to fluctuate with adjustment of intracellular Ψ both in the blue-green and certain primitive red algae (Rhodophyta) (4). This latter algae group is believed by many investigators to contain the photosynthetic eukaryotes most closely related to cyanobacteria (4, 6, 8, 29).

(6) The symbiosis hypothesis proposed by Margulis (49, 50) which states that the ancestor of higher plant chloroplasts was a cyanobacterial cell type provides an important link to photosynthetically mediated osmoregulation in crop plants.

These metabolic capabilities represent then a final justification for use of the cyanobacteria as subjects for the study of osmoregulation as a function of nutritional mode and structural complexity.

Pertinent Literature in Related Fields

A very detailed assessment of the pertinent literature for the purpose of establishing the background and significance of the objectives may be found in the original grant proposal (NAGW-344) already approved for funding.

An update of the pertinent literature was given in the proposal for Phase II (the second eighteen months) submitted on February 23, 1983 and on file.

Since early 1983, several publications have appeared that are relevant to the present study. These are in the area of solutes accumulated in blue-green algae (cyanobacteria) during osmotic adjustment. In addition, an additional article on the osmotic significance of betaine has also appeared. A brief review of these publications follows.

Blumwald and Tel-Or (4a) reported that a salt adapted Nostoc species accumulated sucrose as an osmoticum. Erdmann (25b) and Mackay et al. (48a) reported that certain unicellular marine blue-green algae synthesize glucosylglycerol as an osmoticum. Reed and Stewart (54a) reported the fluctuation of trehalose with salinity in the cyanobacterium Rivularia atra. Reed et al. (54b) most recently reported on a survey of the osmoregulatory carbohydrates in a large number of cyanobacterial isolates.

A summary and greatly simplified interpretation of these recent papers is as follows:

a) Non-reducing carbohydrates play a major role in osmoregulation by cyanobacteria.

b) The three major carbohydrates are glucosylglycerol, trehalose and sucrose.

c) Marine cyanobacteria appear to generally employ glucosylglycerol, while fresh water forms employ sucrose.

d) No clear cut relationship between genus and osmolyte employed by cyanobacteria is now evident.

Our research reported above is supported by these findings. We have, however, found exceptions to many of the conclusions drawn from the above papers.

In addition, an excellent review on the central importance of betaine in osmoregulation by Ruduber et al (56a) has appeared in Science. This gives further justification to our search for the first pathway of betaine synthesis.

The specific objectives of the proposed study are as follows:

(1) To ascertain whether there exists any relationships between mode of nutrition in the cyanobacteria (i.e. photoautotrophic, photoheterotrophic or heterotrophic) expressed under aerobic or anaerobic conditions and the nature of the solutes employed for adjustment of intracellular water potential (osmoregulation);

(2) To determine whether there exists any relationships between structural complexity in the cyanobacteria (unicellular, simple filamentous or multi-seriate branched) and the nature of the solutes employed for adjustment of intracellular water potential;

(3) To conduct the studies on osmoregulation under environmental conditions that correspond to those of the major stages in the evolution of the Precambrian biosphere;

(4) To identify and characterize the uptake and enzymatic mechanisms involved in the production and accumulation of the solutes employed in adjustment of intracellular water potential by representative nutritional and structural types of cyanobacteria, under the environmental conditions proposed in the previous objectives;

(5) To determine whether differences in metabolism underlie the intracellular adjustment to lowered environmental water potential achieved by the addition of sodium chloride or non-ionic (penetrating or non-penetrating) solutes to the growth medium of cyanobacteria;

(6) To ascertain whether genome size and other chemical characteristics of the genetic material influence the nature of the osmoregulatory response elicited under varying environmental conditions in the representative cyanobacteria;

(7) To ascertain whether adjustment of intracellular water potential in cyanobacteria is a multiphasic process culminating in the accumulation of a solute (or solutes) compatible to enzymatic functioning; and to determine whether the degree of complexity in any multiphasic process is a function of metabolic, genetic or structural complexity;

(8) To determine to what extent the inorganic or organic solutes accumulating during osmoregulation account for balancing the external water potential; and to ascertain whether the "sensing" of changes in environmental water potential is by a turgor activated mechanism or not; and

(9) To reconstruct a probable sequence of evolution of osmoregulatory mechanisms as a function of the adaptive interaction between the metabolic capabilities of Precambrian cyanobacteria and fluctuating water potential in ecosystems of a changing Precambrian biosphere.

Time course proposed for research designed to achieve the objectives.

Year 1:

(1) Culture of all structural and nutritional types of cyanobacteria proposed (see Table 2, original proposal for first year, June 15, 1982 - June 15, 1983; this objective must be met before research on the other objectives progresses);

(2) Culture of the above strains under conditions that will elicit photoautotrophy, photoheterotrophy and chemoheterotrophy; this objective will confirm the existence of the appropriate nutritional capabilities of the cyanobacteria, as indicated from the literature.

(3) Determination of the changes in osmoregulatory solutes after upshock for approximately one-third of the strains of cyanobacteria in culture.

Year 2: (Actually the next eighteen months of the three year study)

(1) Completion of the study of the nature of osmoregulatory solutes as a function of structural complexity and nutritional mode (photoautotrophy, photoheterotrophy and chemoheterotrophy).

(2) Identification of the enzymatic mechanisms involved in the production and accumulation of the solutes employed in adjustment of intracellular water potential by representative nutritional and structural types of the cyanobacteria cultured.

(3) To determine to what extent the intracellular solutes accumulating in the cyanobacteria studied account for balancing the external water potential.

(4) To determine whether solutes accumulating in the selected cyanobacteria following upshock serve as compatible solutes.

Year 3: (Actually the final six months of the original timetable and the proposed six months extension)

(1) To conduct the studies on osmoregulation under environmental conditions that correspond to those of the major stages in the evolution of the Precambrian biosphere.

(2) Verification of the metabolic intermediates (i.e. pathways) in the synthesis of the major osmoregulatory solutes in selected cyanobacteria by 13C-NMR.

The research on this objective has been greatly delayed due to the breakdown of our NMR instrument and loss of our NMR operator. This delay was communicated to Dr. Donald DeVincenzi by the University's Director of the Office of Research Administration, Dr. Michael R. Dingerson. This delay has in turn delayed research on certain related objectives. It is for this reason that the

extension is requested. There is no request for additional funds for the extension.

(3) Continuation on the elucidation of the pathways and enzymatic reactions involved in the production of osmoregulatory solutes in the selected cyanobacteria.

Specifically, the following experimentation is planned:

a) Determination of the changes in osmoregulatory solutes in the cyanobacteria, Oscillatoria limetica and Aphanotece halophytica when changed from conditions allowing oxygenic photosynthesis to environmental conditions eliciting anoxygenic photosynthesis (sulfide utilization);

b) Determination of the specific pathway of glycinebetaine synthesis in the cyanobacterium, Aphanothece halophytica; currently three possible pathways have been proposed by two groups of investigators; it is important to ascertain which of these pathways is operative in earth's earliest, oxygenic photosynthesizers;

c) Determination of the effect of salinity (and specifically, potassium ion) and betaine on the synthesis of betaine as the major compatible solute in halophilic cyanobacteria; this will require the study of the key, regulatory enzymes of betaine synthesis and the effect of potassium ion and betaine on their activity; and

d) ^{13}C-NMR studies on the synthesis of glucosylglycerol, betaine, trehalose and sucrose in selected cyanobacteria forced to osmoregulate under condition simulating the Precambrian atmosphere.

Previous results from our laboratory related to the proposed research.

Our laboratory has been engaged in research to determine the mechanism(s) of tolerance for hypersalinity and extremely low water potential in the halophilic cyanobacterium, Aphanothece halophytica for the past fifteen years. This research was supported by grants from the National Aeronautics and Space Administration (NASA-NGR-14-008-026). Details of the research are the subject of twelve publications, twenty-six published abstracts from national meetings and extensive progress reports to NASA's Division of Planetary Biology (19, 41, 42, 46, 51, 52a,b,c, 57, 72, 73, 74, 80, 82, 83-88).

In addition, specific results from experimentation conducted through support from the current grant (NAWA-344) have been reported in a previous progress report (February 25, 1983) to Dr. Donald DeVincenzi, part II (second 18 months) of the three year study (February 25, 1983), and in Part III final six month and six month extension (March 1985) to Dr. Donald DeVincenzi. These three previous progress reports and proposals are given in this final report as appendices I, II, and III, respectively.

Progress in achieving the stated objectives of the grant (NAGW-344).

Excellent progress has been made on achievement of most objectives since the start of the funded research. The following is a summary of this progress and the objectives partially achieved during the course of the research:

(1) Twenty-four (24) of the twenty-nine cyanobacteria proposed for culture (in Table 2 of first year proposal) have been successfully cultured. A list of these is provided as Table 1 of the present proposal.

It is believed that the remaining five cyanobacteria (Synechococcus, ATCC# 29154, Synechococcus, ATCC# 29206) will be eventually cultured in the remaining phase of the grant.

The above cultures are now in actively growing condition, under a regular transfer protocol. This research required considerable experimentation to achieve the proper environmental conditions of light, temperature and physico-chemical characteristics of the culture media.

Our group worked closely with certain staff of the American Type Culture Collection (ATCC) facility. Personal communication with Dr. Stjepko Golubic of Boston University during the First Symposium on Chemical Evolution and the Origin of Life, organized by Dr. Donald L. DeVincenzi, NASA Headquarters also aided in solving some of the ancillary problems.

Three additional blue-green algae ('cyanobacteria') provided by Dr. Lynn Margulis of Boston University during the same Symposium on Origin of Life at Ames Research Center, Moffett Field, California were also successfully cultured.

In addition our group isolated a new halotolerated species of Nostoc from eastern New Mexico in 1983. Finally, Dr. Y. Cohen has provided us with cultures of Oscillatoria limnetica for our studies on osmoregulation during anoxygenic photosynthesis.

These cyanobacteria, in actively growing cultures, are now available for use by other scientists working within the same program funded by NASA. This represents, therefore, a valuable repository of the "organisms of the Precambrian" for immediate use by these scientists. Six scientists in universities in this country have already taken advantage of this opportunity.

A list of these cyanobacteria was recently provided to scientists at NASA-Ames Research Laboratory Moffett Field, CA during a week visit, August 6-11, 1984.

(2) One third of the cyanobacteria (approximately) from the group (group E of Table 1) of facultative chemoheterotrophs are now in large scale culture for use in experimentation involving osmoregulatory solute determination and NMR studies.

(3) The principal osmoregulatory solute (compatible solute as well) of the extremely halotolerant Aphanothece halophytica, has been identified as a betaine by NMR. This solute is found in cellular concentration of five to fifteen times those of all of the soluble carbohydrates and free amino acids combined (Table 2).

We have identified a glucosylglycerol that also fluctuates with changes in external (medium) osmolarity. However, this solute accumulates in concentrations too low to serve a major osmoregulatory role.

The betaine content of A. halophytica not only increases dramatically with increasing NaCl-molarity of the growth medium (Table 2) but also increases in response to rapid changes in medium osmolarity (i.e., upshock). Note that betaine content is higher in organisms grown or upshocked in media containing glycylglycine buffer than in media containing MES buffer. We have also found that betaine content falls with downshock, but not to initial levels. There is preliminary evidence that betaine is metabolized following downshock. Studies on this question are currently in progress.

(4) Betaine synthesis in A. halophytica requires light (87). This organism is an obligate photoautotroph but does slowly deplasmolyze in the dark. However, growth will not continue in the dark.

(5) Betaine is not merely a neutral osmoregulatory solute but also displays counteracting effects on salt-inhibition of enzyme activity. Glucose-6-phosphate dehydrogenase from A. halophytica was assayed in the presence of increasing concentrations of KCl. Approximately 50% inhibition of activity was obtained between 0.3 and 0.4M KCl. This concentration has been shown to occur in A. halophytica (see previous studies). Next, this enzyme was assayed in a medium containing 04.M KCl plus either glycerol, proline or betaine. Although some relief from salt inhibition was obtained with proline and glycerol, betaine clearly was the superior counteracting, compatible solute (52a).

Finally, the effect of the extent of methylation of glycine on protection against salt inhibition of glucose-6-phosphate dehydrogenase was examined. There was found a clear relationship between degree of methylation and extent of salt counteracting effect (52a).

(6) The source (organism) of the enzyme is a factor in the extent of protection against salt inhibition afforded by betaine. Contrary to reports in the literature (82), a compatible, osmoregulatory solute will not protect a particular enzyme regardless of source. We obtained purified glucose-6-phosphate dehydrogenase from an eukaryotic organism, Torula yeast and another prokaryotic organism, Leuconostoc mesenteroides. The enzyme from the latter organism was several-fold more salt tolerant than the enzyme from the former organism. Betaine had dramatically different effects on the degree of salt protection for the enzyme from different sources. The inherently more salt tolerant enzyme was actually inhibited by betaine (52a).

