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The Utilization of Inorganic and Organic Phosphorous Compounds as Nutrients by Eukaryotic Microalgae: A Multidisciplinary Perspective: Part I

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THE UTILIZATION OF INORGANIC AND ORGANIC PHOSPHOROUS COMPOUNDS AS NUTRIENTS BY EUKARYOTIC MICROALGAE: A MULTIDISCIPLINARY PERSPECTIVE: PART 1

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I. INTRODUCTION

The present review is an attempt to link the relatively sophisticated experimentation of algal physiology and cytological research with the results of ecological survey and theory. When applied to phosphorus utilization, the former approaches lead to elaborate generalizations based upon relatively simplified experimental design, using laboratory cultured species with defined past histories, while exercising control over environmental variables. Unfortunately, the results of such experimentation cannot always be validly applied to natural aquatic ecosystems, as species can adapt to laboratory conditions which may not represent an accurate reflection of nature.

From an environmental standpoint, studies focusing on the utilization of phosphorus compounds by natural phytoplankton assemblages tend to be characteristically crude and often involve poor control over variables. Due to the complex nature of aquatic ecosystems, the conclusions reached may be unwarranted oversimplifications, which necessarily ignore small-scale metabolic phenomena. The specific concerns of the algal biochemist/physiologist are not always congruent with those of the phytoplankton field ecologist and modeler. Regrettably, this lack of cross-fertilization has tended to discourage a comprehensive interpretation of phosphorus nutrition of microalgae in dynamic ecosystems.

In this review we propose to integrate metabolic, cytological, and ecological aspects of the utilization of phosphorus compounds (Figure 1) in order to decipher a composite picture of the flow of phosphorus between eukaryotic microalgae and the total aquatic system. However, we must exclude certain aspects in order to limit our treatment within a reasonable scope. Among these peripheral aspects, we will largely ignore: (1) the geophysical and geochemical processes governing phosphorus availability, (2) the ecological effects of phosphorus-dependent eutrophication, (3) the prokaryotic

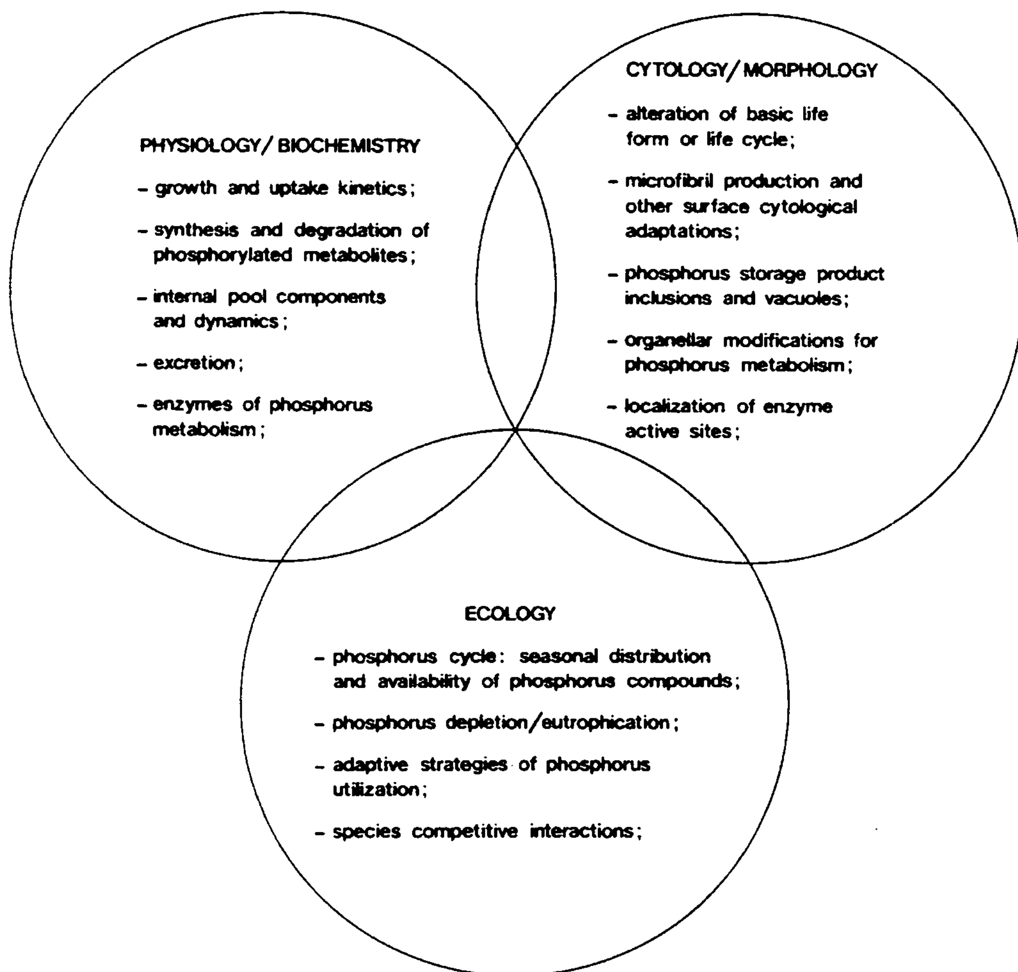

FIGURE 1. Integrated areas in phosphorus research necessary for the proper understanding of the flow of phosphorus in biological systems.

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Cyanophyceae or "blue-green algae", and (4) the multicellular, complex macroalgae. Geophysical and geochemical processes of sedimentation, water turbulence from winds and tides, and consequent mineral recycling need detailed consideration and do not belong strictly to the province of biology. While the study of eutrophication due to input of phosphorus compounds is an active and important research area, particularly with respect to lake management practices, we have chosen not to deal with applied aspects. Although the Cyanophyceae have been particularly well investigated for phosphorus utilization (see Lawry and Jensen[1] and Rigby et al.[2] and references cited therein), the absence of discrete intracellular membrane-bounded organelles and their ability to fix N_2 introduce metabolic complications which deserve special discussion beyond the scope of this review. The macroalgae do not merit inclusion here because they have been scantly studied for phosphorus utilization, and, at best, these investigations have been very superficial despite the complexity inherent in their multicellular structure.

The excellent reviews dealing with the use of phosphorus compounds by algae have been, with notable exceptions such as Hooper's[3] contribution on organic phosphorus compounds, rather polarized towards either a physiological/biochemical treatment,[4-7] or have an ecological bias.[8-10,469] By uniting the efforts of both groups, we hope to

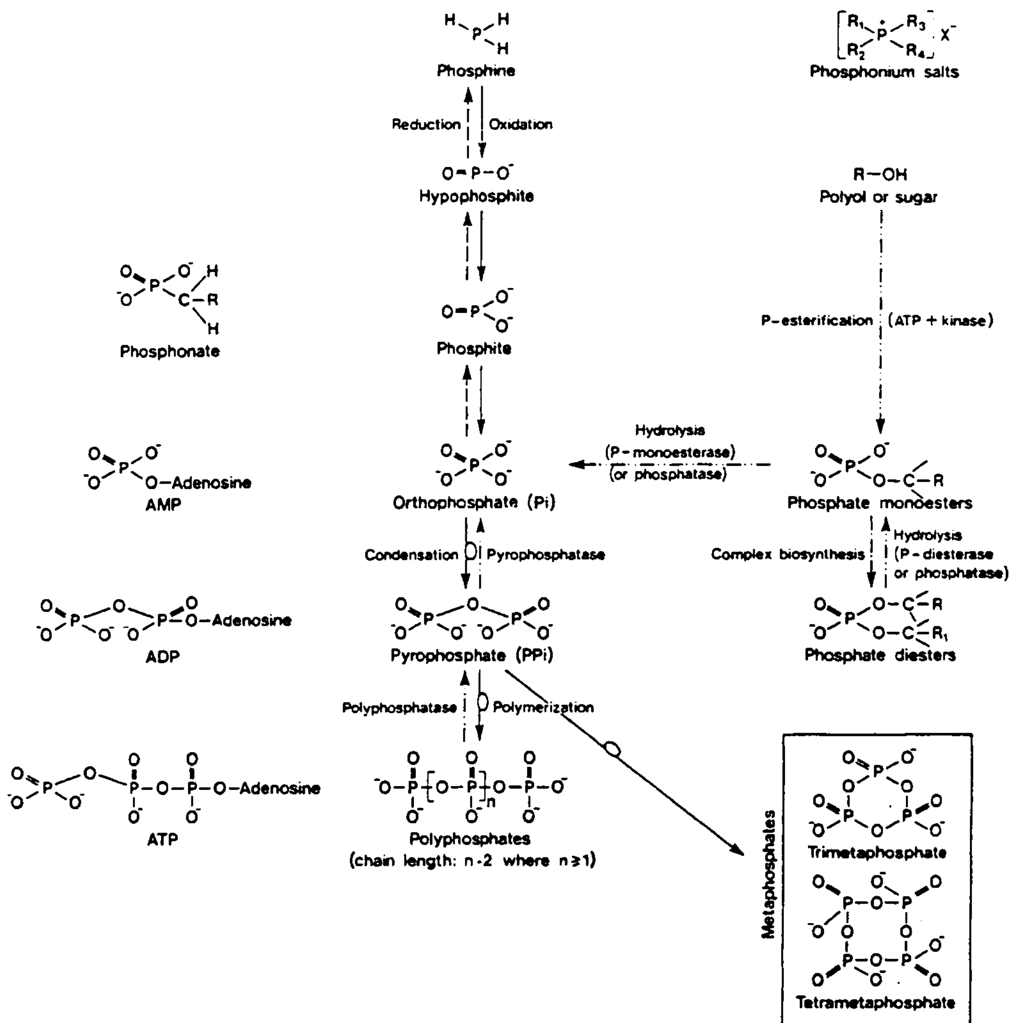

FIGURE 2. Formulation of some of the phosphorus compounds considered in this review. Symbols used: AMP — adenosine 5'-monophosphate; ADP — adenosine 5'-diphosphate; ATP — adenosine 5'-triphosphate; R, R_1, R_2, R_3, R_4 denote organic residues or groups which may be similar or dissimilar; X — halide ion. The *phosphate monoesters* of biochemical importance include the polyol or sugar phosphates, mononucleotides, O-phosphoserine, O-phosphocholine, phosphoproteins, etc. The *phosphate diesters* of biochemical importance include phospholipids, cyclic AMP, dinucleotides and polynucleotides, DNA, RNA, etc.

produce a holistic picture of the relationship between phosphorus metabolism in eukaryotic microalgae and primary production. Such an objective was commendably achieved by Fogg[11] in a concise review, which unfortunately is incomplete in details of intracellular phosphorus metabolism and related cytological manifestations.

II. EXTRACELLULAR PHOSPHORUS COMPOUNDS AVAILABLE FOR UTILIZATION

A. Environmental Phosphorus Compounds and Their Characterization

Of the large variety of P compounds described in chemistry texts, we shall consider only those known or likely to occur in the aquatic environment (Figure 2). From this list, it appears that orthophosphate, pyrophosphate, polyphosphate, and their respective

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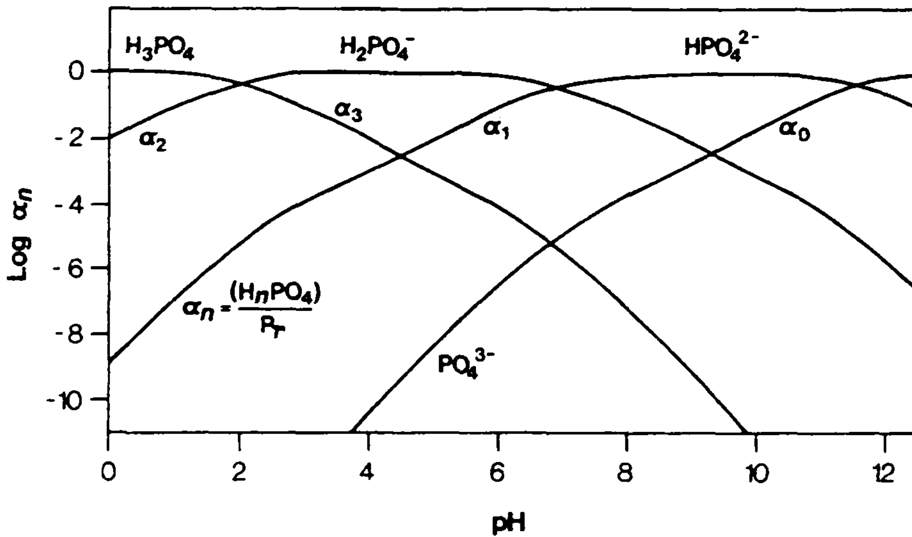

FIGURE 3. Orthophosphate ionic species activity as a function of solution pH. P_T represents total phosphate, α denotes ion activity. (From Stumm, W. and Morgan, J. J., *Aquatic Chemistry*, Wiley-Interscience, New York, 1970, 514. With permission.)

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organic esters are the commonest. Reduced P compounds, such as phosphite, appear to be rarely reported in the biological literature, presumably because they were overlooked in reducing environments where they might be expected to play a role in a phosphorus oxidation-reduction cycle similar to those known for sulfur and nitrogen. In keeping with this concept, both aerobic and anaerobic utilization of hypophosphite and phosphite have been recorded for certain soil bacteria,[47,48] and this utilization appeared to occur by the stepwise enzymatic oxidations to phosphite and phosphate esters depicted in Figure 2. Similar studies have not been reported for any eukaryotic alga. Although theoretically possible, neither the metabolic production nor utilization of phosphine and phosphonium compounds is known for any bacterium or alga. In view of these circumstances, this section will focus primarily on the characterization of exogenous inorganic phosphates and their organic derivatives. One must bear in mind that the above mentioned organic derivatives do not, strictly speaking, include the phosphonates: organophosphorus compounds with a carbon-phosphorus bond[471] (see Figure 2). Their widespread (probably ubiquitous) occurrence in algae,[12,13] protozoa,[14] and a wide assortment of marine invertebrates[15-17] indicates a high probability that these extracellular compounds with intact C-P bonds will be found in the water.