(7) Betaine may exert its "counteracting effects" on salt inhibition not by "restoring activity" of salt inhibited enzymes, but by lowering the Km of the enzyme per se. We had reported earlier that KCl inhibits enzyme activity in halotolerant blue-green algae by causing an increase in Km. Betaine appears to "allow" enzyme function in the presence of high internal K^+ by greatly increasing the affinity of the enzyme for its substrate--for enzymes from certain, but not all, organisms (52a, 52b).

(8) The changes, both quantitative and qualitative, in amino acids following upshock of the halotolerant, Aphanothece halophytica, have been completely documented. The following major points can now be made concerning these changes:

a) The predominant free amino acids in the pool are glutamic>serine>glycine>aspartic>alanine. Whereas the total free amino acids do not increase as the salinity of the growth medium increase (Table 2), an apparent increase occurs in this organism upon upshock in medium containing glycylglycine buffer. No similar increase is observed in upshocked cyanobacteria in medium containing MES buffer. The increase under the former buffer condition appears in the "leucine fraction" of the analysis obtained by ion exchange within the Beckman 19CL Amino Acid Analyzer. However, we have now shown this "amino acid" to be the buffer glycylglycine by using High Voltage Paper Electrophoresis. It is obvious that the dipeptide glycylglycine itself is utilized by the alga during upshock. There are reports of other bacteria utilizing the amino acids of their growth media under upshock conditions. Of even more interest to us is the consequence of glycylglycine accumulation during upshock. The amino acid proline accumulates in MES buffered-organisms following upshock. This is a major difference involving an amino acid known to be involved in the osmoregulatory mechanism (perhaps as a compatible solute) of eukaryotic and prokaryotic organisms. It should be noted also that glycylglycine is taken up from the upshock media under light, dark and dark anaerobic conditions. Another major difference is that lysine greatly increases in cyanobacteria upshocked in glycylglycine, but not in cyanobacteria upshocked in MES buffered-medium. These results were reported in 1983 (87).

b) The amino acids changing to the greatest extent with upshock are serine and glutamic acid. These are also the major amino acids of the free pool in this organism. The glutamic acid always decreases to a major extent and the serine concomitantly rises. The relationship is believed by the investigators to indicate a metabolic relationship between the two in which glutamic acid may serve as an amino donor for serine biosynthesis. In addition, it should be noted that the relationship occurs independent of type of buffer present in the upshock media (87). We feel that these findings are of particular importance in view of the fact that serine is the precursor to betaine and that this amino acid is "triggered" to

accumulate with increasing salinity under all environmental conditions tested. The significance of this observation to osmoregulation in cyanobacteria and to evolution of osmoregulatory mechanisms will be discussed in detail below.

(9) The changes in total soluble carbohydrates, reducing carbohydrates, total amino acids and individual amino acids following upshock in light and dark or three different structural types of cyanobacteria have been determined. These were the unicellular Synechocystis the filamentous, LPP; and the branched Fisherella. The major findings of this experimentation are as follows:

a) No betaine accumulated following upshock in NaCl (sufficient for plasmolysis and recovery) in any of the three structural types of cyanobacteria. These results and several months of preliminary experimentation to "find" betaines in other cyanobacteria, have led us to tentatively conclude that betaine is a compatible, osmoregulatory solute formed in halotolerant and halophilic oxygenic photoautotrophs, but not in fresh water organisms of the same type. All three of the structural types so tested were of fresh origin.

b) Quantitatively speaking, the solute class increasing to the greatest extent in the Synechococcus, LPP (Oscillatoria type) and Fischerella was that of the non-reducing carbohydrates. Non-reducing carbohydrate amount is obtained by subtracting reducing carbohydrates from total soluble carbohydrates in our analysis protocol.

Clearly, the evolutionarily most advanced Fischerella, contains the greatest amount of non-reducing carbohydrates. In addition, the non-reducing carbohydrate fraction (we believe one molecular type) more than doubles with upshock after only seven hours. Furthermore, this increase occurs in both light and dark. The carbohydrate(s) synthesis then proceeds without an immediate light requirement. There is little or no increase in reducing sugars following upshock in the Fischerella.

A much different effect of upshock is seen in the less evolutionarily advanced LPP. In this case, non-reducing carbohydrates (again, we believe one chemical species) more than doubles after six hours of upshock, but, only in the light. Deplasmolysis is also very much slower in the dark. Again, there is no appreciable increase in reducing sugars with upshock.

Finally, the unicellular cyanobacteria Synechocystis behaves much the same way as the filamentous LPP with respect to total carbohydrate. However, under light conditions only, the reducing carbohydrates (but not non-reducing fraction) increase with upshock.

It should be noted that only the Fischerella is capable of growing in the dark on a carbohydrate source (i.e. chemoheterotrophically).

c) The changes in free amino acids in the three fresh-water cyanobacteria did not correlate with those found for the carbohydrates. The greatest changes occur in the unicellular Synechocystis and filamentous LPP. No increase in total amino acids were observed for the Fischerella.

(10) Objectives concerned with the determination of relationship between structural and nutritional modes (i.e. photoautotrophy, photoheterotrophy and chemoheterotrophy) and the nature of the solutes employed for adjustment of intracellular water potential in cyanobacteria (objective 1 and 2) are largely achieved.

The unicellular structural type, Synechocystsis (ATCC #27178) was grown in all three nutritional modes and forced to osmoregulate (after upshock) in each mode. The filamentous structural type, Calothrix (ATCC #27914) was likewise treated in the photoautotrophic nutritional mode. The most advanced structural type (i.e. branched), Chlorogloeopsis (ATCC #27181) was forced to osmoregulate in all three nutritional modes. Previous studies (of growth alone) of cyanobacteria in all three nutritional modes involved prior growth (easier and more rapid) in photoautotrophic conditions followed by a few days transfer to the other two modes prior to experimentation. The present study employed cyanobacteria completely grown under three nutritional modes.

Carbohydrates rather than amino acids are the principal solutes increasing during osmotic adjustment in Synechocystsis under photoautotrophic conditions (almost a 10-fold difference). Increases of both solutes were greater in the light than in the dark. (Figure I, Appendix III) Furthermore, the rise in amino acids after 24-hours was followed by a rapid fall within the next 24-hours. This fall did not occur in the carbohydrates. Subsequent analyses revealed that the carbohydrate fraction increasing was almost entirely non-reducing disaccharides (see progress report phase II, for details).

The principal carbohydrate is glucosylglycerol, followed by sucrose. Only sucrose accumulated in the dark. (Figure 2, Appendix III) The principal amino acids changing with osmotic adjustment in this alga were glutamic acid and proline (Figure 3 of Appendix III). As in the carbohydrate fractions, amino acid changes were more marked in the light than in the dark.

Osmotic adjustment in Synechocystis under photoheterotrophic conditions differed somewhat from that under photoautotrophic conditions. Both carbohydrates and amino acids increased in the dark (almost as much as in the light) (Figure 4, Appendix III). Again, the carbohydrates were quantitatively the more important solute. Gas chromatographic analyses (Figure 5 of Appendix III) of the carbohydrate fraction revealed that both sucrose and

glucosylglycerol increased with the former carbohydrate predominating. Likewise, differences were noted in the amino acid increases during osmotic adjustment. Glutamic acid increased, as it did in the photoautotrophically grown cyanobacteria, but proline did not. Moreover, in this nutritional mode, lysine and valine dramatically increased following upshock. The much greater (than in photoautrophy) increase in carbohydrates in dark upshocked cells was seen to be due to sucrose, not glucosylglycerol (Figures 3, 6 and 7, Appendix III).

Osmotic adjustment in Synechocystis under chemoheterotrophic conditions (dark only) was achieved basically in the same manner as in the other two nutritional modes. The striking difference was in the much greater amino acid content of the osmotically adjusting, chemoheterotrophically-grown cells (Figure 8 of Appendix III). These levels were over twice those found in cells grown in the other two modes. Sucrose was the principal sugar. In this nutritional mode, glutamic acid is still the principal amino acid, but alanine and serine are also seen to dramatically increase (Figure 9, Appendix III).

Osmotic adjustment in the filamentous structural form, Calathrix (ATCC #27914) in the photoautotrophic mode is similar quantitatively to that of Synechocystis. Carbohydrates (again non-reducing) are about 10-fold greater than amino acids in cells upshocked in the light (Figure 10). Dark levels rapidly decreased following an initial, smaller rise. Trimethylsilyl derivatives of these carbohydrates revealed that unlike Synechocystis, Calothrix employed (Figure 11, Appendix III) the non-reducing disaccharide, trehalose. Sucrose also appreciably increased. Glutamate and arginine account for most of the amino acid increase during osmotic adjustment (Figure 12, Appendix III).

Osmotic adjustment in the most complex structural form of the cyanobacteria (i.e. branched), Chlorogloeopsis (ATCC #27181) was like the other two forms achieved principally by carbohydrates under photoautotrophic conditions (Figure 13 of Appendix III). Light conditions resulted in twice as much carbohydrate as in dark conditions. The absolute levels of carbohydrate was much higher in this structural form than in the previous two forms. Amino acid levels were much lower than in the Synechocystis and Calathrix. Gas chromatographic analysis showed that the principal carbohydrate was trehalose, but that sucrose is also formed during osmotic adjustment (Figure 14 of Appendix III). Unlike the case for Calothrix, large amounts of trehalose were also formed in dark upshocked Chlorogloeopsis (Figure 15 of Appendix III). The nature of the amino acids changing during osmotic adjustment in Chlorogloeopsis different from that of the other two structural forms. Figure 16 shows that glutamic acid falls and two derivatives of glutamic acid, proline and arginine rise dramatically.

Osmotic adjustment in Chlorogloeopsis under photoheterotrophic conditions follows basically the same plan (quantitatively) as in the photoautotrophic mode (Figure 17 of Appendix III). Surprisingly,

however, sucrose rather than trehalose is the predominant carbohydrate (Figure 18). Trehalose is nevertheless present. Again, as in the photoautotrophic mode, proline and arginine rise during osmotic adjustment, while glutamic acid falls (Figure 19 of Appendix III).

Osmotic adjustment in Chlorogloeopsis during chemoheterotrophic conditions (only in the dark), follows the same quantitative plan as in the other two nutritional modes. The amino acid levels remain low (Figure 20 of Appendix III). Trehalose and sucrose are again the principal carbohydrates formed during osmotic adjustment (Figure 21 of Appendix III).

A major change in amino acid response was seen in the chemoheterotrophic mode during osmotic adjustment. Glutamic acid level rose and the proline and arginine levels remained almost constant (Figure 22 of Appendix III). Apparently, light is required in Chlorogloeopsis for the conversion of glutamic acid to proline and arginine.

(11) Research on the pathways of synthesis of the osmoregulatory solutes has focused mainly on the pathway of betaine formation in the halophilic (photoautotrophic mode, only) cyanobacterium, Aphanothece halophytica.

As previously noted we have found that the precursor of the carbon "skeleton" of betaine, serine, increases in A. halophytica during osmotic adjustment, both in light and dark. However, betaine synthesis in this cyanobacterium requires light. Our research has been directed toward the discovery of the nature of this light requirement. Our first approach was to determine the origin of the methyl groups of betaine. Figure 1 provides our interpretation of the pathway of betaine synthesis in the subject cyanobacterium. Note that S-Adenosylmethionine (SAM) is given in this figure as the probable methyl source. This compound is widely considered to be the universal methyl donor in the major biological methylations (25a, 13a). Its methyl group is that of its constituent L-methionine Methionine derives its methyl group from methyltetrahydrofolate (thereby implicating folate metabolism). Tetrahydrofolic acid is the carrier of methyl groups in this reaction. It receives the C-1 (methyl) fragment initially (and before two subsequent reductions) from formic acid (formate). It is also widely held (19a) that one of the principal sources of formate in photosynthesizing plants is the decarboxylation of glyoxylic acid produced by photorespiration (basis of the light requirement?). However, one problem remained. Currently, there is considerable support for the existence of a different photorespiratory pathway in cyanobacteria from that in higher plants. Figure 2 depicts both pathways. In eukaryotes glyosylate is transaminated to glycine and glycine subsequently converted to serine. In cyanobacteria, several prominent investigators (17a, 18a) postulated the existence of an alternate pathway through tartronic acid semialdehyde. This was based partially on the lack of effect of the inhibitor of the enzyme catalyzing the conversion of glycine to serine (higher plant pathway). This inhibitor is INH (Isonicotinic acid hydrazide). If INH is able

to exert its effect, and glycine conversion to serine is blocked, glycine will accumulate. We followed the effects of the inhibitor INH on glycine and betaine formation. It should not block betaine formation if the methyl group is derived from the C-2 atom of glyoxylic acid. In addition, we blocked glycolic acid formation itself by the photorespiration methane alpha-hydroxypyridine methane sulfonic acid (HPMS) (see Figure 2). This should block the formation of glyoxylic acid and therefore, formic acid production from its decarboxylation. In turn, betaine formation should be diminished due to a lack of formate, which is the source of its methyl groups. The data from these experiments is provided in Figures 25-29 of the last progress report (Appendix III).

We consider the above data as clear demonstration of a strong photorespiratory pathway of the type found in higher plants.

The next question concerned the metabolic control of betaine formation following upshock of A. halophytica. A controlling mechanism was sought from the previous data. Note in Table 2 that the betaine levels correspond to the levels of NaCl the cyanobacterium was grown in. The higher the NaCl the higher the internal betaine. We have also shown (see ref 51) that intracellular potassium ion (K^+) increases with increasing external salinity. It would appear then that salinity, perhaps through intracellular K^+, was initiating the synthesis of the compatible, osmoregulatory solute betaine.

A principal regulant of betaine synthesis would be the methylation of ethanolamine (Figure 23). It is widely held (13a) that the methylase enzymes employing A-Adenosyl methionine (SAM) as methyl donors are regulated (feedback inhibition) by the product of the reaction, S-Adenosylhomocysteine (SAH). The reaction is:
3 SAM + ethanolamine → 3 SAH + choline.

Choline is oxidized to betaine. Methylations are then regulated by the intracellular SAM/SAH ratios. Methylation activity, therefore, greatly increases upon the enzymatic removal of SAH.

It is currently held (25a, 74a) that prokaryotes and eukaryotes differ with respect to the mechanism of SAH breakdown. In eukaryotes the enzyme is A-Adenosylhomocysteine hydrolase (SAH hydrolase). This enzyme also functions in the reverse direction (actually in favor of) to achieve the synthesis of SAH from homocysteine and adenosine. In this sense it is a regulatory enzyme of SAH levels and consequently, biological methylations. In prokaryotes the enzyme catalyzing SAH removal is S-Adenosylhomocysteine nucleosidase. The reaction is essentially irreversible.

A potent, specific (as specificity goes) inhibitor of the eukaryotic enzyme SAH hydrolase is the adenosine analog, 3-Deazaadenosine. Consequently, it is clinically widely used (25a, 13a) to inhibit all biological methylations, due to its effect on prevention of SAH breakdown. After numerous experiments to

demonstrate SAH nucleosidase in our cyanobacterium failed, we looked for the enzyme believed to be present only in eukaryotes, SAH hydrolase. We found that at least this cyanobacterium has SAH hydrolase previously believed to not be present in prokaryotes. Figures 30-33 from the report in Appendix III show the data obtained from these experiments. Preliminary studies (not reported here) utilized an assay based upon the disappearance of L-homocysteine occurring upon measurement of synthetic activity. The assays revealed good SAH hydrolase activity. However, later assays were based upon the more specific radiolabelled adenosine incorporation into SAH (synthetic direction). The data given in this report are from this latter assay.

Figure 30 of Appendix III shows the effect of culture age on the activity of SAH hydrolase. Also shown in this figure is the effect of 0.4 M KCl added to the assay medium. Clearly the synthesis of SAH (assay in the synthetic rather than hydrolytic direction) is inhibited by the salt. Figure 31 shows (another experiment) the assay of SAH synthesis from the perspective of adenosine incorporation into SAH. Again 0.4M KCl inhibits this incorporation. Betaine, however, increases the activity of the enzyme even in the absence of KCl salt. Furthermore, betaine clearly relieves to a large extent the KCl-inhibition of SAH synthesis. The net effect of betaine accumulation would theoretically be SAH accumulation, and, therefore shutdown of further methylation. In this manner betaine would regulate its own synthesis.

Figure 32 (Appendix III) depicts the results of an experiment on the effect of the specific SAH hydrolase inhibitor, 3-Deazaadenosine on cyanobacterial SAH synthesis. Inhibition was observed even at 10μm inhibitor, thereby providing additional evidence for the existence of this enzyme in cyanobacteria.

Finally, figure 33 (Appendix III) shows the effect of the SAH hydrolase inhibitor, 3-Deazaadenosine on betaine synthesis in the intact cells following upshock. Betaine synthesis was completely inhibited.

(12) Very extensive studies were performed to determine the effect of betaine on the major kinetic parameters of glucose-6-phosphate dehydrogenase from cyanobacterial species (or strains) varying in salt tolerance as well as from a variety of other microorganisms whose purified enzymes display varying salt tolerance. These studies have been the subject of two Master of Science theses and several presentations which were published (91, 95, 96). Genome size of strains of the same species of cyanobacteria (i.e. Synechocystis and LPP) was also considered as a variable in these enzymatic studies (91). It was found that for enzymes from low (1.79×10^9 daltons) and high (3.5×10^9 daltons) DNA-content cyanobacteria (strains 27184 and 29235, respectively, of Synechocystis) and enzymes from a low (2.5×10^9 daltons) and high (5.2×10^9 daltons) DNA content LPP (strains 29126 and 29344) cyanobacteria, varying amounts of KCl were needed for 50% inhibition (Figure 3). The lower DNA content Synechocystis was much more

tolerant of KCl, as was its glucose-6-phosphate dehydrogenase, than the higher DNA strain and its enzyme. Figures 4-8 show that pH, when used as a probe, has much different effects on these enzymes from the two species of cyanobacteria. The enzymes even from identical species but different strains showing varying salt tolerance varied greatly with respect to pH.

Of far greater importance, however, is the effect of glycine-betaine on enzymatic activity reduced 50% by assay KCl. Figures 9 and 10 show that the greater the sensitivity of the enzyme (from whatever source) to KCl, the greater will be the effect of betaine on negating the KCl inhibition. The converse is also true (Figure 10).

The significance of this can best be seen in Figure 11. Here a number of glucose-6-phosphate dehydrogenases are assayed with respect to their sensitivity to KCl and the effect of betaine on this KCl inhibition. Again, the more inhibited the enzyme by KCl the greater the effect of betaine on "restoring" the activity. The more KCl tolerant enzymes are not affected, or are adversely affected by betaine. "Restoration" is actually a misnomer because studies from this project have shown that betaine independent of salt, lowers Km, while high assay salt causes Km to increase. This is the adaptive value of betaine. These figures are from reference 96.

(13) Recent experimentation in our laboratory has also demonstrated that glycine betaine will increase the tolerance of glucose-6-phosphate dehydrogenase to increasing pH (95). Figure 12 shows this dramatic effect of betaine. The significance of this surprising effect of betaine to the osmoregulatory mechanism will be discussed in a later section.

(14) Our studies on the role of the enzyme S-Adenosylhomocysteine (SAH) hydrolase in cyanobacterial osmoregulation and betaine synthesis have demonstrated that the controlling factor is intracellular potassium ion concentration. Apparently, this enzyme, which was first found in cyanobacteria in this project, is a "one-way" enzyme. This means that as intracellular potassium accumulates during the initial stage of osmoregulation to concentrations inhibitory to most enzymes, the SAH hydrolase still functions uninhibited in the hydrolytic direction. It is inhibited, however, in the favored (Keg.) synthesis direction. As SAH is a powerful inhibitor of methylases, which use S-Adenosylmethionine (SAM) to methylate the precursor to betaine, this effect would allow betaine synthesis to continue to a concentration that would allow the SAH hydrolase to function in the synthetic direction. SAH would then accumulate to shut down betaine synthesis. This is a primitive but efficient, adaptive mechanism that regulates betaine synthesis to achieve salt tolerance. These findings have been reported (90, 92) and accepted for publication in an international journal (97).

Significance of the Research Accomplished During Phases I, II and III

The specific findings reported above are significant with respect to qualification of certain conclusions concerning cyanobacteria and the nature of compatible solutes that have been recently reported in the literature.

First, there exists the problem of too closely grouping the cyanobacteria (or blue-green algae) to other gram-negative bacteria, as is frequently done (17). Gram-negative bacteria (not cyanobacteria however) show increases in glutamic acid and/or proline and gamma aminobutyric acid (GABA) following upshock. Obviously from our data this does not occur. In fact, the halotolerant cyanobacterium Aphanothece, always showed a decrease in glutamic with upshock concomitant to an increase in serine, even as K^+ was increasing. Furthermore, we have been unable to find any GABA, even at the nanomole level in this organism.

Secondly, our data show that proline and glycine, two solutes often found in osmoregulating eukaryotes, do accumulate in certain cyanobacteria under certain environmental conditions. In one case (Aphanothece) both betaine and proline accumulate.

As previously reported we appear to also find, again in certain cyanobacteria, the glucosylglycerol involved in osmoregulation. However, a recent review in Science by Yancey, et al (82) reports only this osmoregulatory solute (osmolyte) for the cyanobacteria. The major thesis of this particular article was the great uniformity (and lack of diversity) of chemicals evolved for the purpose of osmoregulation. We believe, from our initial data, that there is considerable diversity in osmoregulatory solutes employed by the cyanobacteria from adaptive evolution to water and saline stress.

Thirdly, and of considerable importance to non-blue-green bacteria, amino acids in the growth (culture) medium may be taken up (or diffuse in) during upshock. These amino acids may, if the case involving Aphanothece is repeated, influence the nature of the solutes formed during osmoregulation. Early heterotrophic bacteria, which were probably the earth's first prokaryotes, almost certainly had amino acids available to them. One of the most common of these was almost certainly glycine, and perhaps, the dipeptide, glycylglycine. These amino acids may have directed the pathways of osmoregulatory solute production.

Fourth, recent research on the pathway of betaine synthesis in higher plants by Andrew Hanson and his coworkers at Michigan State (35a) confirms that serine is the precursor of betaine. The pathway is as follows:

Serine → ethanolamine → N-methylethanolamine → dimethylethanolamine → choline → betaine aldehyde → betaine

However, these investigators have focused their attention to the steps in this pathway that are responsive to increasing water stress or salinity--after the formation of serine. They further note that betaine synthesis requires light, especially for the formation of one-carbon metabolite derivatives of formic acid. Our data certainly agree with those indicating a light requirement for betaine synthesis. However, we believe that our data show that the decreased water potential and/or salinity trigger the synthesis of serine. Serine

formation is the limiting step for betaine synthesis and later stages in the pathway may or may not be stimulated by decreasing water potential or increasing internal salinity. Furthermore, we believe that the serine, while certainly not of photorespiratory origin, probably comes from the non-phosphorylated, D-glyceric pathway.

Fifth, and finally, our data also challenge, or at least qualify, the statement made by Yancey et al (82, p. 1217) that the "counteracting effects [to salt inhibition of enzymes] [of betaine] are independent of the species source of protein. Mammals, teleost, amphibian and elasmobranch proteins respond similarly in the presence of counteracting solutes, regardless of whether they experience these solutes in vivo" and on p. 1221,

> "Through the use of compatible solute systems, proteins are able to work in the presence of high or variable solute concentrations, and the [genetic] modifications of vast numbers of proteins is avoided."

We agree with the importance of their assessment of the evolutionary role of compatible solutes, as this gives even more justification for the objectives of our proposed research. However, our experience (reported here) with glycine-betaine and glucose-phosphate dehydrogenase from different sources, leads us to call for more experimentation on protein modification in organisms employing greatly different osmoregulatory systems.

Sixth, we now know that the cyanobacteria, once called metabolically uniform, employ a diversity of solutes to achieve osmotic balance with an environment of increasing salinity (or lowered water potential). The unicellular form, Synechocystis employed a glucosylglycerol. The branched and filamentous unbranced species employed trehalose and sucrose. Amino acids proved to be a minor component in the total solute change during osmotic adjustment in all structural forms. Interestingly, proline (a common osmotically important solute) accumulated in only the more advanced structural forms.

Nutritional modes of the cyanobacteria did not appear to greatly influence the nature of the solutes accumulating during osmotic adjustment with some possible exceptions. This is significant in view of the greater availability of glucose in photoheterotrophically and chemoheterotrophically grown cells.

Light versus dark incubation during osmotic adjustment did profoundly affect the quantitative and qualitative nature of the solute response. Clearly some of the carbohydrates (e.g. glucosylglycerol) and amino acids (e.g. proline) require light for their maximal synthesis.

Seventh, our demonstration of a "typical" higher plant chloroplast photorespiratory pathway in the halophilic cyanobacterium is significant to the origin of the methyl groups of betaine from formate. There is little doubt that large amounts of photorespiratory carbon passes through glycine and serine in this cyanobacterium.

Eighth, if we are correct in our hypothesis that photorespiratory formate is the source of the methyl groups of betaine then this pathway possesses a definite value to survival of cyanobacteria under conditions of extreme

salinity. However, another question arises. Photorespiration requires elevated oxygen levels. It is, therefore, unlikely that this pathway contributed formate to betaine synthesis in the Precambrian eon, an eon of extremely low atmospheric oxygen. The possibility does exist however, that increased salinity per se may elicit phosphoglycolic acid synthesis from ribulose 1, 5-bisphosphate. This possibility will be tested. Another possibility is the direct synthesis of formic acid from carbon dioxide. Such a synthesis has been previously postulated.