Chemical and physical methods of determining so-called "phosphate" have indicated that total phosphate in aquatic systems consists of a rather heterogeneous group of inorganic phosphate species[29,254] and phosphorus-containing compounds.[18-22] Although physiological ecologists frequently discuss "inorganic phosphate" without regard for speciation, it is crucial to consider the specific forms available for microalgal utilization. Since ionic speciation is determined to a large extent by pH, it is important to note that within the typical aquatic pH range 5 to 9, the dominant free orthophosphate species are HPO_4^{2-} and $H_2PO_4^-$ (see Figure 3). As pH increases from 7.0 to 8.5, the typical seawater range, the ionic equilibrium shifts such that the ratio $[H_2PO_4^-]:[HPO_4^{2-}]:[PO_4^{3-}]$ decreases; nevertheless, HPO_4^{2-} retains its dominance over the other free ionic species.[254] However, as indicated in Figure 4, orthophosphate (P_i) ions form metallo-phosphate complexes and chelates, such that free orthophosphate represents less than one third of the total inorganic phosphate in seawater. Other forms of inorganic phosphate illustrated

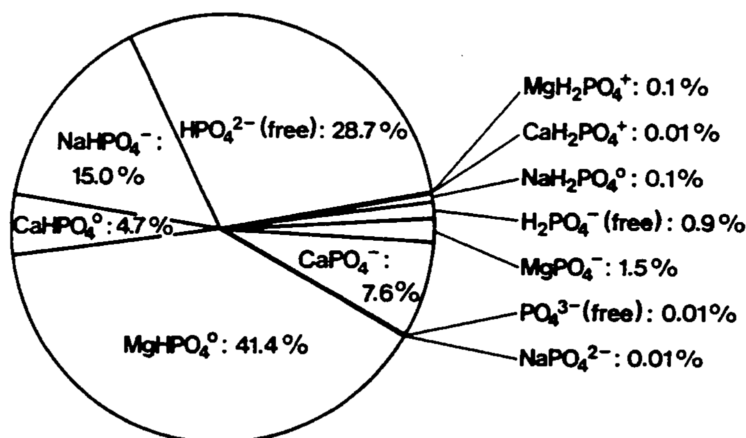

FIGURE 4. Phosphate speciation in seawater at 34.8‰ salinity, 20°C, and pH 8.0. (From Atlas, E., Culbertson, C., and Pytkowicz, R. M., *Mar. Chem.*, 4, 243; 1976. With permission.)

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in Figure 2, e.g. pyrophosphate, metaphosphate, etc., are similarly subject to cation complexation reactions, which may affect their biological availability. Within a given aquatic system, the ratio of inorganic phosphate species is a function of not only pH, but the concentration and relative proportions of cation species and competitive complexing anion ligands, as well as temperature and pressure. Complexation of inorganic phosphates with metal cations, especially Mg^{2+} and Ca^{2+}, resulting in precipitation and surface adsorption onto inorganic particulates, may remove substantial quantities of phosphate from the biologically available nutrient pool. Since coprecipitation of P_i with Ca^{2+} is enhanced with increasing pH in lake water, such as may occur due to high photosynthetic activity,[442] cation interactions may be particularly important in determining phosphate availability in the cellular microenvironment, especially when cell densities are high. Although precipitation of P_i upon Fe-complexes has been cited as a mechanism whereby P_i may become biologically unavailable,[657] for aerobic water from an acidic lake, the interaction between colloidal organic matter and iron was found to decrease phosphate adsorption onto Fe-organic complexes, thus freeing P_i for phytoplankton utilization.[656]

Several investigations[23-27] have attempted to characterize extracellular P-containing compounds into arbitrary categories to properly assess their relative environmental importance. It is of paramount concern to the phytoplankton ecologist to establish the cycling of phosphorus between these categories, and to be able to discriminate between biologically utilizable and nonutilizable forms. The analytical differentiation of the forms of phosphorus present in aquatic ecosystems is usually based upon the relative reactivity with molybdate, ease of hydrolysis, and particle size.[3,21] Those fractions capable of reaction with acid-molybdate to form a heteropoly blue complex are generally termed *reactive phosphate*.[28] Perchloric acid treatment and filtration through a 0.45-μm filter are used to establish ease of hydrolysis and particle size, respectively. It is important to realize that these fractions are operationally defined by the choice of analytical method, and may not represent *in situ* significance with respect to ecological P compartments.

The nomenclature applied to the phosphate fractions requires further standardization in the literature, as terms have sometimes been used carelessly. For example, "soluble reactive phosphate" (SRP) (which passes through a 0.45-μm membrane filter and reacts

with the molybdate reagent), is occasionally used synonymously with "low molecular weight inorganic phosphate", or even "orthophosphate", however, interchanging such designations should no longer be considered excusable. Furthermore, the so-called "soluble organic phosphate" fraction may include considerable amounts of pyrophosphate and polyphosphate, which are technically inorganic.[3] In our nomenclature, the generic term "polyphosphates" covers the cyclic metaphosphates and the linear polyphosphates (see Figure 2), as well as the "ultraphosphates" composed of cross-linked condensed chains of linear polyphosphates, because certain cyclic and linear forms may be artifacts of the extraction procedure.

Based upon the molybdenum blue technique, Strickland and Parsons[21] discriminated between the following phosphorus fractions in aquatic ecosystems: (1) soluble inorganic reactive phosphate (orthophosphate); (2) soluble organic reactive phosphate — low molecular weight organics, such as easily hydrolyzable sugar phosphates; (3) soluble organic unreactive phosphate — not readily hydrolyzable; (4) soluble enzyme-hydrolyzable phosphate — sugar phosphate, linear inorganic polyphosphates, etc.; (5) soluble polyphosphate — inorganic and organic polyphosphates; (6) particulate inorganic unreactive phosphate; (7) particulate organic unreactive phosphate; and (8) particulate reactive phosphate — including both inorganic and organic phosphorus in particulate form. Unfortunately, the characterization of inorganic and organic phosphorus fractions within a scheme such as this is complicated by analytical difficulties.[472-478]

It is now clear that the conventional molybdenum reactivity method of orthophosphate determination in natural water samples may result in severe overestimation (see References 3, 445–446, 473, 478, 533, 671 and references therein). Current experiments on the phosphomolybdate method,[671] indicate that a relatively high fraction of P_i may be "hydrolyzed" from bound sources and returned to solution within seconds, after the addition of acid molybdate. Such problems remain acute in spite of recent procedural modifications to minimize silicate and arsenate interference, to reduce hydrolysis of polyphosphate and organic phosphorus compounds, and to prevent acidic dissolution of metallo-phosphate complexes.

The use of $^{32}P_i$ tracer techniques[96,479] has revealed a discrepancy between the ^{32}P-labeled compartment that organisms and ion exchange resins discriminate as P_i, and the compartment measured chemically as P_i using the molybdenum blue methodology. Concentrations of P_i determined by chemical analysis were generally much higher than estimates from tracer bioassay measurements. From the lack of binding of ^{32}P label to hydrous zirconium oxide in a summer sample of lake water, Rigler[34] postulated the existence of an additional unidentified P compartment that had a stronger binding capacity for P_i than hydrous zirconium oxide; this compartment has since been suggested to represent colloidal phosphorus.[19,20,24] Subsequent investigations[473,478] indicated that reagents of the molybdenum blue method induce acid hydrolysis of organic phosphorus compounds. This release of P_i, mainly from colloidal phosphorus, and the magnitude of the error depends on the ratio of colloidal phosphorus to P_i. The significance of the chemical analysis overestimate is dependent on the ultimate use of the data. If an estimate of biologically available dissolved phosphorus is required, then chemical analysis estimates may not be seriously misleading, in spite of the departure from true P_i concentrations. Considerable amounts of molybdate reactive P, other than P_i, may be degraded by the activity of bacterial and algal phosphatases (see Section IV.), or are perhaps directly utilized, and are thus ultimately available for microalgal P nutrition.

Recently alternative enzymatic methods for determining aquatic P_i concentrations have been proposed.[445,446] One procedure involves the formation of 1,3-DPGA from P_i and glycerate-3-P in the presence of glyceraldehyde phosphate dehydrogenase and NAD;[445] the other method utilizes the ability of P_i to act as a competitive inhibitor of

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alkaline phosphatase hydrolytic reactions.[446] When compared with the molybdenum blue method, enzymatic determinations gave lower results, suggesting that only the P_i fraction was measured.

Measurement of total phosphorus in aqueous samples commonly involves a hot acid-oxidation type digestion procedure, although other methods such as UV irradiation are often used.[21] Recently an improvement in the quantitative conversion of organically bound phosphorus to P_i has been obtained by using autoclave-promoted persulfuric acid digestion.[480]

The differentiation of aquatic P compounds into the neat analytical categories proposed by Strickland and Parsons[21] is not always practical to achieve. The filter pore size (0.45 μm) and filtration pressures (200 to 600 mm Hg) traditionally used to discriminate between dissolved and particulate phosphorus components in water samples appear to be inadequate to retain colloidal phosphorus complexes on the filter.[25] Furthermore, high vacuum pressure may contribute to cell lysis, as well as loss to the filtrate of phosphorus bound to microfibrils at the cell surface.[20,35] Pearl and Lean[20] suggested that, especially for Cyanophyceae, the microfibrils projecting from the cell surface are fragile and may easily be broken off and sucked through a 0.45-μm filter. Since ultrafiltration at low pressure may retain colloids,[25-26] previous interpretations of the excretion and utilization of "dissolved organic phosphate"[42] are complicated and may be partially invalid. Certain researchers[656] have utilized dialysis to discriminate between colloidal and truly dissolved matter in lake water; both P_i and colloidal P will pass through a 0.45-μm membrane filter using standard techniques, however the colloidal fraction can be retained within 4.8 nm dialysis tubing (macromolecules $>$ mol. wt. 12,000 will not dialyze).

In a new approach involving colorimetric analysis, ^{32}P radiotracer, membrane filtration, as well as ultrafiltration or Sephadex gel filtration, Lean[24] demonstrated the existence of four principal P components in lake water: a particulate fraction, an unidentified low molecular weight phosphorus compound (XP), P_i, and colloidal phosphorus. When freshwater algae were incubated with $^{32}P_i$, Lean and Nalewajko[26] obtained further evidence supporting the view that the so-called "dissolved organic phosphate" (DOP) of lake water may be composed of a truly dissolved component of relatively low molecular weight compounds and a colloidal fraction. Examination of filtrate from the freshwater diatom, *Navicula,* obtained by Sephadex-column fractionation, showed that the molecular weight of compounds in the eluted fractions increased with algal incubation time. An unidentified low molecular weight phosphate (XP) was eluted after 21 to 89 hr incubation, followed later by larger molecular weight phosphorylated compounds, and eventually substantial quantities of colloidal phosphate. According to Lean's hypothesis,[24] XP is excreted and combines with a colloidal substance.

An encouraging attempt to partially characterize DOP compounds on the basis of susceptibility to enzymatic hydrolysis was initiated by Herbes et al.[166] These authors used commercial preparations of alkaline phosphatase, phosphodiesterase, and a crude preparation of nonspecific phytase to hydrolyze DOP in lake water samples. No significance quantity of P_i was released from concentrated lake samples treated with either or both alkaline phosphatase or phosphodiesterase, however, phytase hydrolyzed up to 50% of the DOP. Circumstantial evidence from kinetics of hydrolysis and gel filtration suggested that the phytase hydrolyzable fraction may be subdivided into a low molecular weight fraction (possibly inositol polyphosphates), and a high molecular weight fraction, possibly composed of protein, lipid, and/or fulvic acid complexes of inositol phosphates.

Although in most marine and freshwater ecosystems P_i may represent the predominant soluble phosphate fraction,[21] many waters are characterized by high organic production

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and/or low supply rates of P_i, and as a result organic phosphorus compounds are dominant. For example, in Japanese coastal waters, 87 to 98% of the total phosphate was determined to be organic in nature.[30] For a relatively oligotrophic tropical oceanic area with substantial cyanophycean (cyanobacterial) biomass, the concentration of organic phosphates was five to ten times greater than the inorganic fraction.[31]

Rivkin and Swift[32] measured ambient inorganic phosphate concentrations of less than 0.1 μM in the Caribbean and Sargasso Seas, while the organic phosphate concentrations were often higher. For Departure Bay, British Columbia (Canada), more than 75% of the total phosphate in summer surface water was organically combined.[33] Strickland and Austin[33] reported similar high surface concentrations for other northeastern Pacific coastal regions, with the proportion of organic phosphates increasing along with the standing stock of phytoplankton. By contrast, in the aphotic zone of the same area (at depths of 1000 to 2000 m), less than 25% of the total phosphate was organic, confirming that large amounts of organic phosphorus compounds near the surface are correlated with high primary productivity. Downes and Paerl[36] separated SRP compounds from lake water into two fractions, consisting of a reactive high molecular weight fraction (RHMWP) of M.W. > 5000, presumably organically bound, and a fraction corresponding to P_i. In eutrophic New Zealand lakes, RHMWP may represent 70 to 80% of the total SRP compounds, far exceeding the P_i concentration at certain times of the year.[37] A large amount of the phosphorus in lakes may be bound to nonliving components, which usually exceed the living biomass components.[35]

The weakest aspect of the research discussed above and the ecological P-compartment models subsequently developed[23-26] (see Section VII) is the lack of definitive identification of any of the "organic phosphates" claimed to be produced and utilized by algae. Certainly, it is helpful to know that phosphatase-hydrolyzable low molecular weight organic phosphates are phosphate esters, but it would be more reassuring to know the chemical structure of the esterified organic moiety, which could be an alcohol, sugar, nucleoside, or a totally novel product. For instance, it is tempting to speculate that the (XP) component of Lean's model[23-24] may be similar, if not identical, to the "soluble enzyme-hydrolyzable phosphate" of Strickland and Parsons,[21] but the unknown nature of the organic residues makes comparisons uncertain. There is also the question of the "soluble organic unreactive phosphate" fraction of Strickland and Parsons[21] said to be refractory to both enzymatic and acid hydrolysis and therefore unlikely to be composed of phosphate esters. Addition of 2-aminoethylphosphonic acid, a P metabolite identified in several microalgae,[12,13] to seawater samples low in SRP revealed that this compound was neither molybdate reactive nor subject to hydrolysis by commercial alkaline phosphatase.[655] Therefore, we suggest that the "soluble organic unreactive phosphate" fraction is likely to contain the "missing" phosphonates discussed earlier in this section.

To fully appreciate the ecological significance of operationally defined phosphorus compartments in nature, we require further knowledge regarding the chemical speciation of their respective components. Presumably, the same phosphorylated compounds which have been synthesized intracellularly will become temporary exogenous pools upon excretion or cell lysis, however, their persistence and precise chemical nature have not been adequately considered. If microalgal cells are capable of physiological discrimination in their utilization patterns, physiological ecologists must attempt to refine techniques to achieve a comparable scale of resolution.

B. Sources of Extracellular Phosphorus Compounds

Soluble P compounds in marine and freshwater ecosystems are derived from a number of nonbiological sources, including terrigenous runoff and regeneration from sediments. In addition to this, there may be substantial release from suspended colloidal and

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particulate matter in the water column.[443] Further contributions of P_i to lakes have been recently attributed to direct atmospheric input from rain, dust, or snow,[38,481] and to gradual *in situ* dissolution of submerged apatite mineral deposits.[39,40]

Biogenic additions of DOP to natural waters occur as a result of lysis following cell death. In addition to this, considerable recycling may take place through zooplankton[41,441,444] and benthic invertebrate[440] excretion, and the release of DOP by actively metabolizing algal[26,42] and bacterial[43] cells.

In culture, a broad range of phytoplankton species are capable of excreting DOP, particularly as the growth rate declines.[26,42] The appearance of high alkaline phosphatase activity during declining growth phase of phytoplankton blooms is often accompanied by a corresponding increase in DOP in the water.[10] Lean and Nalewajko[26] measured significant DOP excretion from four species of freshwater phytoplankton axenically cultured at approximately environmental P_i concentrations. For several marine algae in axenic culture, particularly diatoms and chlorophytes, Kuenzler[42] noted that maximum DOP excretion occurred at the beginning of the stationary phase of growth, thereby indicating that excretion was not attributable to lysis of senescent or dead cells. Since this export of DOP may be in excess of 20% of the total assimilated phosphorus, it may contribute substantially to the phosphate pool in the natural environment. However, too little is known of the chemical structure of such excretions[252] apart from vague suggestions that they are composed of monophosphate esters.[42] Evidence that a beginning has been made in this direction comes from recent reports on algal release of cyclic-AMP,[198-200,202] which may, however, constitute only a minor fraction of the total excreted DOP. Nevertheless, up to 85% of the total cyclic-AMP synthesized by the chlorophyte, *Chlamydomonas,* was excreted into the growth medium,[199] while the chrysophyte, *Poteriochromonas,* excreted up to 20 times the amount measured in the cells.[198] There is no doubt that precise identification of the other components of DOP excreted by algae is required for better understanding of the reasons for this apparently wasteful process.

As well as organic phosphate esters, it is likely that phosphonate compounds are added to aquatic environments through excretion and organic degradation following cell lysis. In addition to the potentially large contribution made through organic syntheses, particularly by plankton production of amino-phosphonic acid derivatives,[13] and the subsequent release of these compounds, substantial quantities of synthetic organophosphonates may be added to natural systems as pesticide residues and industrial wastes.[44] The significance of such addition has not, as yet, been subject to detailed quantitative investigation.

C. Utilizability Evidenced by Cellular Growth

As a general observation, assimilable P compounds are solubilized prior to crossing the plasmalemma boundary. Presumably, facultative phagotrophic phytoplankton species are able to assimilate phosphorus nutrients following intracellular degradation of ingested particulates, and pinocytosis may also account for some incorporation of macromolecular P nutrients which are not truly dissolved, but such acquisition has not been adequately described. In many dinoflagellate species, vacuole-like pusules, often with abundant vesicular invaginations, may facilitate transfer of nutrients across the plasmalemma to internal vacuole(s), although other functions, including excretion and osmoregulation, have also been attributed to this organelle.[393-395] It was formerly believed that soluble inorganic phosphate, consisting of a single P_i residue, was the only significant biologically available form, due to transport considerations. Smith et al.[40] showed that the leaching of P_i from apatites at environmental pH values was sufficient to maintain growth of mixed algal/bacterial assemblages in the laboratory. However,

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studies which have considered only the concentration of P_i alone have shown this to be an underestimate of total available phosphorus, which does not, therefore, adequately reflect the potential nutritional status of the cells.[46]

Alternative nutritional sources of inorganic phosphate include pyrophosphate, which is known to support growth of the prymnesiophyte, *Prymnesium parvum*, equivalent to growth when P_i is used.[49] Three species of dinoflagellates, including the cultured symbiont, *Symbiodinium (Gymnodinium) microadriaticum*, achieved good growth upon either P_i or pyrophosphate over a wide concentration range of 30 to 300 μM.[50] For freshwater chlorophytes, such as *Chlorella* spp., utilization of linear pyrophosphates consisting of up to 55 residues was demonstrated.[51] These authors[51] also established the presence of a hydrolytic enzyme associated with the cell wall of *C. ellipsoidea* which would facilitate exogenous pyrophosphate utilization.

While there is no conclusive evidence of low molecular weight organic phosphates being transported directly into eukaryotic algal cells, there is at least one reported laboratory observation[52] of transport and metabolism of G-6-P by a cyanophycean species, which was apparently not mediated by extracellular phosphatase. Since rather high external concentrations (2 to 10 μM) were used, it would be unwise to conclude that similar transport occurs in eukaroytic microalgae at lower environmentally realistic levels. However, there is evidence to suggest that natural populations of marine bacteria can take up dissolved cyclic-AMP directly from coastal seawater.[167]

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Data accumulated from many experiments strongly support the contention that utilization of exogenous organic P compounds through enzymatic hydrolysis of terminal phosphate groups[5,53-57] (see Section IV) is important in the nutrition of many species. In an early experiment, Chu[58] showed that the marine diatom *Phaeodactylum tricornutum* grew on either inositol hexaphosphate or glycerophosphate as a P source. Subsequently it was discovered that marine species from the Prymnesiophyceae[59] and Dinophyceae[60] were capable of growth upon a variety of phosphate esters, including glycerophosphate and adenylic, guanylic, and cytidylic acids. This work was further substantiated for other marine species which grew well on glycerophosphate, G-6-P, AMP, CMP, and GMP.[54-55,61] The freshwater chrysophyte, *Dinobryon sertularia*, was also grown on glycerophosphoric, uridylic, or adenylic acids as the sole phosphorus source.[465] It is likely that, in the absence of P_i, glycerophosphate (α or β isomers, or both together) may be utilized by all the algae listed in Table 1, since this organic phosphate has often been recommended as a P source in media designed for general culture of marine algae,[374,483,484] or special media for certain freshwater algae.[367] At least for *Chlorella* spp., phospholipids[62] are also potential P substrates for growth. Therefore, it appears that in addition to P_i, a broad spectrum of dissolved inorganic and organic phosphorus sources are capable of sustaining growth, and that utilization of such compounds is relatively widespread among the microalgae (see Table 1).

The above cited evidence clearly demonstrates the ability of phytoplankton to grow upon organic phosphate sources in culture, particularly when they are provided with such exogenous nutrients in high concentration. However, the contribution of such compounds to phytoplankton nutrition in aquatic environments is more difficult to establish. Kuenzler[42] and Lean and Nalewajko[26] indicated that excreted organic phosphate appeared to be reutilized by the algae themselves, and that the excretion-reutilization was maximal when the medium was nearly depleted of P_i. However, as pointed out by Nalewajko and Lean,[469] results of experiments in which $^{32}P_i$ was used to determine that algae take up DOP[42] must be viewed with caution. Radioactively labeled DOP compounds or colloids excreted by algae growing on $^{32}P_i$ may pass through a 0.45-μm filter. When algae are grown in this filtrate and then refiltered at a later time to determine "uptake", some of the colloidal P may be retained by the filter and consequently mistaken for uptake.

The organically bound Sephadex-separated RHMWP fraction from lake water, when added to P-starved *Chlorella* cultures, was capable of sustaining good growth after 96 hr.[19] Further experiments by White and Payne[37] using lake water showed that the RHMWP fraction supported growth of *Chlorella* equivalent to that upon P_i, although not all of the fraction was utilized.

Although the RHMWP fraction must undoubtedly undergo hydrolysis for uptake to occur, this process seems to be initiated only when P_i is limiting.[19] In view of such potential for organic phosphate utilization, and given the rapid turnover among phosphorus compartments (see Section VII), it seems indefensible to consider external P_i as the only biologically available P source.

Although phosphonate compounds are highly resistant to degradation, the intermediary role of bacteria may serve to render such bound phosphorus ultimately available to phytoplankton. This view is supported by the discovery of bacterial utilization of phosphonates as a P source for growth in culture,[63] but there are no published reports on either the occurrence and distribution, or algal utilization of extracellular phosphonates dissolved in lake or seawater. Obviously, these compounds have been largely ignored by both ecologists and algal physiologists, and a detailed investigation is long overdue.

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III. ORTHOPHOSPHATE UPTAKE

A. Mechanisms

1. The Transmembrane Electrochemical Potential Gradient

The plasmalemma presents a semipermeable barrier to the entry of nutritionally significant ions. Although the obvious primary functions of such a structure are to maintain cellular integrity and to restrict the loss of intracellular metabolities, the ability to selectively transfer exogenous solutes, often against a concentration gradient, is ultimately no less important. Regardless of the membrane model adopted for consideration, the Davson-Danielli-Robertson protein-lipid bilayer,[64] or the more currently favored Singer-Nicolson fluid-mosaic model[65] with globular proteins embedded in a dynamic lipid bilayer, ion transport can be considered in a stepwise manner. The steps involved include: (1) ion diffusion into the cell free-space (the region immediately exterior to the plasmalemma); (2) adsorption and binding of the ion to plasmalemma surface sites; (3) entry of the ion into the plasmalemma; (4) the passage of the ion through the plasmalemma; and (5) intracellular release of the ion.

On the basis of studies with purified membrane lipids, it can be concluded that "membrane-like" lipid bilayers should have low intrinsic permeability and ion-transport affinities. In particular, highly soluble ions, such as P_i, have a high affinity for the aqueous phase, thus, transport would require the endergonic disruption of hydrogen bonding before membrane entry. For all ions considered, the permeability of algal membranes was greater than that anticipated for simple lipid bilayers.[66]

Most current membrane transport models regard the plasmalemma as a mosaic of negatively and positively charged areas,[67] with an overall positive external charge relative to the cell interior. With few exceptions algal cells pump out Na^+ ions while actively taking up K^+ and Cl^-, rendering the cytoplasm and vacuole electronegative with respect to the external medium.[461,462] A transmembrane electrochemical gradient is established by ionic fluxes and Donnan charge activity. The Donnan charges arise through dissociation of H^+ ions from macromolecules which are fixed intrinsically in the cell wall, membranes, or cytoplasm, or are at least not freely diffusible through the plasmalemma. Although membrane surface charges tend to facilitate anion binding, negative internal Donnan charges and progressive membrane hyperpolarization due to anion influx act to reduce anion uptake through electrostatic repulsion. Furthermore, dissociation of H^+

Table 1
LIST OF PHOSPHORUS COMPOUNDS REPORTED UTILIZABLE BY EUKARYOTIC MICROALGAE AS ALTERNATIVE NUTRIENTS IN THE ABSENCE OF ORTHOPHOSPHATE

Algal species	Phosphorus compounds[a]	Ref.
Bacillariophyceae (Diatoms)		
Cyclotella cryptica	G-6-P	54
Fragilaria capucina	GP	374
Phaeodactylum tricornutum	AMP, αGP, G-6-P, phytin, PP,	54, 55, 58
Skeletonema costatum	Phytin	58
Thalassiosira weissflogii	AMP, αGP, G-6-P	55
Chloromonadophyceae (Rhaphidophyceae)		
Chattonella akashiwo	GP	373
Chattonella inlandica	GP, GMP	372
Chlorophyceae		
Chlamydomonas reinhardi	F-1-P, βGP, G-1-P, G-6-P, meta-P,, P-glycol, R-5-P	358
Chlamydomonas spp. (clones 0–5, F–17)	G-6-P	54
Chlorella ellipsoidea	Phospholipids	62
Chlorella pyrenoidosa	PP,, poly-P,	51
Chlorella sp. (clone 580)	G-6-P	54
Volvox globator, V. tertius	GP	367
Chrysophyceae		
Dinobryon sertularia	AMP, GP, UMP	465
Micromonas[b] sp.	AMP, CMP, GMP, βGP	61
Ochromonas danica	βGP, G-1-P, G-6-P	184
Olisthodiscus luteus	AMP, CMP, FDP, GP, G-6-P, meta-P,, PP, RMP, UMP	365
Synura sp.	GP	367
Cryptophyceae		
Rhodomonas lens	G-6-P	54
Dinophyceae		
Amphidinium cartereae, A. rhynchocephalum	AMP, CMP, GMP, GP	60
Ceratium hirundinella	AMP, ATP, CMP, GMP, βGP	359
Crypthecodinium cohnii, Gonyaulax polyedra	GP	364, 643
Gymnodinium nelsoni	G-6-P	54
Gyrodinium californicum, G. resplendens, G. uncatenum	AMP, CMP, GMP, GP	60
Heterocapsa niei	GP	366
Katodinium rotundatum	AMP, CMP, FDP, GP, G-6-P, meta-P,, PP, RMP, UMP	365
Prorocentrum micans	AMP, CMP, FDP, GP, G-6-P, meta-P,, PP,, UMP	365
Protogonyaulax tamarensis	GP	371
Pyrocystis noctiluca	AMP, βGP, G-1-P, G-6-P, phytin, PP,, poly-P,	557
Symbiodinium microadriaticum	AMP, CMP, GMP, GP	644
Woloszynskia limnetica	GP	367
Euglenophyceae		
Phacus pyrum	GP	367
Eustigmatophyceae		
Clone *GSB Sticho*	G-6-P	54
Monodopsis subterranea	AMP, FDP, GP	379
Prasinophyceae		
Pyramimonas sp. (clone Pyr 2)	G-6-P	54

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Table 1 (continued)
LIST OF PHOSPHORUS COMPOUNDS REPORTED UTILIZABLE BY EUKARYOTIC MICROALGAE AS ALTERNATIVE NUTRIENTS IN THE ABSENCE OF ORTHOPHOSPHATE

Algal species	Phosphorus compounds[a]	Ref.
Prymnesiophyceae (Haptophyceae)		
Emiliania huxleyi	AMP, CMP, GMP, GP, G-6-P	54, 59
Hymenomonas carterae	AMP, αGP, G-6-P	55
Hymenomonas sp.	AMP, CMP, GMP, GP	59
Isochrysis galbana	AMP, CMP, GMP, GP, MEP, RMP	363
Ochrosphaera neapolitana	AMP, CMP, GMP, GP	59
Pavlova gyrans	AMP, CMP, GMP, GP	59
Pavlova lutheri	AMP, CMP, GMP, GP, MEP, RMP	363
Phaeocystis pouchetii [b]	Phytin	58
Prymnesium parvum	AMP, CMP, GMP, GP, MEP, PP_i, RMP	49, 363
Syracosphaera sp.	AMP, CMP, GMP, GP	59
Rhodophyceae		
Asterocytis ramosa	GP	382
Porphyridium aerugineum	GP	383, 384
Tribophyceae (Xanthophyceae)		
Vaucheria dichotoma	αGP, βGP	378

[a] Abbreviations used: AMP — adenosine-(3′ or 5′)-monophosphate; CMP — cytidine-(3′ or 5′)-monophosphate; F-1-P — fructose-1-phosphate; FDP — fructose-1,6-diphosphate; GMP — guanosine-(3′ or 5′)-monophosphate; GP — glycerophosphate (mixture of α and β isomers; where either isomer was tested individually, it is indicated as αGP or βGP); G-1-P — glucose-1-phosphate; G-6-P — glucose-6-phosphate; MEP — monoethylphosphate; meta-P_i — metaphosphate; P-glycol — phosphoglycolate; phytin — myoinositol hexaphosphate; PP_i — inorganic pyrophosphate; poly-P_i — inorganic polyphosphate; RMP — riboflavin monophosphate; R-5-P — ribose-5-phosphate; UMP — uridine-(3′ or 5′)-monophosphate.
[b] This genus belongs strictly to the Prasinophyceae,[369] but the alga under investigation was stated to be a chrysomonad;[61] it appears that the alga was either misidentified or misclassified.