Ninth, betaine synthesis in a representative of earth's earliest oxygenic photoautotroph is of considerable evolutionary importance. Given the possibility that higher plant chloroplasts arose from cyanobacteria (endosymbiotically), and the recent demonstration that betaine synthesis may occur within plant chloroplasts (35b) our research on this pathway directly relates to the origin of a very important pathway.

Of even greater importance is the possible mechanism of regulation of betaine synthesis through K^+ and betaine levels. This mechanism is made possible by our demonstration that SAH hydrolase does in fact exist in a prokaryote, the cyanobacteria. Briefly our hypothesis is:

(a) With increasing environmental salinity the cyanobacterium accumulates K^+ ion; this ion stimulates SAH hydrolase in the hydrolytic direction thereby breaking down the methylase inhibitor SAH; increased methylation results in increased betaine synthesis; betaine protects enzymes in the presence of the increased intracellular KCl.

(b) Increased KCl inhibits the SAH hydrolase in the direction of SAH synthesis; this inhibition results in the decreased synthesis of the methylase inhibitor SAH; this results in more betaine synthesized.

(c) As betaine accumulates it protects SAH hydrolase from KCl inhibition of its synthesis direction; SAH will accumulate once again and inhibit methylase enzyme resulting in a cessation of betaine synthesis.

Tenth, a recent publication in Science (22a) deals with the universal association of trehalose with protection against dehydration of biological membranes. In the presence of trehalose membrane lipids completely dehydrated had the same transition temperature as fully hydrated membrane. We feel that this is the role of trehalose in osmoregulation by cyanobacteria. Osmotic adjustment must also require protection of desiccated membranes.

Currently we feel that osmotic adjustment to hypersaline conditions can only be achieved in those cyanobacteria capable of betaine synthesis. Betaine fulfills three roles of a compatible solute. These are: a) preservation of membranes from dehydration injury during osmotic adjustment; b) preservation of enzyme functioning in the presence of high intracellular salt; and c) balance of the intracellular water thermodynamically with that of the external environment.

All other cyanobacteria capable of osmotic adjustment to much lower salinities (generally up to 1M NaCl) employ compatible solutes that merely allow enzymatic functioning, not protection against high salt. In this sense

betaine is more than a compatible solute, it is a protective solute or osmolyte. Trehalose will serve two of the three functions, that is, protection of membranes and allowance of enzymatic function.

The significance of the betaine effect on KCl-inhibited enzymes lies in the inherent salt sensitivity of the enzyme (in this case, glucose phosphate dehydrogenase). It would appear that there is an evolutionary bifurcation in adaptation to high intracellular salt. Either the prokaryote evolved salt (high) requiring enzymes, as did the halobacteria, or the metabolic pathways for the synthesis of betaine. One conclusion from this study is that without either structural salt tolerance (requirement) or betaine synthesis, a cyanobacterium is greatly limited in its ability to tolerate salt.

The significance of the betaine effect on shifting the pH optimum upward lies in the probable increase in intracellular pH during osmoregulation. This is the case in organisms possessing a proton (out)-potassium ion (in)-pump. Previous experiments in our laboratory have shown that Aphanothece which accumulates potassium ion during osmoregulation also synthesizes betaine. Hence the connection.

Finally, perhaps the most significant of all of our findings involves that of the relationship between intracellular potassium accumulation to offset lowered water potential due to salt stress and the enzymatic synthesis of glycine-betaine. Our research has definitely shown that an important regulatory step in betaine synthesis is that of the methylation of phosphorylethanolamine using S-Adenosylmethionine as the methyl donor and a specific methylase to catalyze the reaction. The products of the reaction, choline and eventually betaine, and S-Adenosylcysteine (SAH) are regulated by SAH which inhibits the methylase. High potassium ion concentration drives the hydrolysis of SAH by allowing the hydrolytic direction of the SAH hydrolase to function while inhibiting the synthetic step which leads to SAH formation.

This results in increased betaine synthesis up to a concentration that will "protect" SAH hydrolase in the synthetic direction. The increased SAH resulting will thereby shut down betaine synthesis by inhibiting the methylase.

It appears that our project has elucidated the most primitive mechanism of salt-induced betaine synthesis in cyanobacteria. This may, if correct, prove to be highly significant by virtue of the postulated (by some) cyanobacterial ancestry of the chloroplasts of higher plants.

C. Presentations, Activities and Publications Resulting from the Research Since 1985 Proposal and Progress Report

1. Publications and Abstracts:

Pavlicek, K.A. and J.H. Yopp. 1985. Influence of KCl and betaine on soluble and membrane NAD(P)H dehydrogenases from the halophilic cyanobacterium, Aphanothece halophytica. Plant Physiol. 77:647-S.

Sibley, Marion H. and J.H. Yopp. 1985. Occurrence and osmoregulatory role of S-adevosylhomocysteine hydrolase in betaine synthesis by the

halotolerant cyanobacterium, Aphanothece halophytica. Plant Physiol. 77:771-S.

Myers, G.O. and J.H. Yopp. 1985. Salt inhibition and betaine restoration of activity of glucose-6-phosphate dehydrogenase from cyanobacteria differing in genome size. Plant Physiol. 77:650-S.

Sibley, Marion H. and John H. Yopp. 1986. Unidirectional inhibition of cyanobacterial S-Adenosylhomocysteine hydrolase by potassium chloride: proposed role in osmoregulatory betaine synthesis. Plant Physiol. 80(4):699.

Hawkins, Lynda, D. Ritter and John H. Yopp. 1987. Proposed additional role for glycinebetaine in the adaptation of halophilic cyanobacteria to hypersalinity: protection against pH-induced loss of enzymatic activity. Plant Physiol. In press.

Sibley, Marion and John H. Yopp. 1987. Regulation of glycinebetaine in the halophilic cyanobacterium Aphanothece halophytica: proposed role of SAH hydrolase. Archives of Microbiology. Accepted for publication, January.

Yopp, J.H., K.A. Pavlicek and Marion H. Sibley. 1985. "Evolutionary Significance of Osmoregulatory Mechanisms in Cyanobacteria." Proc. Second Sympos. on Chem. Evol. and Origin of Life. 2:74, NASA, Ames Res. Ct., Calif.

Yopp, J.H. 1985. "The role of sulfur in osmoregulation and salinity tolerance in cyanobacteria, algae and plants." In: The Global Sulfur Cycle, Dorion Sagan, (ed.); Life Sciences Division, NASA Office of Space Science and Applications, Washington, D.C., pp. 83-86.

2. Presentations:

Pavlicek, K.A. and J.H. Yopp. 1985. Influence of KCl and betaine on soluble and membrane NAD(P)H dehydrogenases from the halophilic cyanobacterium. Aphanothece halophytics Annual Meeting of the American Society of Plant Physiologists, Brown University, Providence, Rhode Island. June 23-28, 1985.

Sibley, Marion H. and J.H. Yopp. 1985. Occurrence and osmoregulatory role of S-adevosylhomocysteine hydrolase in betaine synthesis by the halotolerant cyanobacterium Aphanothece halophytica. Annual Meeting of the American Society of Plant Physiologists, Brown University, Providence, Rhode Island. June 23-28, 1985.

Myers, G.O. and J.H. Yopp. 1985. Salt inhibition and betaine restoration of activity of glucose-6-phosphate dehydrogenase from cyanobacteria differing in genome size. Annual Meeting of the American Society of Plant Physiologists, Brown University, Providence, Rhode Island. June 23-28, 1985.

Yopp, J.H., K.A. Pavlicek and Marion H. Sibley. 1985. Evolutionary significance of osmoregulatory mechanisms in cyanobacteria. Second Symposium on Chemical Evolution and the Origin of Life. National Aeronautics and Space Administration, Ames Research Center. Moffett Field, California. July 23-26, 1985.

Sibley, Marion H. and John H. Yopp. 1986. "Unidirectional inhibition of cyanobacterial S-Adenosylhomocysteine hydrolase by potassium chloride: proposed role in osmoregulatory betaine synthesis." Annual Meeting of the American Society of Plant Physiologists at Louisiana State University, Baton Rouge, Louisiana. June 8-12, 1986.

Invitation by coordinators of the NASA-San Jose State (California)--SPONSORED SUMMER RESEARCH PROGRAM (ANNUAL), to present two papers entitled "The role of oxygenic photoautotrophs in the sulfur cycle: from sulfate assimilation to dimethylsulfide production," on August 3, 1984; and, "The role of sulfur in osmoregulation and salinity tolerance in cyanobacteria, algae, and plants," on August 2, 1984; Drs. Lynn Margulis and Ellen Weaver, coordinators; San Jose State University.

3. Master of Science Theses Supported by the NASA Grant--Completed Under Direction of John H. Yopp:

Lynda Kaye Hawkins. "The Effects of Glycinebetaine, pH and Potassium Chloride on the Physico-Chemical Properties and Function of Glucose-6-Phosphate Dehydrogenase (E.C.1.1.1.49) from Two Strains of Synechocystis spp. Differing in Genome Size and Salt Tolerance." August, 1986.

Myers, Gerald O. "Properties of Glucose-6-Phosphate Dehydrogenase (E.C.1.1.1.49) from Strains of the Cyanobacteria Synechocystis spp. and LPP spp. Differing in Genome Size." April, 1985.

4. Related Accomplishments from NASA-Supported Research (Grant NAGW-344)

Appointment to the Society of Sigma Xi Lectureship. Midwest Lecture Circuit (1984-1986): Topic was NASA-sponsored research entitled "Adaptations by Plants to Extreme Environments." (Lectures at various universities, e.g. Augustana, University of Louisville.)

Appointed as a Visiting Scholar and Program Speaker in the Oak Ridge Associated Universities Visiting Scholars Program, 1986-1987, by Dr. James Gumnick, Program Director. Topic: Physiological and Biochemical Mechanisms of Resistance to Hypersalinity by Photosynthetic Microorganisms. (NASA Research) 1971-1986.

Invitation to present lecture entitled, "Salt Resistance Mechanisms in Photosynthetic Organisms and Chloroplasts of Higher Plants." Pedro Urena National University, Santo Domingo, Dominican Republic at the Invitation of Rector, Dr. Jaime Vinas Roman, August 15-21, 1986.

Presentation of lecture and workshop entitled "The Need to Integrate Chemistry, Physics, Biology and Evolution into a Treatment of the Origin of the Universe and Life for High School Science Students" to the Master Biology Teachers of the Honors Institute in Microbiology. Sponsored by the National Science Foundation (grant to Dr. Isaac Scheckmeister), 1984.

Science project advisor and instructor for two Carbondale Community High School students (Todd Martin, Michelle Yee) to aid in development of NASA space shuttle experiment, Spring, 1984.

Presentation of 2 lectures and workshops entitled: "The Need to Integrate Chemistry, Physics, Biology, and Evolution into a Treatment of the Origin of the Universe and Life for High School Science Students" and "Isolation of Extreme Organisms," to the Master Biology Teachers of the Honors Institute in Microbiology. Sponsored by the National Science Foundation (grant to Dr. Isaac Schechmeister), January 26, 1985; March 1985; and twice in 1986.

5. Other Major Accomplishments and Continuing Activities

Darryl Ritter, a current Ph.D. student has now (1986-1987) isolated DNA from Aphanothece halophytica and LPP strain (ATCC) #29126 and made clone banks using the Escherichia coli plasmid pBr 322. These banks are currently being screened for the expression of key cyanobacterial enzymes with subsequent characterization of gene and promoter-terminator sequences encoding for glucose-6-phosphate dehydrogenase.

Southern Illinois University at Carbondale has now purchased a 300 MHz wide bore NMR to finish the NMR C-13 studies initially proposed. Support will be provided totally by the university.