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ions from fixed carboxyl groups associated with cell surface carbohydrates gives the cell wall a net negative charge.[488] How, then, can we account for high intracellular P_i levels and relatively rapid uptake against an electrochemical gradient?

The movement of ions through the plasmalemma is subject to physical driving forces, including: (1) the concentration gradient existing across the membrane and (2) the presence of an electrochemical potential gradient established by differential passive ion diffusion, Donnan charge interactions, and active ion transport. The electrochemical driving force across the membrane, or Nernst potential (E_j) attributable to a given ion, j, at passive flux equilibrium may be described by the Nernst equation:

$$E_j = \frac{R\,T}{z_j\,F} \ln a_j^o / a_j^i \qquad (1)$$

where R = the universal gas constant (joules deg^{-1} $equivalent^{-1}$), T = temperature in absolute degrees (° K), F = the Faraday constant (coulomb $equivalent^{-1}$), z_j = the valence of the ion, and a_j^o and a_j^i represent, respectively, the ion activity external and internal to the membrane. The net transmembrane electrochemical potential gradient at equilibrium is, therefore, the sum of E_j values for all ions involved, modified by the permeability coefficient (P_j) for each ion. Due to active transport the measured transmembrane potential may differ significantly from the Nernst potential established by passive influx. A detailed consideration of the biophysical ion interactions is beyond the scope of this

review, therefore, the reader should refer to more comprehensive treatments in the literature.[66,68-71,455,461-463,676] We offer only a cursory overview of the major ion transport mechanisms, with references to their possible involvement in P_i ion uptake in microalgae.

2. Passive Diffusion

Passive diffusion is considered to be a "downhill" transfer, with solutes moving along an electrochemical gradient only until thermodynamic equilibrium is achieved, and without the expenditure of metabolic energy. Occasionally it is mistakenly believed that passive diffusion of ions can only proceed in response to a concentration gradient, however, as the Nernst equation implies, if the electrochemical gradient is favorable, net diffusion can occur even against an unfavorable concentration gradient. Since no hypothetical carriers or discrete numbers of transport binding sites are involved in passive diffusion mechanisms, they are nonsaturable, with solute flux proportional to the electrochemical gradient. Consequently, metabolic inhibitors have no direct effect on diffusive flux. Given that internal P_i concentrations may be several orders of magnitude greater than typical external concentrations in the environment, and based upon the evidence from inhibitor and electrochemical studies (see Section III.A.4 and III.B) as well as kinetic arguments (see Section III.C), it is unlikely that passive diffusion accounts for more than a small fraction of total P_i influx.

3. Facilitated Diffusion

Facilitated diffusion resembles passive diffusion in that transport must be energetically "downhill," requiring a favorable electrochemical gradient. However, transport rates by facilitated diffusion may be in excess of that predicted merely for random passive diffusion. Frequently, hypothetical carrier mechanisms, or "permeases", are invoked to explain facilitated diffusion. Such carriers are considered to bind the substrate at the membrane surface by conformational alterations of relatively fixed carrier molecules, or by the physical diffusional transfer of the carrier across the membrane. It has also been suggested, although not proven, that ion flux may be enhanced by endoosmotic transport through a system of dynamically charged membrane "pores". However, since polyvalent ions such as P_i have a large hydrated radius, and given the fact that hypothetical membrane "pores" are considered to be predominantly negatively charged,[71] P_i anions are poor candidates for pore-mediated transport. Facilitated diffusion mechanisms have many properties characteristic of enzymatic involvement: (1) they can be saturated, exhibiting a rectangular hyperbolic transport rate vs. solute (substrate) concentration curve; (2) they exhibit stereospecificity with respect to the solute (substrate); and (3) they are susceptible to both competitive and noncompetitive inhibition. However, in contrast to active transport mechanisms any energy expenditure required for transport must be indirect. It has been difficult to assess the degree to which transport systems for other ions affect P_i transport and particularly to determine how closely energy expenditure is coupled to the uptake mechanism(s). The role of facilitated diffusion in P_i uptake in microalgae has not been clearly established.

4. Active Transport

Active transport refers to transmembrane accumulation of solute against an electrochemical gradient, requiring the direct expenditure of metabolic energy. Such metabolically "uphill" transport is considered to be carrier mediated and predominantly unidirectional. There are some similarities between the "enzymatic" character of active transport and facilitated diffusion, however, active transport mechanisms are more temperature sensitive and are inhibited by metabolic poisons.

Since external concentrations of inorganic phosphate are typically in the micromolar range (Raven[66] and references cited therein), passive diffusion along an electrochemical

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gradient alone is insufficient to account for the observed intracellular phosphate concentrations, except at environmentally unrealistic levels. However, to conclusively demonstrate active transport across the semipermeable plasmalemma, it is necessary to establish the following: (1) a cellular energy requirement for uptake; (2) a change in the uptake rate after addition of an inhibitor or a change in temperature; (3) uptake saturation kinetics typical of enzymatic systems; (4) electrochemical evidence that the transport rate exceeds that predicted from the permeability and electrochemical gradient; and (5) that the steady-state electrochemical potential is not in equilibrium across the membrane.

Based upon electrochemical measurements (in giant algal cells), net P_i influx occurs even when vacuolar ion activity exceeds that of the medium, strongly suggesting active uptake, presumably at the plasmalemma.[66] Although comparable electrochemical determinations are rarer for relatively nonvacuolate microalgae, by measuring internal and external P_i concentrations and applying the Nernst equation, active P_i uptake was similarly indicated for *Chlorella*.[485,486]

In the case of a primary active transport system, the hypothetical permease (see Mengel and Kirkby[68] for details) may be phosphorylated for substrate binding, with intracellular release of the substrate by phosphatase cleavage, and subsequent regeneration of the active binding form at the expense of ATP. Only a few ATPase activities have been identified for algal membranes,[72,209,451-454] and of these, clear evidence of ion transport significance was presented only for the diatoms *Nitzschia alba*[453] and *Skeletonema costatum*.[454]

When transmembrane movement of one ion is contingent upon transport of a different ion, the transport systems are considered to be coupled. Coupled transport may result from transport linked by distinct but dependent systems, or from the simultaneous transport of two different ions in one direction bound at separate sites of the same carrier (symport). Alternatively, coupled transport may arise from the transfer of different ions by the same carrier in opposite directions (antiport). In many cases, an active transport system is linked to a facilitated diffusion mechanism, and is, therefore, a secondary mechanism driving ion uptake. After active efflux, the energetics of downhill Na^+ entry are apparently insufficient to drive P_i influx via secondary active transport, however, interactions between P_i and cations at low internal electrochemical potential may serve to enhance P_i transport.[66]

It has been well established that electrogenic ion pumps are also involved in ion transport, whereby net electrical charge is transferred across the membrane via active transport, although the precise mechanisms are seldom clearly distinguishable. Hodges[73] proposed a 'reverse chemiosmotic model', in which the cleavage of ATP generates an excess of protons which are then actively pumped out, thereby creating a pH gradient capable of driving ion uptake. The progressive depolarization of the charophyte *Nitella clavata* plasmalemma when the external H^+ concentration was increased, as well as the generation of large passive H^+ influxes with the membrane potential difference "clamped" at the K^+ equilibrium potential by applying a negative feedback voltage, suggested to Kitasato[456] that electrogenic H^+ extrusion had occurred. Spanswick's work on *N. translucens*[457] also supported the existence of an electrogenie H^+ pump, although his explanation for the changes in membrane conductance differed from that of Kitasato.[456] The actively pumped out H^+ ions of Hodges' model[73] would create an intracellular pool of nondiffusible anion equivalents, thereby establishing a transmembrane electrochemical potential gradient. Extruded H^+ ions would not only serve to neutralize electronegativity at the external plasmalemma surface, thus promoting anion binding, but may also be cotransported with anions down an electrochemical potential gradient by facilitated diffusion. Based upon the relative effects of metabolic uncouplers, fatty acids, the ionophore valinomycin, and high external K^+ concentrations, on the P_i

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uptake systems in P-starved *Chlorella*, Jeanjean[678] concluded that P_i uptake at neutral pH may be driven by the proton gradient and by the membrane potential at alkaline pH. Despite cautious suggestions, there is little evidence to conclude that the influx of P_i is mediated solely by the proton gradient.[460]

A variety of neutral ion pumps may be involved in ion transport, which unlike the proposed electrogenic mechanisms do not depend upon the direct alteration of transmembrane potential for ion flux. In these circumstances, cations and anions may be transported in the same direction (possibly bound to the same carrier) in electrochemically equivalent ratios, or ions of the same charge may be exchanged across the membrane in an electrically neutral one-to-one correspondence. Hodges' model[73] suggests that the reaction of anion equivalents with water would yield excess hydroxyl ions, which when extruded may serve to drive anion uptake by exchange diffusion.

In addition to the generation of hydroxyl groups through ATP hydrolysis connected with the proton pump, exchangeable hydroxyl groups may be produced through intracellular reduction of nitrate and the decarboxylation of organic acid anions.[73] Although a link with P_i uptake has not been established for either higher plants or algae, it is also conceivable that excess intracellular bicarbonate ion may be expelled in exchange for inorganic nutrients anions. Regrettably, most of the work on algal anion uptake mechanisms has focused on the intertidal macroalgae and green algal coenocytes from fresh to brackish waters with large vacuoles (which have rather specialized osmoregulatory characteristics[458,459]), at the expense of more ecologically important (especially marine) microalgal species. The lower C:P ratio and lower intracellular concentrations of the primary osmoregulatory ions, K^+, Ca^+, Mg^{2+}, and Cl^-, noted for relatively nonvacuolate microalgal species such as *Euglena* and *Chlorella*, compared to vacuolate coenocytes such as *Nitella* and *Hydrodictyon*, may be indicative of substantial differences in the respective kinetics and mechanisms of P_i transport.

Furthermore, in terms of membrane transport mechanisms and the establishment of electrochemical transmembrane potential, more detailed attention has been directed toward the 'nonnutritive' ions with high flux rates, Na^+, K^+, Cl^-, and SO_4^{2-},[461-463] instead of the nutritionally significant ions, NH_4^+, NO_3^-, and P_i. With the possibility that P_i transport mechanisms may be subject to coupled interactions with other ions, and given the knowledge that the fluxes of osmoregulatory ions such as Na^+, K^+, Cl^-, etc. have a pronounced effect on the membrane potential, and, thus, anion transport, it is imperative to carefully consider multiple ion effects to properly describe P_i uptake systems.

A P_i active transport protein (M.W.42,000 ± 1000) has been identified for *Escherichia coli*,[88] for which the P_i binding constant approximates the half-saturation constant (K_s) of uptake. However, it is significant to note that although there is ample evidence of active P_i transport from inhibitor studies, bioenergetic arguments, hyperbolically saturable uptake kinetics, and influx against an electrochemical gradient, a specific ATP-mediated P_i permease has yet to be isolated and characterized from any eukaryotic microalga.

B. Energy Transduction for Active Orthophosphate Uptake

The active transport of P_i into algal cells has been demonstrated for many species, including *Nitella translucens*,[74] *Euglena*,[75] and the chlorophytes — *Scenedesmus*,[76,77] *Hydrodictyon africanum*,[78] and *Chlorella pyrenoidosa*.[79] Based upon cellular energetics, the two primary bioenergetic candidates for active P_i transport driving forces are high energy nucleotide triphosphates generated by photophosphorylation in chloroplasts and oxidative phosphorylation in mitochondria, and the electron transport system of these organelles. Several investigators[80,462,464] have proposed hypothetical anion carriers which could be alternatively oxidized and reduced by electron flow, driven by either the

photosynthetic or respiratory electron transport chains. A major theoretical problem facing hypotheses of anion transport directly contingent upon electron transport, such as Lundegårdh's suggestion[464] that anion transport is powered by redox reactions of the cytochrome systems, is that such systems are associated with mitochondria and chloroplasts, but are notably absent from the plasmalemma. It has, therefore, been difficult to substantiate the presence of reasonable mechanisms linking organellar electron transport with uptake at the plasmalemma. While there is evidence of Cl^- influx dependent upon photosynthetic electron transfer (see reviews in references[461-463]), electron transfer hypotheses, when applied to P_i uptake, are now considered erroneous, since under conditions which inhibited mitochondrial electron transport (treatment with CN^- or antimycin A[81,82]) and/or photosynthetic electron transport (exposure to CN^-, DCMU, no CO_2[82]), P_i uptake was generally only mildly affected. In a current review, Poole[460] claims that *all* anion uptake in plants is ATP dependent, however, this may be overstating the case.

By manipulation of experimental conditions and the use of metabolic inhibitors, it was possible to substantiate the central role of ATP in the transmembrane P_i uptake process (see Table 2). Uncouplers of oxidative phosphorylation, such as DNP and CCCP, inhibit not only ATP production, but P_i influx as well.[78,79,597] Similarly, DCCD, which affects ATP synthesis in chloroplasts as well as in mitochondria, is particularly effective in simultaneously suppressing ATP production and, thus, P_i uptake.[78,79] Results obtained by treating illuminated cells with DCMU, in order to block noncyclic photophosphorylation, have not always been unequivocal (and are frequently contradictory); therefore, cautious interpretation is required.[50,74,77,79,597] In the light, cultures of *C. pyrenoidosa,*[79] *Scendesmus,*[77] and the dinoflagellates *Symbiodinium (Gymnodinium) microadriaticum*[50] and *Pyrocystis noctiluca*[597] showed depressed P_i uptake in the presence of DCMU, however, uptake in *Nitella translucens*[74] was unaffected; for the dinoflagellates *Amphidinium carterae*[50] and *A. klebsii,*[50] P_i uptake was actually stimulated. The pronounced decrease in P_i uptake with DCMU treatment, while intracellular ATP levels were only slightly depressed, led Jeanjean[79] to conclude that there was probably no direct relationship between ATP synthetis and P_i uptake. However, it seems more likely that the impact of DCMU treatment upon intracellular ATP pools may be ameliorated by contributions from ATP not derived from noncyclic photophosphorylation. If P_i uptake in illuminated cells were primarily dependent upon ATP generated through noncyclic photophosphorylation, then the impact of DCMU on ATP-mediated uptake would still be substantial. Nevertheless, the unsuccessful attempts to correlate P_i transport rate with intracellular ATP levels or adenylate energy charge (EC), in a direct quantitative manner demonstrate the elusiveness of the relationship.[136,676]

The evidence that light is generally capable of stimulating P_i uptake[74,76,78,83,87,94,97,116,597] directly implicates photophosphorylation as a major source of ATP for this transport process. In the light, P_i influx is much less affected by inhibition of oxidative phosphorylation than of photophosphorylation.[76,78] However, Sundberg and Nilshammar-Holmvall[84] showed that conditions favoring both oxidative and photophosphorylation (light and O_2), supported higher uptake than conditions permitting only photophosphorylation (light and N_2). Therefore the oxidative pathway apparently does make a contribution. Further support for this suggestion is derived from experiments with *Chlorella vulgaris,*[85] which clearly indicated that higher P_i uptake rates were maintained in the presence of both light and air than under either light or dark N_2-purged regimes.

Under conditions selected for P_i limitation, i.e. P_i-limited batch and cyclostat cultures, P_i uptake in *Euglena gracilis* was completely inhibited by CN^-.[75,86] This suggests that for this species oxidative pathways may take precedence under P_i-limited circumstances in

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Table 2
EFFECTS OF METABOLIC INHIBITORS ON ORTHOPHOSPHATE UPTAKE

Species	Inhibitor[a]	Experimental conditions[b]	Effect on orthophosphate uptake	Ref.
Charophyceae				
Nitella translucens	CCCP	I	Decrease	74
	DCMU	I	No effect	
Chlorophyceae				
Chlorella pyrenoidosa	DCMU	I	Decrease	79
	DNP	I	Decrease	
	CCCP	I	Decrease	
	DCCD	I	Decrease	
Chlorella pyrenoidosa	CN^-	II	No effect	81
Hydrodictyon africanum	DNP	I	Decrease	78
	DNP	III	Decrease	
	CCCP	I	Decrease	
	DCCD	I	Decrease	
Hydrodictyon africanum	Antimycin A and CN^-	I	Slight decrease	82
Hydrodictyon africanum	Ethionine	I	Decrease	66
Scenedesmus sp.	Menadione	IV	Slight decrease	76
	Menadione	II	Decrease	
	DNP	II	Very slight decrease	
	DNP	IV	Decrease	
Scenedesmus sp.	DCMU	V	Decrease	77
	DCMU	VI	No effect	
	DCMU	VII	No effect	
Scendesmus sp.	CN^-	II	No effect	81
Dinophyceae				
Amphidinium carterae	Arsenate	I	Increase	50
	DCMU	I	Increase	
	DCMU	III	No effect	
	CCCP	I	Decrease	
	CCCP	III	Decrease	
Amphidinium klebsii	Arsenate	I	Increase	
	DCMU	I	Increase	
	DCMU	III	Decrease	
	CCCP	I	Slight decrease	
	CCCP	III	Decrease	
Pyrocystis noctiluca	CN^-	II or IV	Great decrease	597
	DCMU	II or IV	Decrease	
	CCCP	II or IV	Decrease	
Symbiodinium (=Gymnodinium) microadriaticum	Arsenate	I	Decrease	50
	DCMU	I	Decrease	
	DCMU	III	No effect	
	CCCP	I	Decrease	
	CCCP	III	Slight decrease	
Euglenophyceae				
Euglena sp.	DNP	II	Decrease	75
	Arsenate	II	Decrease	
Euglena gracilis	CN^-	VIII	Complete inhibition	86

[a] Abbreviations and metabolic effects of inhibitors used: antimycin A — an inhibitor of oxidative and cyclic photosynthetic phosphorylation; arsenate — a competitive inhibitor of P_i uptake; CCCP — carbonyl cyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative and photosynthetic phosphorylation; CN^- — cyanide, an inhibitor of oxidative electron transport; DCCD — N,N'-dicyclohexyl carbodiimide, an inhibitor of oxidative and photosynthetic phosphorylation; DCMU — 3-(3,4-dichlorophenyl)-1,1 dimethyl urea, an inhibitor of Photosystem II and noncyclic photophosphorylation, induces cyclic photophosphorylation; DNP — 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation; ethionine — an adenylate trapping agent; menadione — an inducer of cyclic photophosphorylation.

[b] Experimental conditions: I — P-sufficient, light, presence of O_2 and CO_2; II — P-starved, light, presence of O_2 and CO_2; III — P-sufficient, dark, presence of O_2 and CO_2; IV — P-starved, dark, presence of O_2 and CO_2; V — P-starved, light, presence of N_2, no CO_2; VI — P-starved, dark, presence of N_2, no O_2; VII — P-starved, dark, presence of O_2, no CO_2; VIII — P-limited, phased cyclostat, light, presence of O_2 and CO_2.

the light. Similar complete inhibition of P_i uptake by CN^- was also observed for *Pyrocystis noctiluca* in P-starved batch culture after 48 hr.[597] However, it is significant to note that CN^- treatment had little effect on P_i uptake for two chlorophytes in P-limited batch culture.[81]

When *Hydrodictyon africanum* cells were exposed to conditions which allowed only cyclic photophosphorylation of Photosystem I, and inhibited oxidative phosphorylation and noncyclic electron flow (high light, DCMU, and no CO_2 or O_2), P_i influx was adequately maintained, but at a reduced rate.[78] Similar results were obtained by treatment with DCMU and far red light, which stimulates only Photosystem I cyclic electron flow.[78] Smith's experiments[74] with illuminated *Nitella translucens* in the absence of O_2 and CO_2 indicated that $^{32}P_i$ influx was rarely inhibited and could even be stimulated,[74] thereby supporting the role of cyclic photophosphorylation as the primary source of ATP for transport in this species. However, the relative contribution of cyclic photophosphorylation to the P_i transport ATP pool may have been overemphasized, given the fact that in the natural environment actively photosynthesizing microalgal cells are rarely (if ever) exposed to conditions in which disssolved O_2 and CO_2 concentrations approach zero, and electron transport between the two Photosystems is blocked. Given the problematic interpretation of studies involving DCMU treatment (see Table 2), the quantitative contribution of noncyclic photophosphorylation to P_i transport in the light remains uncertain. As the amount of ATP required for transport represents only a small fraction of the total cellular ATP, and is perhaps below detectable limits, it is difficult to attribute ATP for uptake to a particular pathway. However current evidence[66] favors the noncyclic photophosphorylation pathway as the major source. Under aerobic conditions, both in the presence and absence of CO_2, preillumination enhanced short-term dark uptake of P_i in the chlorophyte *Ankistrodesmus braunii*.[87] Furthermore, this preillumination enhanced dark uptake effect was more pronounced when the reaction centers of both Photosystems I and II were illuminated, than when only Photosystem I was stimulated. Further research on *Ankistrodesmus braunii* indicated that P_i uptake was nitrate-linked in the light.[487] Such experiments provide additional evidence supporting the involvement of noncyclic photophosphorylation in P_i influx.

In *Hydrodictyon africanum*, Raven and Glidewell[82] showed that pseudocyclic photophosphorylation, with ATP synthesis coupled to noncyclic electron flow using O_2 as the electron acceptor, was sufficient to maintain substantial P_i transport. However, these authors acknowledge that this particular pathway may not contribute significantly to the transport ATP pool under normal circumstances, since the conditions required for the isolation of pseudocyclic photophosphorylation from alternate pathways (light, N_2, high O_2, and no CO_2, addition of antimycin A and CN^-), are highly artificial.

In the dark, ATP for P_i uptake may be obtained via mitochondrial oxidative phosphorylation, with perhaps a relatively small contribution from substrate level phosphorylation. The enhanced dark uptake effect due to preillumination,[87] indicates that ATP pools saturated through photophosphorylation may be used to power uptake during postillumination darkness. Although P_i transport rates are typically reduced in darkness, an experiment on three P-saturated dinoflagellate species[50] revealed that uptake was fastest in the dark, with intermediate light intensity usually yielding the slowest uptake. For *Pyrocystis noctiluca*, high P_i transport rates were maintained in the dark for P-sufficient cultures, as well as for P-starved cultures.[597] Since dark uptake in *Amphidinium carterae* approximated that in the presence of light and DCMU, the authors[50] suggested that uptake may be independent of light, or that electron transfer from Photosystem II may inhibit uptake. For *A. carterae* and *A. klebsii*[50] and *P. noctiluca*[597] the pronounced inhibition of uptake in the dark by CCCP, which uncouples both oxidative and photosynthetic phosphorylation, indicated the large contribution of

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oxidative pathways to dark uptake. Since many of the data obtained by these authors[50,597] for dinoflagellates are not in accord with those obtained for other nondinoflagellate species (see Table 2), and are not always consistent between experiments,[50] further confirmation is required to determine whether or not these observations have taxonomic significance.

The interpretation of information obtained from using metabolic inhibitors, electrochemical measurements, and environmental manipulation to study P_i uptake mechanisms and energy transduction is complicated by the diversity in experimental regimes selected by various investigators. Differences in irradiance, temperature, O_2 and CO_2 concentration, severity of nutrient limitation, inhibitor concentration, as well as variations in the length of culture preconditioning and incubation period, can alter the kinetics and conceivably the mechanisms of P_i uptake. A species-specific, or clone-specific, response should also be anticipated, with some organisms more heavily dependent upon a particular energetic pathway for P_i transport (i.e., photophosphorylation in the chloroplast vs. mitochondrial oxidative phosphorylation).

The use of metabolic inhibitors and manipulation of environmental conditions to discriminate between alternative uptake mechanisms presupposes that inhibition will be relatively specific and not result in general metabolic impairment, and also that such selective inhibition will be of sufficient magnitude to be readily detectable. In spite of the fact that these criteria are not always met, the overwhelming bulk of evidence supports the existence of active P_i transport mechanisms in microalgae, driven by energy transduced into ATP.

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C. Kinetics

1. Evidence for Monophasic and Multiphasic Uptake Kinetics

The net uptake of nutrients by cells must be interpreted as a series of regulated processes involving several kinetic rate parameters, including those pertaining to: (1) the rate of synthesis/degradation of nutrient transmembrane carriers, (2) the rate of substrate binding and release from these hypothetical carriers, (3) the rate of the transmembrane influx/efflux, and (4) the kinetics of intracellular enzymatic assimilation and conversion of transported nutrient into temporary storage pools or cellular structural material. All of these metabolic processes may potentially be regulated by feedback control. Thus, simplified descriptions of nutrient uptake kinetics do not always adequately define the true series of uptake events. However, as a generalization, the P_i uptake kinetics for many microalgal species[57,75,86,90-95,368,434] appear to follow the rectangular hyperbolic relationship predicted from the Michaelis-Menten enzyme kinetic model:

$$V = \frac{V_{max} P_i}{K_s + P_i} \qquad (2)$$

where V, the specific phosphate uptake rate (h^{-1}), is defined in terms of V_m, the maximal specific uptake rate (h^{-1}), P_i, the ambient concentration of P_i in the medium (μM), and K_s, the half-saturation constant for uptake, i.e., the substrate concentration (μM), where $V = V_m/2$. However, deviation from simple Michaelis-Menten kinetics has been reported by Brown et al.[111] and others.[97]

Although there is no apparent shortage of kinetic parameter measurements in the literature (see Table 3), certain precautions are necessary in comparing data obtained by different methods, and extrapolating such data to the aquatic environment. There are

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Table 3
KINETIC PARAMETER VALUES FOR ORTHOPHOSPHATE UPTAKE

Species	K_s (μM)	V_m (μmol cell^{-1}h^{-1} × 10^{-9})	Culture conditions[a]	Ref.
Bacillariophyceae				
Asterionella formosa	1.90—2.80[b]	9.9—13.2[b]	I	98
	0.60	91.4	I	99
	0.70	15.0	I	482
	0.60	42.9	II	100
	0.34	2.6—16.1[c]	II	450
	0.06	18.0	II	658
Cyclotella meneghiniana	0.80	5.5	I	98
	0.75	5.5	I	99
Diatoma elongatum	2.80	8.0	I	101
Fragilaria crotonensis	1.06	3.4	II	100
	0.03	132.3	II	658
Nitzschia actinastroides	1.02	—	II	92
Thalassiosira pseudonana				
Clone 3-H (neritic)	0.58	8.6	II	9
	0.70	—	II	57
Clone 13-I (oligotrophic oceanic)	0.70	17.3	II	57
	0.50	2.8	III	57
Clone 66-A (oligotrophic oceanic)	0.65	—	II	57
Thalassiosira weissflogii	1.72	250.0	II	9
Chlorophyceae				
Ankistrodesmus braunii	2.27—6.45[d]	—	V	94
Ankistrodesmus falcatus	3.95	32.5	II	100
Chlorella pyrenoidosa	0.68	48.0	II	95
	2.00—4.10[e]	—	V	102
Pediastrum duplex	0.90—1.48[f]	5.8—51.3[f]	V	558
Scenedesmus sp.	0.60	4.8	II	93
Selenastrum capricornutum	2.58	—	II	95
Volvox aureus	0.17—0.96[c]	0.2—2.8[g]	V	555
Volvox globator	0.19—4.20[c]	0.1—2.1[g]	V	555
Chrysophyceae				
Dinobryon cylindricum	0.72	2.0	IV	465
Olisthodiscus luteus	1.00—1.98[h]	228.0—433.2[h]	I	103
Cryptophyceae				
Cryptomonas erosa	0.13	2.2	I	659
Dinophyceae				
Amphidinium carterae	0.01	41.1	IV	50
Amphidinium klebsii	0.01	47.4	IV	50
Peridinium sp.	6.30	559.0	IV	465
Prorocentrum minimum	1.96	167.0	I	655
Protogonyaulax tamarensis	0.40	299.0	I	655
Pyrocystis noctiluca	1.90	2.9×10^4—10.5×10^4[i]	II	597
	2.70	9.1×10^4	I	597
Symbiodinium (= Gymnodinium) microadriaticum	0.01	5.4	IV	50
Euglenophyceae				
Euglena gracilis	0.70—2.80[j]	0.9—4.7[j]	VII	97
	1.40	106.2—150.6[j]	VI	86
Prymensiophyceae				
Pavlova lutheri	0.38—0.63[i]	19.8—30.0[i]	II	91

Table 3 (continued)
KINETIC PARAMETER VALUES FOR ORTHOPHOSPHATE UPTAKE

Species	K (μM)	V_m (μmol cell^{-1}h^{-1} × 10^{-9})	Culture conditions[a]	Ref.
Mixed natural phytoplankton populations				
Central North Pacific	0.14—0.18[k]			368
California current	0.40			368
Jordan River	0.18			104
Lake Kinneret, Israel	0.21—0.79[k]			104
Lake Wingra, Wis.	0.45			111
Mirror Lake, N.H.	0.10—0.27[l]			465
Lake Memphremagog, Canada	0.01—0.08[k]			658

[a] Culture conditions: I — P-limited batch; II — P-limited chemostat; III — N-limited chemostat; IV — P-sufficient batch; V — P-starved batch; VI — P-limited phased cyclostat; VII — P-sufficient synchronized batch.
[b] Uptake kinetic values for two different cultures of the same strain, experiments 2 months apart.
[c] Kinetic values over a range of incubation and preconditioning temperatures.
[d] Lower value in presence of K^+, higher value in presence of Na^+.
[e] Variation between batch culture experiments.
[f] Range in values for different levels of P starvation.
[g] V_m per colony, rather than per cell.
[h] Values for different cultures at different stages in the culture cycle, K_t and V_m increased with time.
[i] Range in kinetic values for different dilution rates.
[j] Circadian oscillation in kinetics coupled to photoperiod.
[k] Uptake kinetics determined for different populations in different seasons.
[l] Morning vs. afternoon determinations.