Table 1

Nutritional and Morphological Types of Blue-Green Algae (Cyanobacteria) Successfully Cultured in Defined Nutrient Medium from June 15-December 30, 1982

Nutritional type and strain genus	Morphological type, mean genome size[1], and mol% GC	Taxonomic Group	Culture collection(s)[3] and collection number
A. Obligate photoautotrophs			
Synechococcus	unicellular, 1.57 x 10^9 daltons; 47-56 mol% GC	Chroococcaceae; Section I	PCC #6311; ATTC #27145
Synechococcus	unicellular, 2.4 x 10^9 daltons; 66-71 mol% GC	Chroococcaceae; Section I	PCC #7009; ATTC #29203
Aphanothece halophytica	unicellular	Chroococcaceae; Section I	Botany Dept.; SIU, isolate YT-2
Oscillatoria	filamentous; 4.38 x 10^9 daltons; 40-50 mol% GC	Oscillatoriaceae; Section III	PCC #6304; ATCC #27930
LPP Group	filamentous; 2.63 x 10^9 daltons; 42-52 mol% GC	Oscillatoriaceae; Section III	PCC #6703; ATCC #27907
LPP Group	filamentous, 5.19 x 10^9 daltons; 42-52 mol% GC	Oscillatoriaceae; Section III	PCC #7408; ATCC #29344
B. Obligate photoautotrophs-nitrogen fixing			

Table 1 (continued)

Gloeothece	unicellular; 5.02 x 10^9 daltons; 40-43 mol% GC	Chroococcaceae; Section I	PCC #6501; ATCC #27151 UTEX 1938
LPP Group	filamentous; 3.77 x 10^9 daltons; 44-52 mol% GC	Oscillatoriaceae; Section III	PCC #7408; ATTC #29344
Anabaeana	filamentous heterocystous; 3.17 x 10^9 daltons; 38-44 mol% GC	Nostocaceae Section IV	PCC #7122; ATCC #27899 UTEX B 629
Nostoc	filamentous, heterocyctous; 4.00 x 10^9 daltons; 39-45 mol% GC	Nostocaceae Section IV	PCC #6719; ATCC #29105
Nostoc	filamentous, heterocyctous; 6.42 x 10^9 daltons; 39-45 mol% GC	Nostocaceae Section IV	PCC #7422; ATCC #29312
C. Facultative photoheterotrophs			
Synechocystis	unicellular; 1.79 x 10^9 daltons; 35-37 mol% GC	Chroococcaceae; Section I	PCC #6803; ATCC #27???
Synechocystis	unicellular; 3.50 x 10^9 daltons; 42-48 mol% GC	Chroococcaceae; Section I	PCC #7509; ATCC #29235
LPP Group	filamentous; 2.58 x 10^9 daltons; 53-59 mol% GC	Oscillatoriaceae Section III	PCC #7407; ATCC #29126

Table 1 (continued)

D. Facultative photoheterotrophs-nitrogen fixing			
LPP Group	filamentous; 3.3 x 10^9 daltons; 42-52 mol% GC	Oscillatoriaceae; Section III	PCC #6409; ATCC #2911?
Calothrix	filamentous; heterocystous; 5.23 x 10^9 daltons; 40-44 mol% GC	Nostocaceae; Section IV	PCC #7103, ATCC #27905
Calothrix	filamentous; heterocystous; 8.58 x 10^9 daltons; 40-44 mol% GC	Nostocaceae; Section IV	PCC #7102; ATCC #27901
Fischerella	branched-filamentous, heterocystous; 3.62 x 10^9 daltons; 42-46 mol% GC	Stigonemataceae; Section V	PCC #73103, ATCC #29114 UTEX 1301
E. Facultative chemoheterotrophs			
Synechocystis	unicellular; 2.34 x 10^9 daltons; 42-48 mol% GC	Chroococcaceae; Section I	PCC #6714; ATCC #27178
Synechocystis	unicellular; 1.79 x 10^9 daltons; 42-48 mol% GC	Chroococcaceae; Section I	PCC #6803; ATCC #27184
Oscillatoria	filamentous, 3.86 x 10^9 daltons; 40-50 mol% GC (nitrogen fixing)	Oscillatoriaceae; Section III	PCC #6412; ATCC #29??? UTEX 1546

Table 1 (continued)

Calothrix	filamentous, heterocystous; 5.23×10^9 daltons; 40-44 mol% GC	Nostocaceae; Section IV	PCC #7103; ATCC #27905
Calothrix	filamentous, heterocystous; 7.75×10^9 daltons; 40-44 mol% GC	Nostocaceae Section IV	PCC #7101, ATCC #27914
Chlorogloeopsis	filamentous, division in more than one plane 5.24×10^9 daltons; 42-43 mol% GC (heterocystous)	Stigonemataceae; Section V	PCC #6718; ATCC #27181

[1] Genome size and mol% GC is given in Rippka et al and Herdman, et al.

[2] Taxonomic group is according to classical position (Family) and Section number given by Rippka et al which was determined by developmental DNA and physiological characteristics.

[3] Pasteur Culture Collection (PCC) and American Type Culture Collection (ATCC) numbers according to Rippka et al.

[4] Placed by Rippka et al in Section I, Chroococcaceae.

Table 2

Major Solute Content of A. halophytica Grown in Media of Increasing NaCl Salinity

NaCl Molarity of Medium	Solute Class*			
	total free amino acids	total soluble carbohydrates	total carbohydrates	glycine-betaine
Glycyl-glycine buffered	mg x 10^{-7} per cells†	mg x 10^{-7} per cell††	mg x 10^{-7} per cell	mg x 10^{-7} per cell
1	2.83	2.83	0.51	28.0
2	3.31	3.01	0.53	44.0
3	4.36	3.37	0.59	123.0
4	7.53	**	____	130.0
MES buffered				
1	2.56	____	____	36.2
3	1.23	____	____	67.6

* all values are averages of triplicate determinations
** not determined
† values are leucine equivalents
†† values of all carbohydrates are glucose equivalents

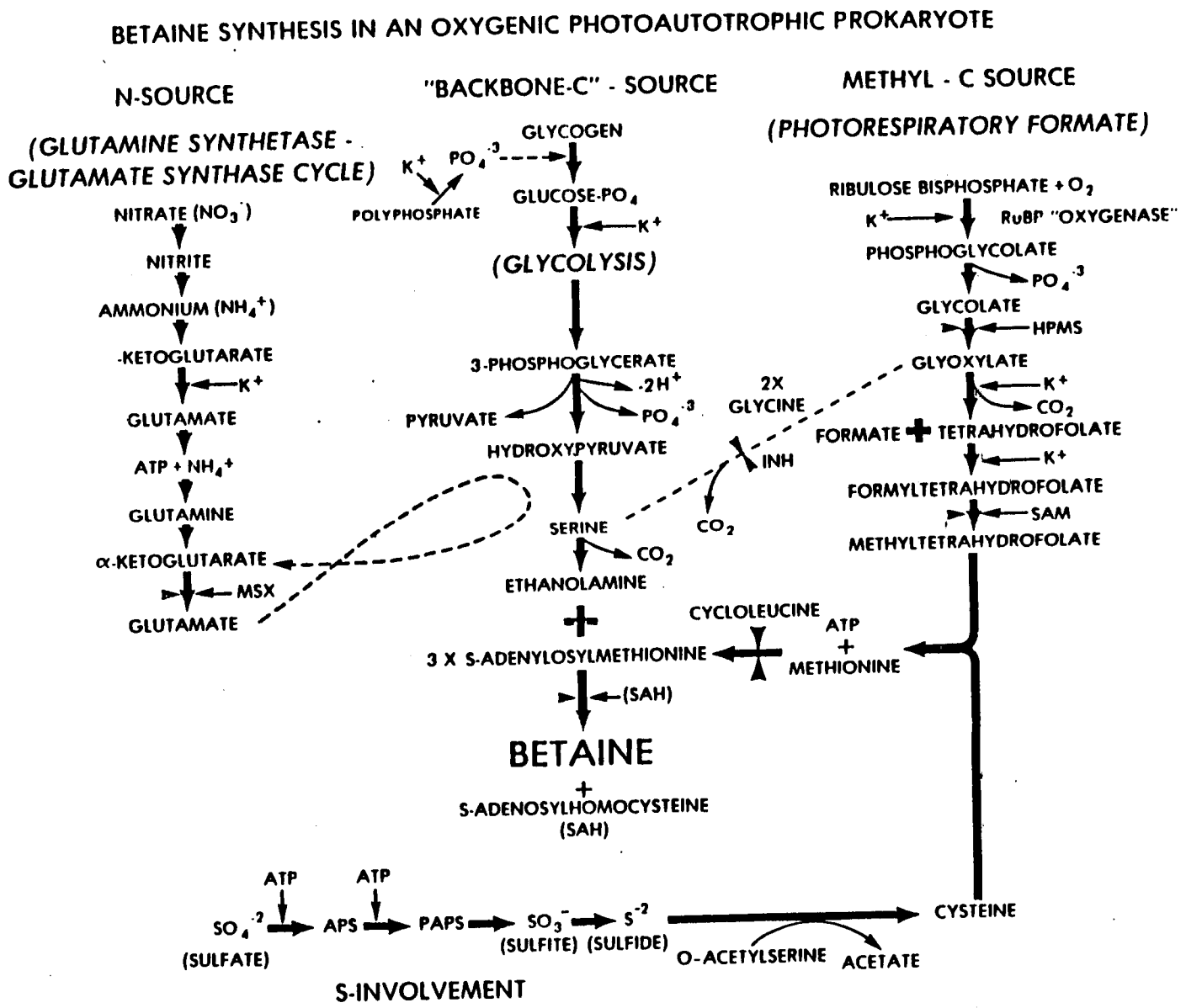

FIGURE 1.

PHOTORESPIRATORY PATHWAYS IN CYANOBACTERIA

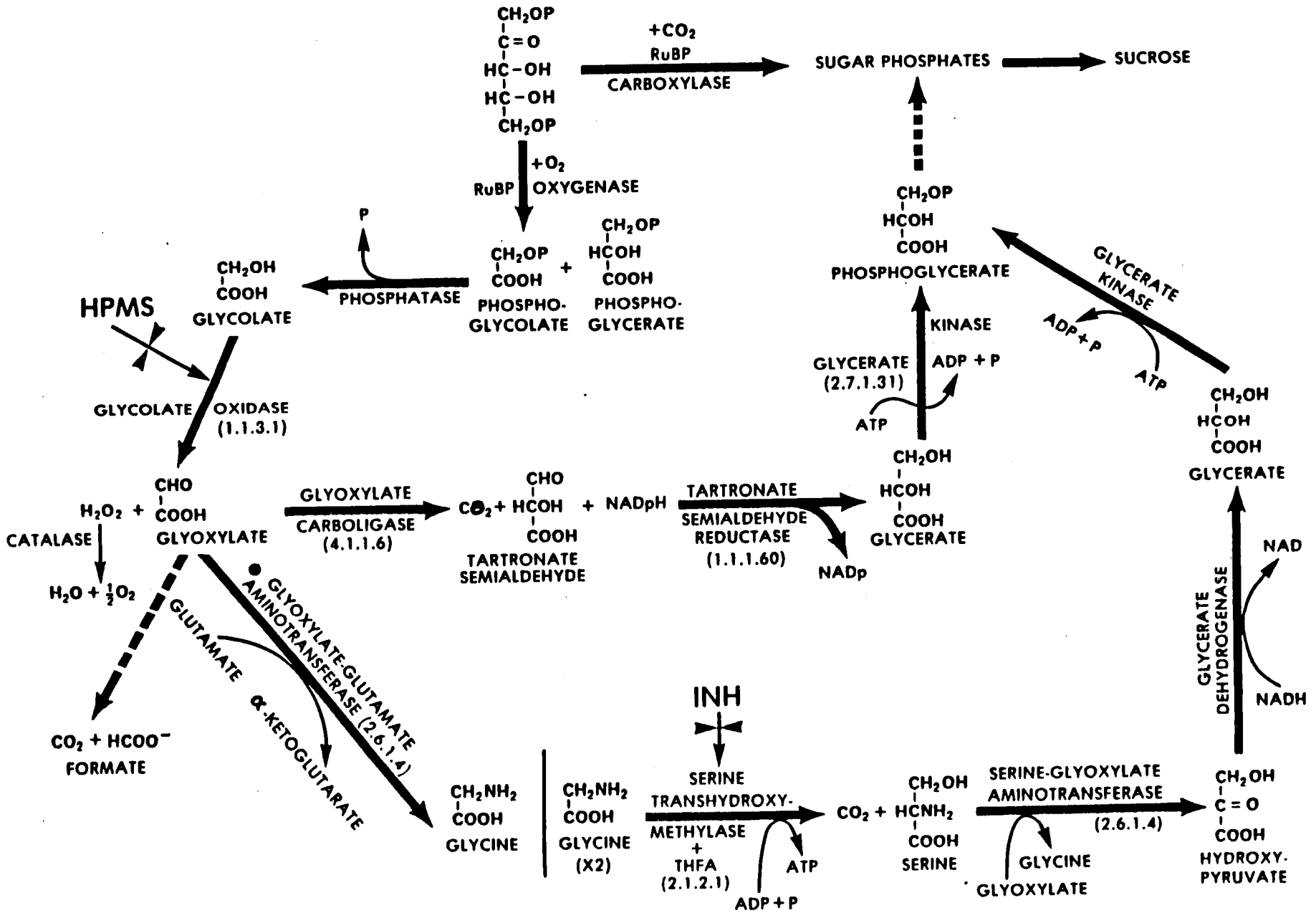

FIGURE 2.