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two standard methods of determining P_i uptake: (1) measurement of the accumulation rate of a radioactive phosphorus tracer, such as ^{32}P or ^{33}P, and (2) measurement of the rate of loss of P_i (as soluble reactive phosphate) from the medium, the 'disappearance' method. As pointed out by Nalewajko and Lean,[469] both these methods of measuring uptake rates have problems. With the radiotracer method, when the sample is filtered, labeled dissolved or colloidal organic phosphorus, as well as labeled particulate phosphorus, is retained by the filter, and in natural samples, adsorption by detritus may also occur. P_i which is only bound extracellularly or in the periplasmic space may be retained at the cell surface and regarded as 'uptake' if cells are not properly washed. Using the disappearance method can result in an underestimate of phosphate uptake because the molybdenum blue method measures some dissolved organic and colloidal phosphorus in addition to P_i. In both cases, choice of incubation time may be very important in order to estimate true maximal uptake rate, free of any possible feedback inhibition effects. This can be easily checked by determining a time course of uptake rates at different incubation times.

The linearity or nonlinearity of nutrient uptake in time course experiments is a function of the magnitude of the nutrient perturbation relative to the cell density, as well as the relative nutritional status of the cells. Thus, while some researchers have a reported linear P_i uptake over incubation periods ranging from minutes to a few hours for laboratory[91,93,100,501] populations, and over 6 hr[562] and 24 hr[57] for natural populations, others showed nonlinear uptake in laboratory[96,97,112] and natural[25] populations, within the

first few hours after nutrient addition. In addition to the effects of incubation time on estimates of V_m, it may also be important in determining whether gross or net uptake is being measured. Generally, uptake rates measured over a few minutes should represent gross uptake; however, Robertson and Button[538] have shown that excretion or leakage can take place within 2 min in the marine yeast *Rhodotorula rubra*. Nevertheless, if an assessment of growth is desired from uptake measurements, then incubation periods of several hours should estimate a *net* flux, in which excretion or leakage of phosphorus compounds[26,42,105] has been allowed to occur. Additional sources of error in uptake measurements could result from use of high vacuum pressures, resulting in the breakage of delicate surface-bound microfibrils, or leakage due to membrane disruption which would tend to underestimate uptake rates.

Further complications in interpreting estimates of uptake kinetic parameters based on $^{32}P_i$ or $^{33}P_i$ uptake may occur in oligotrophic areas. Since ambient P_i levels are frequently at or below the limit of detection, one does not accurately know what concentration of substrate (P_i) to use in plotting the uptake rate vs. substrate concentration curve, and, consequently, determinations of K_s (in particular) may be inaccurate.

As well as the pronounced effect of environmental manipulation upon uptake kinetics (see Section III.D), there may be substantial variability in uptake kinetic measurements between experiments even when the experimental regime is rigidly standardized. In the past, interexperimental variability was frequently not acknowledged. However, recent efforts to report standard errors of kinetic parameters constitute a welcome trend. It should also be recognized that uptake kinetic parameters are calculated as a gross average for millions of cells, and that individual variability, which may be substantial, must simply be ignored. Fuhs and Canelli[596] proposed the use of ^{33}P autoradiography to determine differential P_i uptake by individual cells, allowing for a high degree of resolution and specificity. This approach appears to be very useful for mixed populations.

The clonal variability within and between subpopulations must also be taken into account when considering P_i uptake responses. Physiological modifications in uptake parameters are characteristic short-term responses to environmental perturbations. However, persistent environmental pressure may result in the selection of favorable genotypes for optimum survival. In long-term laboratory cultures, where the regime is highly artificial, genetically fixed uptake characteristics may become manifest, which are not typical of the natural environment from which the cells were isolated. Even in carefully controlled populations there may be considerable variability in uptake responses between clones of the same species.

It is interesting to note from Table 3 that, except for the anomalously high values for *Ankistrodesmus braunii*[94] and the dinoflagellate *Peridinium* sp.,[465] the K_s for P_i uptake is generally $<4\ \mu M$ for most species. Given the wide variability in experimental regimes adopted, this is at least circumstantial evidence that the fundamental mechanisms (presumably active transport) involved in P_i uptake are similar for different algal classes growing under a range of environmental conditions. It may be significant that K_s values from natural assemblages generally lie within the lower range of values obtained from laboratory cultures. Perhaps long-term culture produces clonal selection artifacts, resulting in eventual departure from original *in situ* kinetic parameters. The wide range of values evident within a given algal class renders it imprudent to conclude that K_s values have a strict taxonomic correlation. Therefore, the apparent trend towards higher K_s values for the Chlorophyceae cannot as yet be definitively attributed to taxonomic status, as all species investigated are from freshwater. It is probably incorrect to attribute the high K_s values obtained for *Ankistrodesmus braunii*[94] and *Peridinium* sp.[465] to feedback

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inhibition of the internal phosphate pools upon the uptake process, due to the use of P_i saturated cultures. Current information suggests that K_s values for P_i uptake are not particularly responsive to changes in the nutritional status of the cells, at least in the short term.[57,91,93,597] Alternatively, it is possible that such species possess multiple uptake systems, and that these K_s estimates correspond primarily to the low affinity mechanism.

Regression analysis of values in the literature for kinetic parameters and P-cell quotas indicated that marine species have higher V_m values for P_i uptake than freshwater species of comparable size (quota).[658] This tendency is rather paradoxical given that P is more often considered the primary limiting nutrient in freshwater, rather than marine, environments. Furthermore, these authors[658] did not confirm the significant differences between V_m and V_m/K_s for diatoms and cyanophytes which have previously been assumed.[512]

Although most studies of P_i uptake in microalgae tend to support a monophasic uptake model,[57,86,95,98–99,368] at least within the confines of environmentally realistic external P_i concentrations (typically $<5\ \mu M$), there is evidence of multiphasic uptake systems worthy of serious consideration, particularly for high P_i concentrations. In P_i limited chemostat cultures, the chlorophyte *Selenastrum capricornutum* exhibited P_i-uptake responses consistent with the existence of a steady-state constitutive system, supplemented by a secondary system which functioned primarily during a transient response to a pulsed addition of P_i.[105] Tomas[103] also suggested the presence of dual P_i-uptake systems for the chrysophyte *Olisthodiscus luteus*, to explain anomalous differences in uptake rate between 6.0- and 10.0-μM external P_i concentrations. For *Pyrocystis noctiluca*, P_i uptake was triphasic between 0.1 and 100 μM in P_i-depleted batch cultures and biphasic between 0.2 and 25 μM in P_i-limited continuous cultures.[597] The apparent oscillation in K_s values for uptake of low P_i concentrations by synchronized *Euglena gracilis* batch cultures was interpreted to indicate the operation of a saturable uptake system, along with a system which was nonsaturable over the concentration range examined.[97] In view of this recent evidence of multiphasic uptake for several species, a judicious use of the Michaelis-Menten parameters for each phase is required to more accurately reflect the flexibility in uptake capacity. Unfortunately, all of the above evidence remains inconclusive due to the lack of sufficient biochemical information.

It should be recognized that kinetic evidence for multiphasic uptake does not necessarily imply the existence of multiple biochemically distinct carrier molecules, although this possibility is certainly not precluded. The low affinity system may include a large (if not exclusive) passive diffusion component. Alternatively, low affinity and high affinity P_i transport may be the result of substrate binding to multiple binding sites of the same carrier sites which differ in P_i affinity. Since detailed biochemical and biophysical characterization of dual P_i uptake systems is generally lacking in the literature on eukaryotic microalgae, it is instructive to examine the kinetic evidence from bacteria[360] and fungi.[106,361,362] A comprehensive investigation of the P_i transport system kinetics in *Escherichia coli*, using induced mutations, revealed the existence of a low affinity uncoupler-inhibited transport system operating in conjunction with a repressible high affinity system requiring a P_i binding protein.[360] A dual uptake feedback regulated model has been proposed for the fungus *Neurospora crassa*,[106] which deserves careful consideration in view of the possibility that analogous systems may be operative in eukaryotic microalgae. In this system, a high affinity derepressible transport system predominates at starvation P_i levels, but is irreversibly repressed at high external concentrations when a low affinity reversibly inhibited system is dominant. An intensive study of the P_i uptake mechanism(s) in *Chlorella pyrenoidosa*[102,539] over a wide external

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P_i concentration range (5.0×10^{-7} M to 1.25×10^{-2} *M*) indicated the presence of two transport systems. An arsenate-inhibited high affinity transport system with a K_s value of 2.0 to 4.1 μM was determined at low concentrations, and a second value of 200 to 310 μM was measured at millimolar substrate levels. However, it can be successfully argued that the concentrations at the high end of the spectrum are scarcely representative of conditions in the natural environment.

The obvious advantage of multiphasic uptake mechanisms with multiple sites of differing substrate affinity on the same carrier or on separate carriers involves the linkage of uptake kinetic parameters to ambient nutrient concentrations in the most bioenergetically favorable way, thereby allowing cells to maximize transient uptake upon encountering nutrient-rich water and, as well, to achieve a high sustainable uptake rate in nutrient-depleted areas.

In spite of the obvious plasticity of uptake kinetic parameters, particularly V_m, in response to short-term environmental change, there is an apparent molecular conservatism in the transport of externally available phosphate. The typical stability of K_s values within a given phase with respect to relatively short-term changes in cell nutrient status,[57,91,93,597] and perhaps light,[597] argues against modifications in phase-specific substrate affinity as a physiological adaptation to short-term environmental change. Nevertheless, K_s values for P_i uptake by phytoplankton in the central North Pacific,[368] where P_i is chronically in short supply, were typically lower than those from either the California Current[368] or Chesapeake Bay[396] phytoplankton where the environment is comparatively P_i-rich, suggesting the possibility of long-term genetically fixed adaptation. Such results may indicate that in a high P_i regime, low substrate affinity and low transport rate will suffice to supply the cellular nutritional requirements, while minimizing metabolic requirements for P_i carrier synthesis and mobilization. When ambient P_i concentrations are low, higher substrate affinity and/or increased uptake rate, requiring a relatively greater energetic investment in transport, may be needed to support continued growth and metabolism.

2. Cellular Nutrient Status

a. Luxury Uptake and the P-Starvation Induction Effect

Although Michaelis-Menten kinetics are typically used to describe P_i uptake kinetics as a function of external concentrations, observations that P_i uptake is inversely related to internal P-pool concentrations[85,93,100,113,406] are evidence of the coupling of uptake kinetics to cellular nutrient status. It has long been known that microalgal cells are capable of "luxury uptake", whereby surplus P is accumulated in excess of that required for immediate growth.[93,98,101] The enhancement of phosphate uptake under a nutrient-limited regime is a well-documented phenomenon in the literature. However, direct kinetic comparisons are not always valid, as such physiological responses are highly subject to preconditioning effects. In phased P_i-limited stationary phase batch and cyclostat cultures of *Euglena gracilis,* uptake was linear with time and could be adequately described by Michaelis-Menten kinetics.[86] In contrast to this, a P_i-sufficient batch culture exhibited triphasic uptake kinetics between 0 and 100 μM P_i, with Michaelis-Menten kinetics applicable only between 0 to 3 μM P_i.[97] Although the intracellular phosphate levels are frequently cited[111] as the major factor controlling uptake kinetics,[9,75,78,93,108-110,516] Perry[57] demonstrated that P_i uptake rates for P_i-loaded cells from a P_i-limited chemostat tend to reflect the nutritional past history, rather than the instantaneous internal concentrations, at least in the short term. Such results underscore the necessity of establishing a clearly defined nutritional preconditioning period of several generations before uptake kinetic measurements are attempted.

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When the above factors have been considered, there remains a readily apparent relationship between P_i uptake rate and the degree of P_i limitation. The transient increase in V_m noted under P_i-limiting conditions has been conclusively established;[9,57,86,93,100,105,111,112,406,465,597] however, this enhancement effect is explicable either in terms of a single inducible uptake mechanism, or as the induction of a secondary transport mechanism. Under a short-term starvation regime of several days, maximal P_i uptake rates for *Olisthodiscus luteus* showed a progressive increase with time.[103] Perry noted significantly that K_s values for P_i uptake by chemostat cultures of clones of the marine diatom *Thalassiosira pseudonana* remained essentially the same regardless of the cellular nutritional status, although V_m values were markedly enhanced by P_i limitation.[57] Similar observations were made for *Pavlova lutheri,*[92] *Scenedesmus,*[93] and *Pyrocystis noctiluca.*[597] Nalewajko and Lean[540] found trends of increasing V and K_s during starvation of three species, whereas Brown et al.[111] reported increasing V and decreasing K_s for the chlorophyte *Selenastrum.* In the turbidostat experiments of Azad and Borchardt,[112] V_m for P_i uptake in two chlorophytes continued to increase with starvation to a maximal value. However, beyond this critical starvation point where the ratio of cell P-quota (q) to q_m, the cell P concentration at which growth is no longer limited, was less than 0.3, uptake declined rapidly, with a concomitant increase in the lag period before significant influx occurred. Chisholm and Stross[86] also noted a short lag period before steady-state uptake was resumed by batch-cultured *E. gracilis* that was not P_i starved. This effect was attributed to a presumed lag in induction of the permease system. However, results under prolonged P_i depletion are indicative of severe metabolic, and perhaps genetic, impairment of the uptake mechanisms. The induction effect of P_i starvation was shown to increase uptake rates for P-limited cells by one to two orders of magnitude greater then that for P_i-sufficient cells.[57,86,100,112] Under limiting conditions, feedback upon the uptake mechanism(s) may be reduced to accommodate increasing influx. Early work on *Scenedesmus,*[93] and later on other microalgal species, showed that P_i uptake kinetics resembled those of a noncompetitive type of enzyme inhibition where

$$V = V_m/(1 + K_s/P_i)(1 + i/K_i) \quad (3)$$

i is the inhibitor concentration (μM) and K_i is the constant expressing the degree of inhibition (μM). In this case, the inhibitor does not affect the substrate affinity, but V_m is modified by the term $1 + i/K_i$. Rhee[93] considers the noncompetitive inhibitor to be phosphorylated internal pool components which can depress uptake by inactivation of permease binding sites. For several species in P_i-limited chemostat culture, K_s for P_i uptake did not vary with the growth rate; however, V'_{max}, the apparent maximal uptake rate, increased with decreasing growth rate, or cell P quota.[100,406] Nevertheless, feedback control of P_i uptake as described by Rhee[9,100] was not evident for certain other species,[91,95] and, thus, may not be a universal phenomenon. Since short-term high uptake rates were maintained in Perry's experiments[57] even after high intracellular P levels were achieved after P_i enrichment, she concluded that the starvation induction effect was more likely the result of induced production of new P_i binding sites, rather than the derepression of formerly unavailable sites. Perry's[57] interpretation of the short-term starvation response is, therefore, not congruent with Rhee's view,[93] which proposes a rapid noncompetitive repression/derepression effect regulated by intracellular levels of phosphorylated metabolites. In summary, there are two possible alternative means by which P_i starvation may influence uptake kinetics and lead to an increased transport rate: (1) the synthesis of carrier protein may be induced *de novo*, thus providing more membrane binding sites for transport; and (2) derepression of already synthesized carrier

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system protein may facilitate increased transport activity. These alternatives are not theoretically incompatible, and both may exert their effects on uptake events. As Perry[57] suggested, the short-term effects of P_i readdition to starved cells may be attributed to reduced carrier protein synthesis, with longer term effects due to both the progressive inhibition of new binding site production, as well as direct repression of already available carrier binding sites. Use of metabolic inhibitors affords the opportunity to partially resolve this controversy. Unfortunately, microalgal physiologists have generally neglected such detailed biochemical investigation into the nature of the inducible transport system, in the pursuit of simple kinetic parameter measurements.

A notable exception to the above statement is the work of Jeanjean and co-workers,[79,102,391,400-402,539,678] using *Chlorella pyrenoidosa* as a model organism to study P_i transport kinetics and mechanisms in detail. The typical increase in P_i transport rate following short-term starvation, which was maximal after approximately 4 hr in P_i-free medium, was effectively eliminated by the addition of inhibitors of protein synthesis.[391,400-402] Cycloheximide, which inhibits protein synthesis on eukaryotic 80S cytoplasmic ribosomes,[403] when added to *Chlorella* cultures at the beginning of the starvation period, suppressed the transient increase in P_i uptake rate.[401] However, the timing of inhibitor addition is critical, as cycloheximide treatment after 4 hr starvation resulted in a slow decline in uptake velocity to a level still above unstarved preconditioning rate. The suppressive effect of transient P_i readdition to starved cells was rapidly manifest, and as a consequence uptake declined promptly to the prestarvation level.

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That these inhibitory effects were due to the inactivation of the transport system by interference with protein synthesis, rather than feedback inhibition, was supported by the observation that cells starved for 2 hr, then incubated in high P_i medium for a short time sufficient to insure P loading, and ultimately placed again in P_i-free medium, exhibited an increased uptake rate shortly after, *except* in the presence of cycloheximide. P_i-loaded cells would be expected to exhibit repression of short term uptake rate due to TCA-soluble polyphosphate accumulation, if Rhee's hypothesis[93,113] is correct, but this was not apparent from these experiments. On the other hand, if the loaded P was not assimilated as polyphosphate, short-term repression effects would not necessarily be expressed.

The inhibitory effects of the base analogue 6-methylpurine, which interferes with functional RNA synthesis, was tested by addition at the beginning of the starvation period.[401] The initial depressive effect of 6-methylpurine on uptake velocity was not as marked as for cycloheximide, although it was more effective in reducing uptake when added after 4 hr of P starvation.

Use of selenate, which interferes with incorporation of sulphur-containing amino acids into protein, resulted in a starvation induced uptake rate in *Chlorella* only 50% as great as that of untreated controls.[402] In the same series of experiments, a similar reduction in uptake was achieved in starved cells exposed to darkness and the absence of CO_2. As the author suggests,[402] such conditions would tend to limit production of carbon skeletons and reduce the rate of sulphate and nitrate reduction involved in protein synthesis, including carrier protein. However, the metabolic effects of CO_2-limited dark treatment are quite nonspecific and these results may be partially (or completely) attributed to factors other than lack of carrier protein, such as reduced energy transduction.

The sulphydryl reagents pCMB and NEM were used in conjunction with cycloheximide to further elucidate the starvation induction effect in *Chlorella*.[391] Treatment with low levels of pCMB did not result in uptake inhibition for P_i-saturated cells, however, reduced uptake was observed for starved cells. The inhibitory effect of pCMB was due to an interference with *de novo* protein synthesis, since cycloheximide

treatment after 2 hr starvation effectively eliminated transport sensitivity to pCMB. In spite of these provocative observations, such inhibitor studies require interpretation with due restraint since the susceptibility of the transport system to reduction of starvation-induced uptake was a function of the inhibitor used, the inhibitor concentration, the pH of the incubation medium, the degree of osmotic shock to which the cells were exposed, as well as the nutritional status of the cells.

In summary, P_i uptake is not only a function of external concentration, but it may also be controlled from the TCA-soluble polyphosphate pool by a feedback regulation mechanism.[113] In these cases, the simple Michaelis-Menten equation is not sufficient to describe the uptake rate, which is a function of the external P_i concentration and the size of the TCA-soluble polyphosphate pool. If Rhee's relationship in Equation 3 is generally applicable, then values of K_s and V_m do not suffice in describing uptake capacities of microalgae, since uptakes rates may be substantially modified by the magnitude of K_i and i, particularly for species where the ratio K_i/i is large. Species possessing high K_s values for P_i uptake may achieve some compensation through correspondingly high K_i values, and thus suffer no competitive disadvantage.

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b. Interaction of Other Nutrients or Trace Elements with Orthophosphate Uptake

Research in the area of nutrient interactions has largely been neglected, and only within the last few years have concerted efforts been made to examine these important effects. Phosphorus and vitamin B_{12} interactions were examined in steady state chemostat cultures of the prymnesiophyte *Pavlova lutheri*.[108] Uptake of both limiting and nonlimiting nutrients was found to be controlled by internal as well as external substrate concentrations. Thus there is a limit to luxury consumption of one nutrient when growth is limited by another, because the uptake of the nonlimiting nutrient is considered to depend on both its external concentration and on the rate of uptake of the limiting nutrient.[108] While Droop's experiments[108] indicated that the intracellular concentrations of both limiting and nonlimiting nutrients increase as a function of the growth rate, Rhee[113] examined P_i uptake for nitrate-limited continuous cultures of *Scenedesmus* sp., and found that cell P levels remained almost constant regardless of growth rate, and the N:P ratio in the inflow or reservoir medium, possibly because P_i uptake was controlled through a feedback mechanism. Similarly, in P_i-limited cultures cellular N levels remained constant, with nitrate uptake being regulated by feedback from free amino acids.[508]

The enhanced uptake rate phenomenon seems to be highly specific for P limitation. Maximal P_i uptake rates for N-limited chemostat cultures remained consistently an order of magnitude lower than for P_i limited cells.[57,113] Perry[57] regards this as evidence against a nitrogen-mediated repression/depression system which would impinge upon the P_i uptake mechanism. However, Rhee[113] argues that the measured accumulation of an acid-soluble polyphosphate fraction under N stress may function as a noncompetitive inhibitor to repress P_i uptake. In an earlier report, Ketchum[114] commented that the rate of P_i uptake by the diatom *Phaeodactylum tricornutum* was a function of external concentrations of both nitrate and phosphate. Cells were able to take up P_i in the absence of nitrate; however, the stimulative effect of nitrate upon P_i absorption tends to substantiate Rhees' view.[113]

Further nutrient interaction studies were conducted for three marine diatoms, in which P was the nonlimiting nutrient under ammonium or silicate limitation.[534,542] Under either ammonium or silicate limitation, short-term uptake of the nonlimiting nutrient, P_i, was frequently reduced, while the uptake of the limiting nutrient (ammonium or silicate) was enhanced when the nutrient-limited culture was given a spike addition of the limiting

nutrient. Under ammonium or silicate starvation this trend was more pronounced. For example, under ammonium starvation, the nonlimiting nutrients, P_i and silicate, were not utilized during the first 8 hr of the experiment.[542] Enrichments with ammonium or nitrate of potentially N-limited samples of natural assemblages from the central North Pacific, where P_i levels were low to undetectable, resulted in no stimulation or inhibition of P_i uptake during a 24-hr incubation.[368] For nitrate-limited natural populations off the west coast of Africa, suppression of the uptake rates for nonlimiting nutrients, P_i and silicate, occurred when a pulse of nitrate was added to the N-limited cultures, while nitrate uptake was enhanced.[543]

The recent interesting results of Terry[551,552] are further evidence that nutrient interactions deserve more attention in the future, as the evidence of interactive effects has not always been consistent. *Pavlova lutheri* was grown in continuous culture at different dilution rates and N:P supply ratios.[551] When subsamples from the continuous culture were given nitrate only, P_i only, or nitrate plus P_i, at saturating levels, he found that the short-term P_i uptake rate was not affected by the presence of nitrate; however, nitrate uptake was reduced in the presence of P_i. The percent reduction in V_N due to the P_i addition was positively correlated with both the N:P supply ratio (i.e., inhibition was least when cells were most N limited) and the preconditioning growth rate. Percent inhibition of nitrate uptake was a hyperbolic function of P_i concentration, with a maximum of 50% inhibition achieved. Determination of uptake kinetic parameters showed that V_m for nitrate uptake was reduced by the presence of P_i, but the K_s was not affected. Terry[552] has recently extended this work to include two marine diatoms, and carbon and ammonium as well as nitrate interactions with P_i. In addition to the previous findings, he found that P_i uptake was reduced in the presence of ammonium. A note of precaution is in order for researchers examining nitrate-phosphate interactions, because high P_i concentrations may interfere with the copper-cadmium column used in nitrate analysis, resulting in a serious underestimate of nitrate concentrations.[544]

Interaction of P_i uptake with elements other than N and Si has also been studied.[545,546] Arsenate is a chemical analog of phosphate and, therefore, it competes for transport into the cell. Arsenate and arsenite were readily taken up by *Skeletonema costatum* and they inhibited primary productivity at near ambient concentrations of 67 n*M* arsenate when P_i concentrations were low.[545] A P_i enrichment of greater than 0.3 μM alleviated this inhibition; however, the arsenate stress caused an increase in the cell's P requirement.

Interactions between P_i uptake and trace elements have not been studied. Based on very interesting recent results of interactions in microalgae between silicate and copper or zinc,[547,548] between ammonium and mercury,[548] and between boron and phosphate in higher plants,[550] this area of research certainly warrants attention.

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D. Modifying Factors

1. Light

The evidence regarding light stimulation of P_i uptake is frequently far from unequivocal. Furthermore, there are no data on how light transients affect the uptake process. Natural phytoplankton assemblages collected from oligotrophic central North Pacific waters and incubated at 0 to 90% of surface irradiance exhibited no significant differences in P_i uptake rates with varying irradiance or depth.[57,368] Similarly, Mackereth[115] reported little or no effect on uptake attributable to variations in the light regime for the diatom *Asterionella formosa*. P_i-limited continuous cultures of the diatoms *Thalassiosira pseudonana* and *T. weissflogii* showed a comparable lack of light influence upon uptake rate.[9] However, evidence from laboratory experiments frequently suggests light-enhanced P_i uptake, including work on species such as *Hydrodictyon africanum*,[78,82,83]

Nitella translucens,[74] *Ankistrodesmus braunii,*[87,94] *Scenedesmus,*[76,81,116] *Chlorella pyrenoidosa,*[79,81,553] *Euglena gracilis,*[86,97] and *Pyrocystis noctiluca.*[597] From the natural environment, coastal marine phytoplankton assemblages[117] appeared to exhibit similar light-enhanced uptake rates. By compiling data on natural phytoplankton assemblages, Reshkin and Knauer[118] described a Michaelis-Menten-type hyperbolic relationship relating P_i uptake rate (V) to percent surface irradiance:

$$V = V_m \frac{I}{K_L + I}, \tag{4}$$

where I is the irradiance and K_L is the half-saturation constant, i.e., the irradiance where $V = \frac{1}{2} V_m$. Application of this equation to experimental data yielded a K_L value of 4.2% of surface irradiance. For Lake Superior phytoplankton assemblages, P_i uptake kinetics were light dependent at low light levels below or near I_k, the irradiance at the intersection of an extension from the initial slope and the maximum photosynthetic rate of the plot of photosynthetic rate vs. irradiance.[553] Furthermore, for dinoflagellates from the Rhode River estuary, Correll et al.[8] concluded, on the basis of microautoradiographic evidence, that ^{33}P assimilation occurred only when sufficient light was provided. However, much more experimental work is needed to determine how the degree of P limitation and/or the degree of light limitation affects the relationships between irradiance and P_i uptake; such experiments have already been initiated for nitrate and ammonium.[554]

Using *Chlorella* in a dark experiment without inhibitors, Schneider and Frischkneckt[119] demonstrated a depressed influx of P_i when compared with illuminated controls. Anomalously, in synchronized cultures of *Scenedesmus,* the P_i uptake maximum actually occurred during the *dark* period of the growth cycle.[84] A similar pattern of higher P uptake during the dark period was also observed for batch cultured P-saturated dinoflagellates;[50] therefore, this is not an isolated occurrence. This may reflect the higher demand for phosphorylated intermediates required for cell division during the dark period, with energy for uptake derived from oxidative phosphorylation or residual ATP generated during the light period.