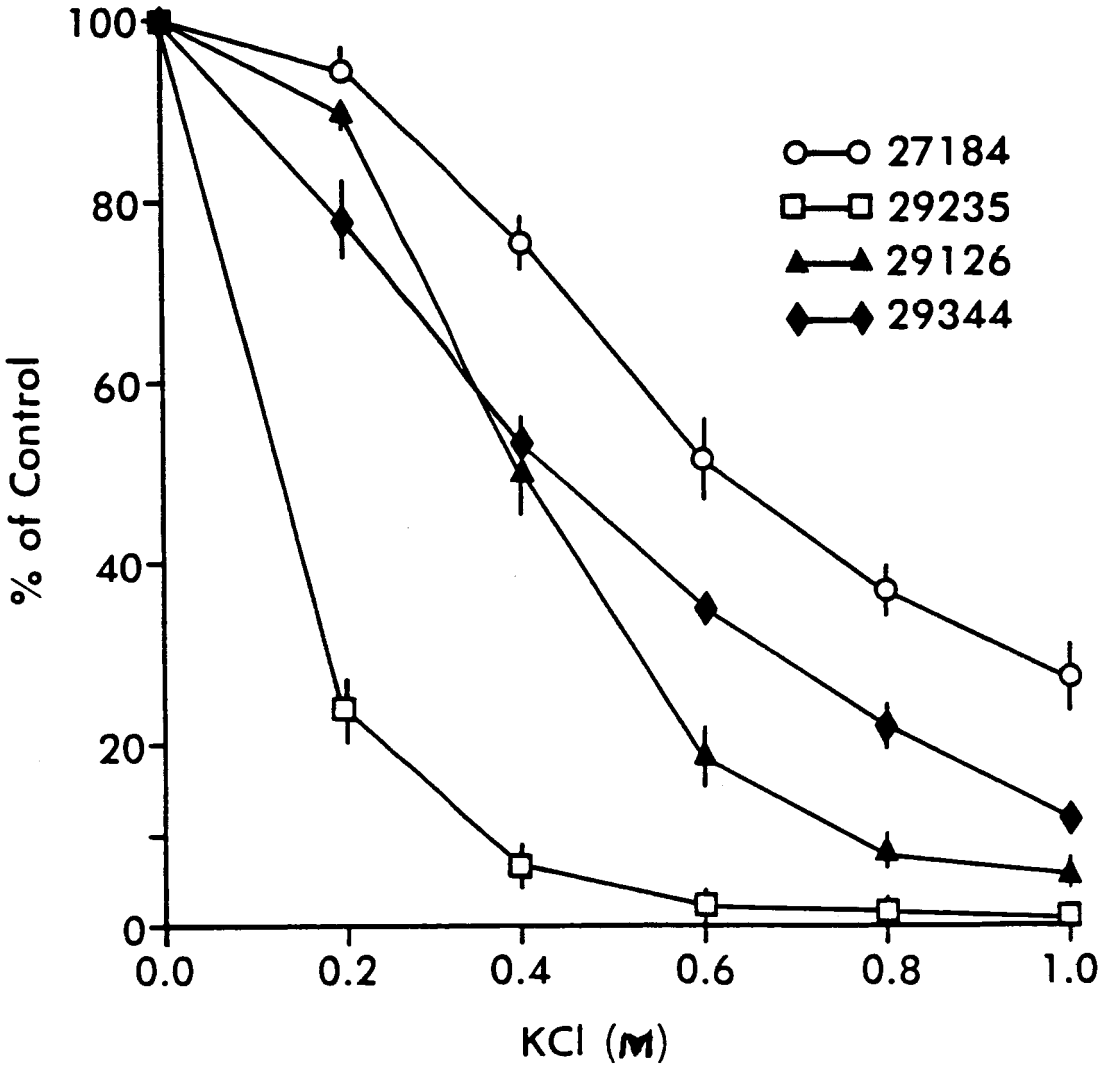

FIGURE 3. KCl inhibition of Synechocystis and LPP spp. glucose-6-phosphate dehydrogenase.

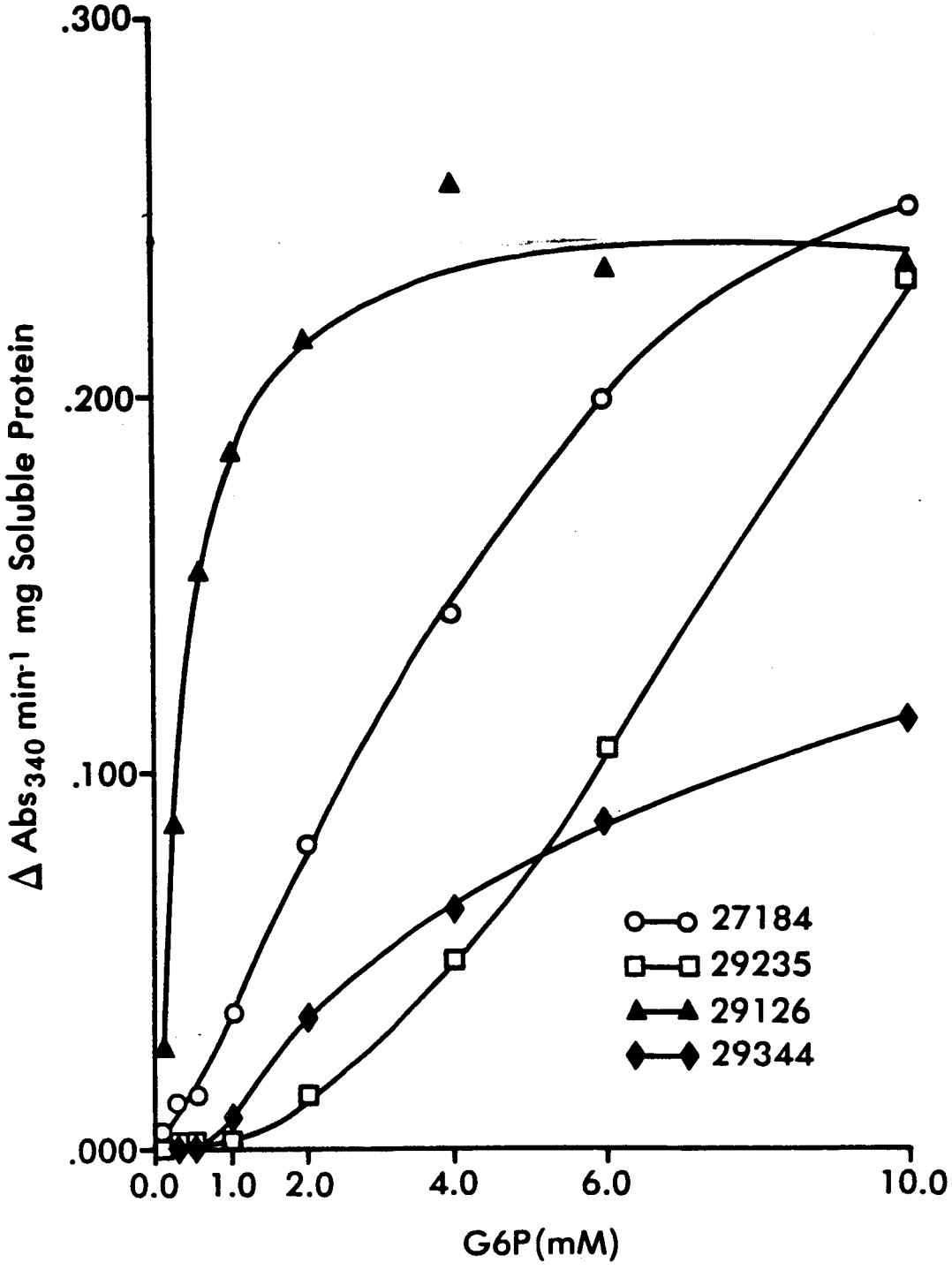

FIGURE 4. Substrate saturation curves for Synechocystis and LPP spp. glucose-6-phosphate dehydrogenase at pH 6.4

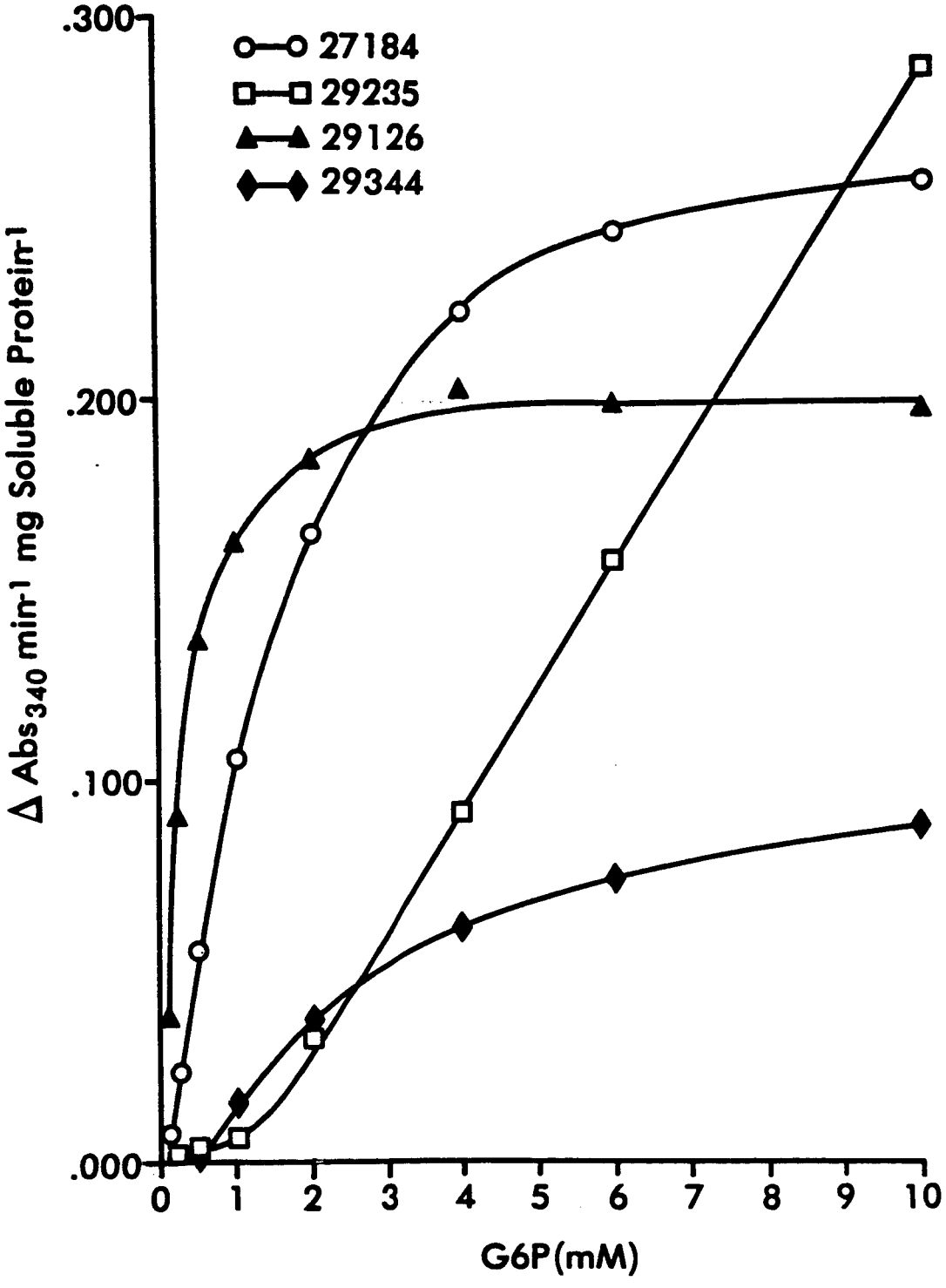

FIGURE 5. Substrate saturation curves for Synechocystis and LPP spp. glucose-6-phosphate dehydrogenase at pH 6.9

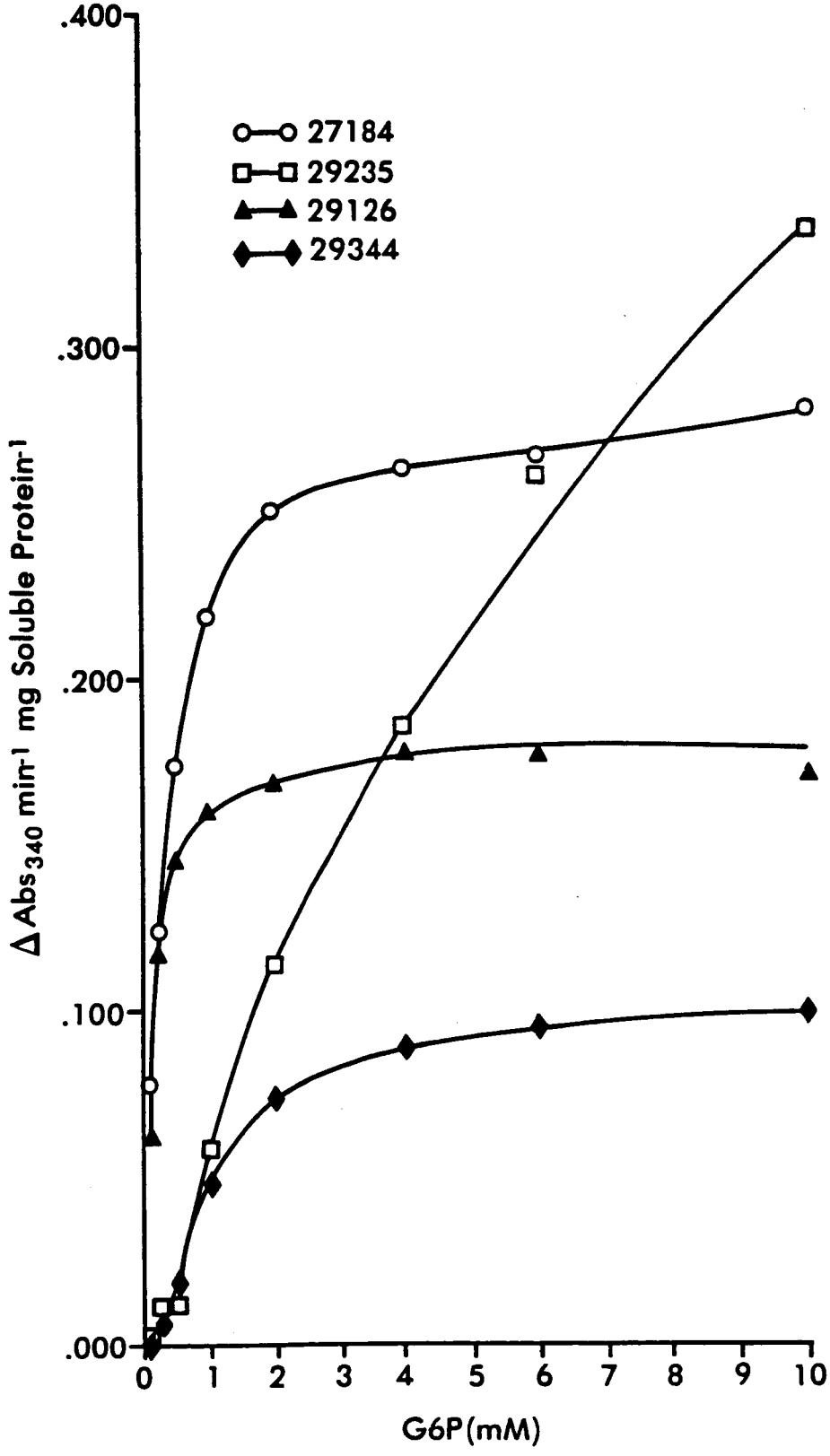

FIGURE 6. Substrate saturation curves for Synechocystis and LPP spp. glucose-6-phosphate dehydrogenase at pH 7.4

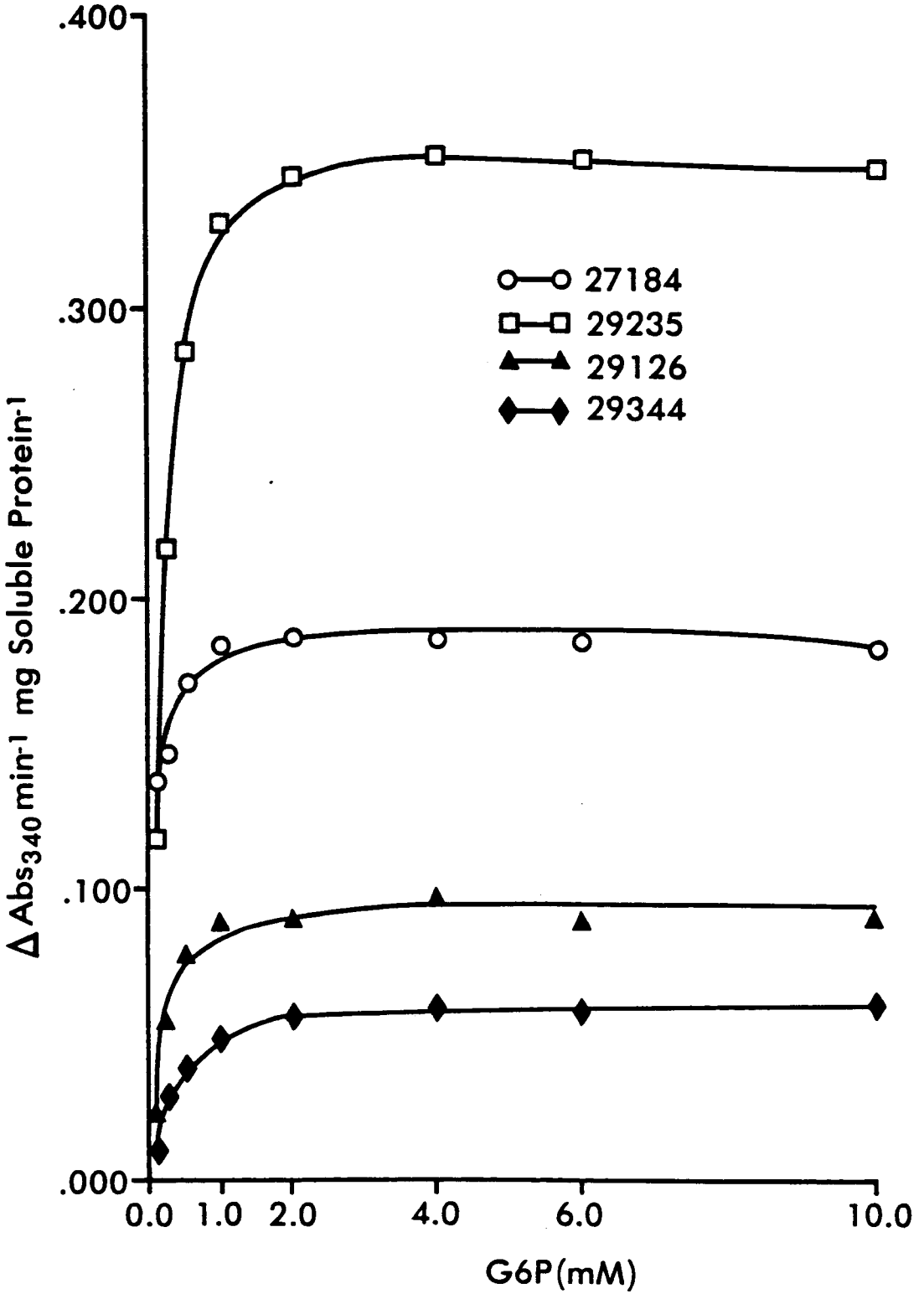

FIGURE 7. Substrate saturation curves for Synechocystis and LPP spp. glucose-6-phosphate dehydrogenase at pH 7.9

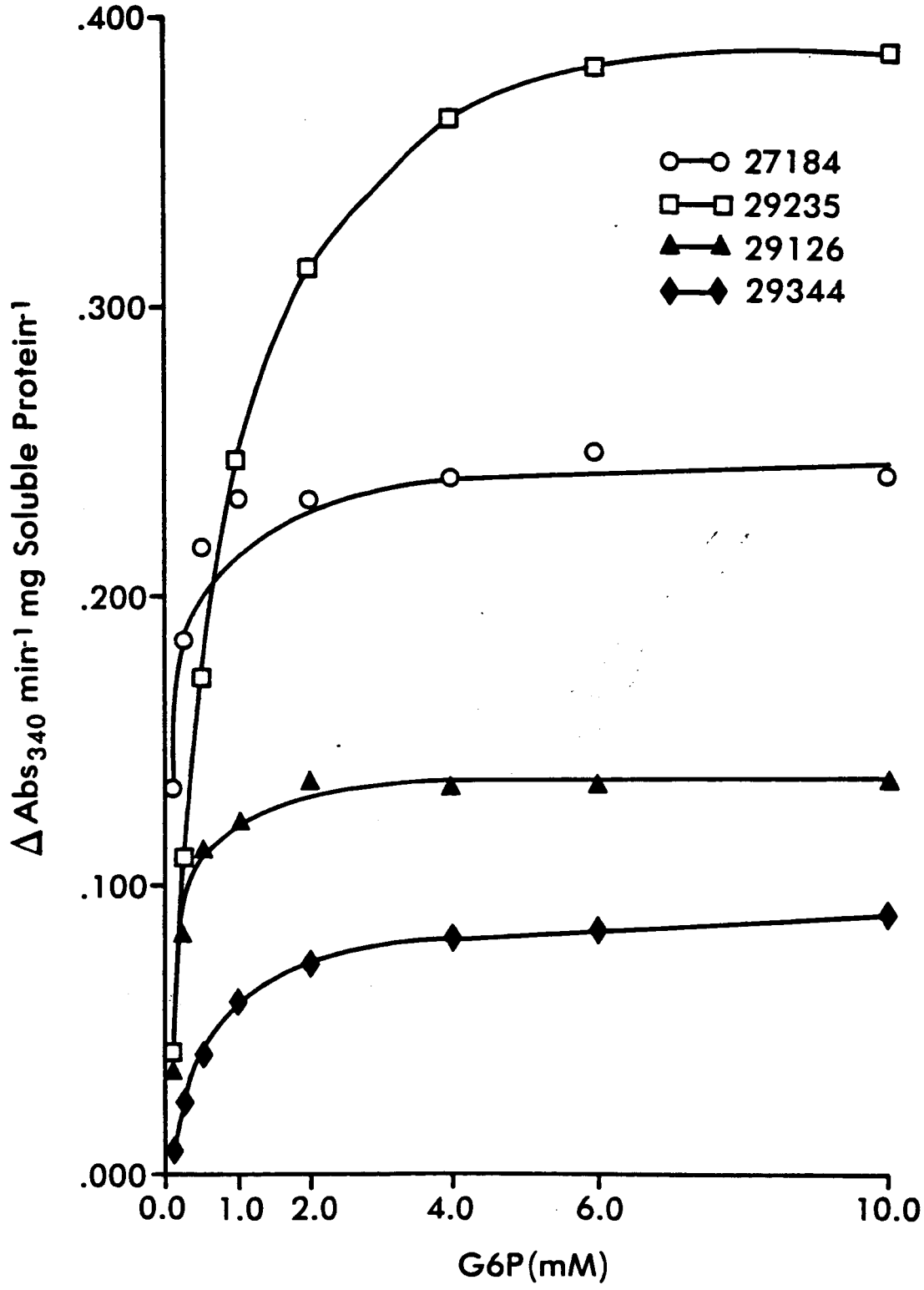

FIGURE 8. Substrate saturation curves for Synechocystis and LPP spp. glucose-6-phosphate dehydrogenase at pH 8.4

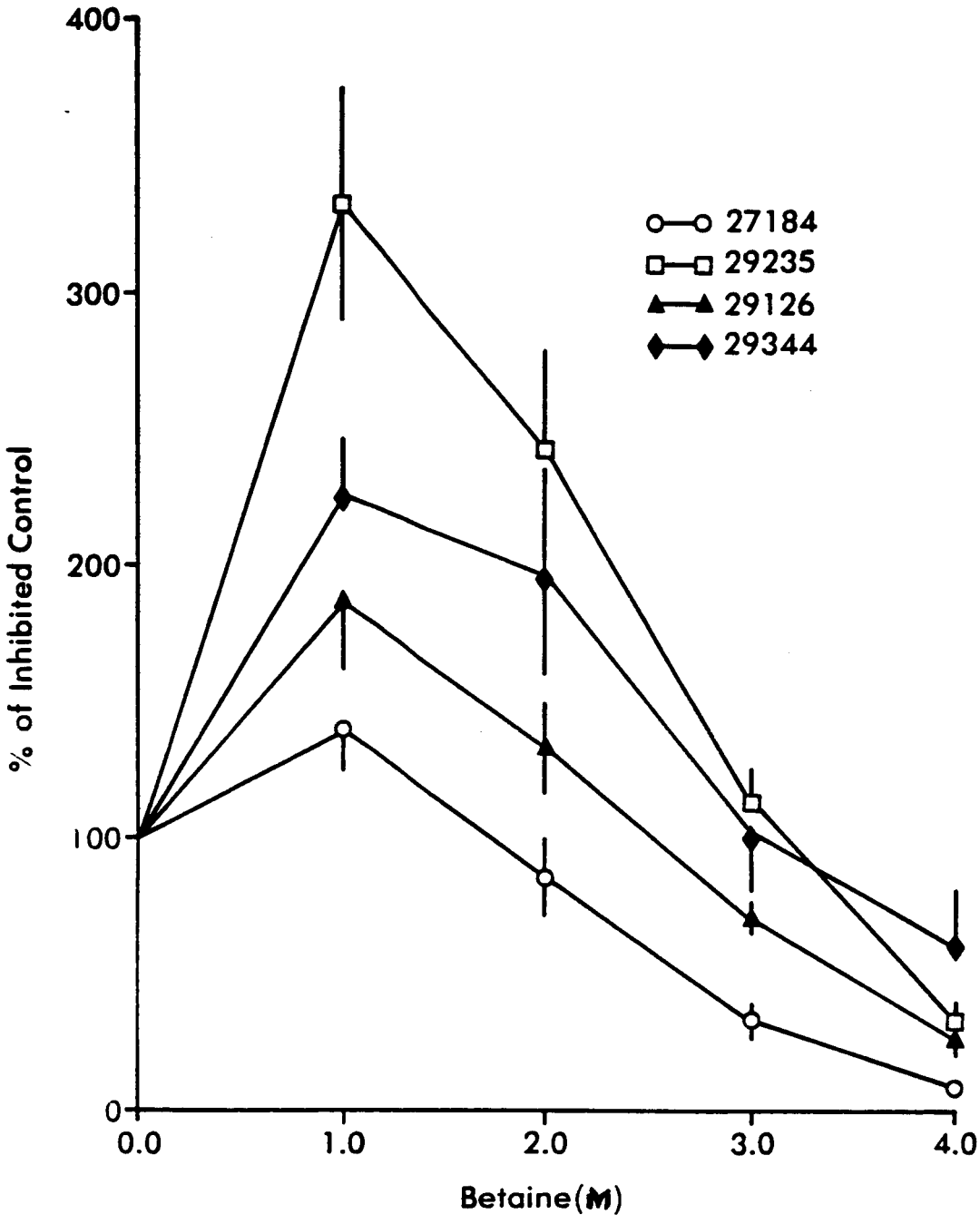

FIGURE 9. The effect of glycinebetaine on KCl inhibited glucose-6-phosphate dehydrogenase of Synechocystis and LPP spp. as a percent of inhibited control.

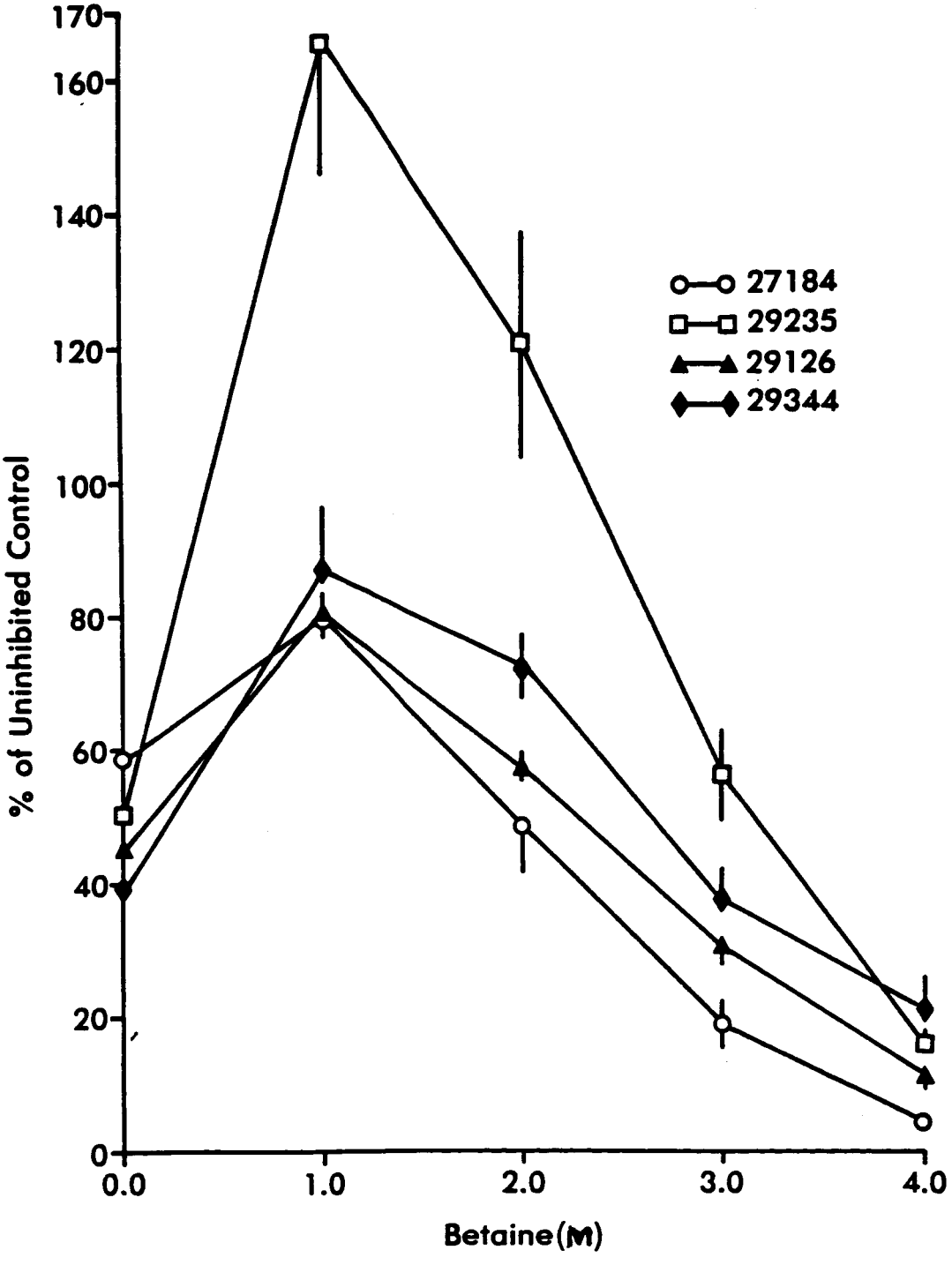

FIGURE 10. The effect of glycinebetaine of KCl inhibited glucose-6-phosphate dehydrogenase of Synechocystis and LPP spp. as a percent of uninhibited control.

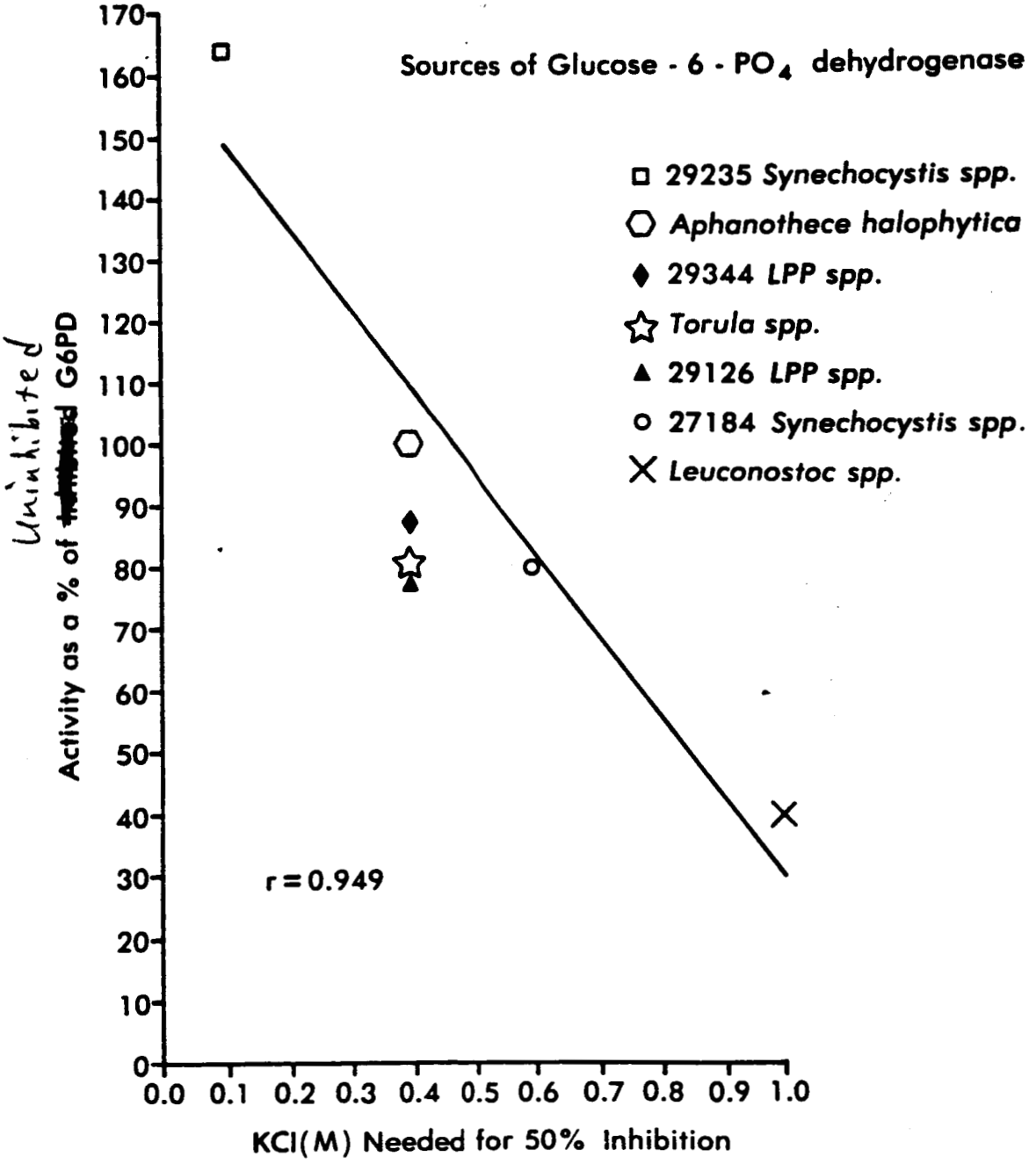

FIGURE 11. The effect of 1M glycinebetaine on KCl inhibited glucose-6-phosphate dehydrogenase from several genera.

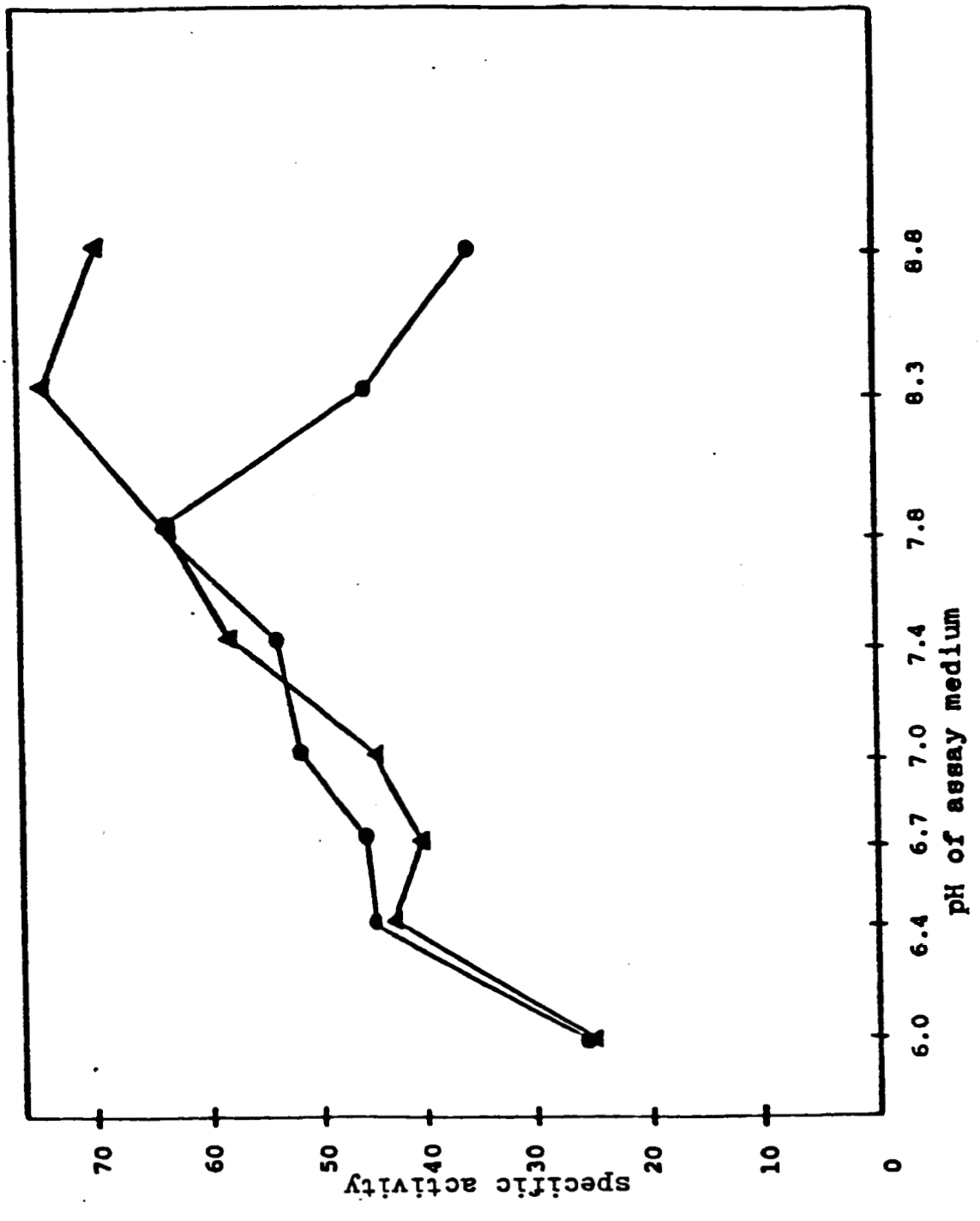

FIGURE 12. The effect of pH alone and with glycine-betaine in the assay medium on the specific activity (umol NADPH formed/min/mg protein) of G6PDH from Synechocystis spp. 27184. (with 1 M glycinebetaine; without glycine-betaine)

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