To reconcile the apparent contradictions regarding the influence of light on P_i uptake, it is important to recognize that the primary driving force for active transport may be derived from a variety of transduction systems, depending upon the experimental conditions. Presumably the organisms have adapted somewhat differently to utilize these alternative systems most efficiently. It may be significant that several studies which indicated no light stimulation of P_i uptake[9,57,115] involved "natural" species grown at very low ambient P_i concentrations. Other studies[78,81,87,116] which tended to contradict this interpretation used "lab" species and P_i concentrations that were much higher than environmental levels. One plausible explanation for these differences is that at high external P_i concentrations excess photosynthetic energy is expended on luxury uptake.

In a comprehensive study of P_i uptake kinetics in *Euglena gracilis,* Chisholm and Stross[86,97] showed that the influence of light upon uptake was a function of cellular metabolic status. P_i-sufficient batch cultures synchronized to a 14:10 light/dark cycle exhibited an almost complete inhibition of P_i uptake in the dark; whereas phased P_i-limited cyclostat and stationary phase batch cultures showed a reduction in dark uptake of only about 35%. This seems to indicate a closer dependence of luxury P_i uptake to photosynthetic activity, than is the case when this nutrient is limiting.

2. Temperature

From a physicochemical standpoint it is conceivable that temperature could indirectly alter cellular uptake kinetics by affecting ambient water properties, the ionic speciation

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ratios of transportable P_i, the rate of diffusion into the cellular boundary layer, and the frequency of molecular collision with cell-surface P_i receptors. Munk and Riley[122] have considered the theoretical effects of temperature on the properties of the aquatic microenvironment surrounding phytoplankton cells, with reference to nutrient uptake. The dependence of chemical reaction rates upon temperature may be expressed in the Arrhenius relationship

$$k = Ae^{-E/RT} \tag{5}$$

where k, the reaction rate, is defined in terms of A, the constant indicating the frequency of reaction complex formation, −E, the activation energy (cal mol^{-1}), R, the universal gas constant (cal °K^{-1} mol^{-1}), and T, the temperature in absolute degrees (°K). Presumably, the conformation of plasmalemma protein and lipid constituents is also temperature sensitive, and this may consequently affect carrier binding properties, and, thus, transport rate. Since enzymatic reaction rates can be profoundly affected by temperature due to the relatively high kinetic activity of the reactants, the rates of carrier protein and ATP synthesis for transport, as well as enzymatic conversion rates of phosphorylated intermediary metabolites, which may alter feedback inhibition on uptake, will be modified. Many enzymatically regulated reactions are susceptible to the so-called temperature coefficient (Q_{10}) effect, whereby reaction rates approximately double in response to a temperature increase of 10°C. However, particularly in vivo, such a relationship may only be applicable within a relatively narrow species-specific temperature tolerance window, beyond which metabolic impairment, often in the form of enzyme denaturation, results. Given the fact that P_i uptake has been shown to be an active process (presumably enzymatically regulated), and since seasonal variations of greater than 10°C are not uncommon in aquatic ecosystems, it is paradoxical that relatively little effort has been expended in examining temperature effects on uptake.

In freshwater algae it was observed that at lower temperatures the production of biomass equivalent to that obtained at higher temperatures required higher ambient P_i concentrations,[405] but the effect could not be conclusively attributed to either a direct impact upon cellular metabolism leading to growth or to the impairment of the P_i uptake mechanism(s). Recent work on P_i-limited continuous cultures of *Scenedesmus* sp. and *Asterionella formosa,*[450] as well as *Pavlova lutheri,*[449] showed that the cell quotas (q) of both limiting and nonlimiting nutrients increased with decreasing temperature, suggesting that reduced growth rates at low temperatures are not primarily the result of decreased P_i transport rate.

Fuhs et al.[9] found that P_i-limited chemostat cultures exhibited increased short-term P_i uptake rates in response to higher temperature, as expected. For *P. lutheri* in P_i-limited chemostat culture at a low steady-state growth rate, uptake rate on a per-cell basis was approximately double for cells maintained at 15°C, as compared to cells grown at 18.8°C and 23°C, although uptake by the total cell population of a fixed external P_i supply remained approximately constant throughout this temperature range due to lower cell quotas at the higher temperatures.[449] For the cyanophyte *Anabaena,* cultured at 22 to 23°C, initial P_i uptake rates increased with temperature to a maximum of 35°C; however, such high uptake rates were not always sustained over longer time periods at the highest temperatures.[110] Since many cyanophytes are known to be relatively eurythermal, compared to eukaryotic microalgae, it is premature to consider such a response as necessarily typical. Potential adaptive effects of prolonged growth at a particular permissive temperature which is typical of neither the habitat from which the species was isolated nor the previous culture regime, and the resultant effect upon uptake rate remain to be established.

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The influence of temperature upon nutrient uptake kinetics may be expressed as not only a species-specific, but also as a nutrient-specific response. There is a scarcity of data on how a transient in temperature affects the P_i uptake rate. Recent results by Rhee and Gotham[450] suggest that these studies might be interesting to pursue. They used steady-state cultures growing at a fixed dilution rate under P or N limitation and various temperatures to measure transient P_i or nitrate uptake rates. In *Scenedesmus* sp.[406,450] the optimum temperature for nitrate uptake (15°C) was clearly uncoupled from the optimal growth temperature (20 to 25°C), and similar results for nitrate uptake were reported for *Chlorella pyrenoidosa*[408] and the green macrophyte *Codium fragile*.[407] In contrast to this tendency, the maximal P_i uptake rate for *Asterionella formosa* was observed at 20°C, the temperature at which maximal growth was achieved.[450] However, for P_i-limited chemostat cultures of *Pavlova lutheri* the increase in cell P quota at extreme temperatures demonstrated that uncoupling of uptake and P-dependent growth had occurred.[449] For two colonial chlorophycean *(Volvox)* species, maximum growth rates were obtained at 20°C, with a decline at higher temperatures.[555] However, V_m values for P_i uptake by short-term P_i-starved cultures increased consistently from 5 to 30°C. This trend was not matched by the K_s values for uptake, which were minimal at 10°C, and rose sharply within a 5° interval on either side of this temperature. In batch cultures, the K_s for nitrate uptake for *Skeletonema costatum*,[409] the chlorophyte *Dunaliella* sp.,[410] and the dinoflagellate *Gymnodinium splendens*[410] increased with maintenance temperature. However, in N- and P_i-limited continuous cultures, K_s values for uptake showed either unclear or undetectible variations in response to temperature.[450] Thus, it appears that temperature effects on P_i uptake are not reducible to simple generalizations based upon broad spectrum theoretical models of cellular metabolism.

3. pH Effects

As is the case for salinity, information regarding the effects of pH upon P_i uptake is woefully inadequate. However, it has been established for the cyanophyte *Synechococcus*[1] and for a few eukaryotic algal species[75,83,94,391,556] that P_i uptake is a pH-dependent phenomenon. This is not surprising given the fact that ionic speciation is a function of pH, with $H_2PO_4^-$ and HPO_4^- species dominant throughout the typical environmental pH range from pH 5 to 9[29] (see Figure 3). Ullrich-Eberius[94] concluded that only $H_2PO_4^-$, which predominates at lower pH, is transportable, however, the wide optimal pH ranges for P_i uptake for most species suggest that HPO_4^{2-} is transported as well, even though, generally speaking, increasing ionic charge makes passage through the membrane more difficult.[69]

P_i influx into *Hydrodictyon africanum* was maximal between pH 6 to 8; however, influx dropped off markedly at the environmental extremes of pH 5 and 10.[83] Similar maximal uptake rates at approximately neutral pH have been noted for other species.[1,75,94,391] The influence of preconditioning and incubation P_i levels on pH-dependent uptake must be considered. Transfer of phototrophic *Euglena gracilis* from pH 6.8 to 4.2 in a high P_i medium resulted in severe growth inhibition, as well as a fourfold increase in total cell P, due to a massive influx of P_i from the medium. The author[556] speculated that the inhibitory effect of light upon cell division may have occurred in response to light inactivation of the monobasic P_i carrier at low pH, thereby allowing passive diffusion to swamp the cell's internal P_i equilibrium. When incubated at 10^{-5} *M* P_i, nonstarved *Chlorella* cells exhibited only a single maximal uptake peak at pH 6 to 7, whereas starved cells displayed two maximal uptake peaks, one at neutral and one at alkaline pH; at 10^{-3} *M* P_i, both starved and unstarved cells showed maximal uptake at approximately neutral pH, with a shoulder at approximately pH 8.[391]

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The author[391] suggested two possibilities for these pH effects: (1) that starved cells may synthesize a secondary transport system active at alkaline pH, in addition to one operative at neutral pH, which may be induced or repressed depending upon the relative nutritional status of the cell; or, alternatively, (2) a single transport mechanism may merely become active over a broader pH range in response to nutrient depletion.

At higher (millimolar) P_i levels a larger component of diffusive flux is expected, which may exhibit markedly different pH dependence when compared with active transport at lower (micromolar) concentrations. As yet it has not been possible to specifically discriminate between the pH susceptibility of multiphasic P_i transport mechanisms, with any degree of certainty regarding the biochemical nature of the pH-induced response.

4. Salinity and Ionic Effects

In most plant cells electrochemical and flux data suggest that inorganic anions, including $H_2PO_4^-$ and HPO_4^{2-}, are actively transported inwards at the plasmalemma, while cations, such as H^+ and Na^+, are usually actively transported out (Raven[66] and references therein). These observations are consistent with the postulated existence of electrogenic and/or neutral ion pumps.

Although the phenomenon of cation stimulation of P_i uptake has been more extensively examined in cyanophycean species,[2,110,120] there are examples of similar enhancement shown for certain green algae. For *Hydrodictyon africanum, Chlorella,* and *Scenedesmus* (Raven[83] and references therein) and *Ankistrodesmus braunii,*[94,121] Na^+ ion was apparently more simulatory than K^+ ion. The evidence from cyanophytes[2,110,120] would suggest that maximal P_i uptake rates are obtained in the presence of divalent cations, such as Ca^{2+} or Mg^{2+}, however, the synergistic relationships between mono- and divalent cations have not been adequately investigated for eukaryotic microalgae. In one anomalous case, all monovalent cations tested caused uptake inhibition ($Li^+ > K^+ > Na^+$) in P_i-deprived *Euglena.*[75] However, since laboratory conditioning was rather extreme, involving streptomycin bleaching, axenic growth with ethanol as the sole carbon source, and high cation concentrations (60 m*M*), such an effect may have been induced by membrane or osmoregulatory disruption.

Since P_i uptake was shown to be an ATP-mediated phenomenon (see Section III.B), it is instructive to note that all four categories of ATP-hydrolytic activity associated with membranes of the diatom *Nitzschia alba* displayed a divalent cation requirement, usually for Ca^{2+} or Mg^{2+}, with some Mg^{2+}-dependent activity stimulated by monovalent cations.[72] There are several theoretical mechanisms of cation enhancement to be considered,[2] including: (1) enhanced P_i uptake due to the cation neutralization of membrane surface negative charges; (2) facilitated P_i transport, coupling influx with a cation symport or antiport system; (3) the requirement of active P_i transport enxyme(s) for cations as co-factors or allosteric effectors; and (4) a direct P_i uptake enhancement due to cation interactive effects upon active transport enzymes(s) to which the cation is not bound. A major objection against the facilitated diffusion symport coupling hypothesis is that the energetics of direct coupling of uphill P_i influx to downhill Na^+ influx are unfavorable.[83] Further indirect evidence against symport or antiport explanations is that while Ca^{2+} ion had a marked effect upon P_i uptake in the cyanophyte *Synechococcus leopoliensis,* there were no comparable P_i-mediated effects upon Ca^{2+} ion influx or efflux.[2] Although caution must be exercised in attempting to generalize from a single prokaryotic species to eukaryotic species, it is significant that for thermodynamic reasons, and the absence of a sigmoidal uptake vs. substrate concentration curve, these authors[2] rejected hypotheses involving electrostatic masking of surface membrane charge and cations functioning as allosteric effectors.

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Given the known influence of ions on membrane transport, it is somewhat surprising that so little effort has been expended on investigating the effects of salinity upon nutrient ion uptake. There has not been to date a concerted attempt to determine what effect (if any) high ion concentrations in seawater have upon the rate of P_i uptake. Along with potential direct effects of ions upon membrane transport, there may be osmoregulatory interactions between external ion concentrations and intracellular metabolities which might indirectly impinge upon nutrient transport. It has been shown for *Chlorella pyrenoidosa* that P_i uptake is sensitive to osmotic shock at neutral and alkaline pH.[391] Such effects may be significant in coastal environments characterized by high tidal flux, or where catastrophic freshwater runoff results in a rapid salinity change. In view of the typical ionic ratios present in seawater, it is scarcely conceivable that low cation concentrations could restrict P_i uptake, however, in freshwater and low salinity estuarine environments, such potential effects warrant further investigation.

5. Physical Cellular Properties

a. Turbulent Effects and Motility

Planktonic cells are, by definition, distributed primarily by the bulk transport of the water masses within which they reside. Macroscale physical factors, such as the wind-driven and geostrophic circulation patterns, largely determine the horizontal spatial distribution of phytoplankton. In coastal regions, diel tidal flux may be an important factor governing this distribution.

Horizontal flow patterns, as well as upwelling and convective overturn, also transport required nutrients and are responsible for their replenishment as they are exhausted by phytoplankton consumption. In addition, large turbulent eddies with an upward vertical component can serve to maintain sinking cells in the euphotic zone.[122]

The importance of smaller scale turbulent mixing upon nutrient availability should not be underestimated. Although it has become common laboratory practice to mechanically agitate cultures, particularly for nonmotile species, both in the preconditioning and experimental time interval of nutrient uptake and growth studies, little is understood about the physiological impact of such turbulence. The justification for this practice is the attempt to mimic natural turbulent influences and the effect of sinking. This may be important to eliminate localized microzones of nutrient depletion which could potentially occur in dense cultures. A recent study of small-scale turbulent effects[345] indicated that the uptake rate of dissolved reactive phosphate by P_i-limited *Phaeodactylum tricornutum* and the marine chlorophycean flagellate *Brachiomonas submarina* in lag phase was maximal at the lowest agitation rate, and that this maximal rate was sustainable over a longer time interval than when the agitation rate was increased. However, since the minimal uptake rate was not directly a function of increasing agitation, the author[345] suggested that dissolved reactive phosphate uptake was supplemented by uptake of other phosphorylated compounds at high agitation rates. This conjecture cannot be substantiated, as there is no information available regarding the effects of turbulence on organic phosphorus excretion rates. It is conceivable that metabolic disorientation resulting from high shear forces may stimulate organic phosphorus excretion, and that the return of such compounds to the medium from lysed cells and other particulates to a free soluble state available for reutilization may also be enhanced.

From the viewpoint of nutrient acquisition, motility may be regarded as a twofold strategy: (1) by physical displacement of the organism towards nutrient richer medium, new nutrients become accessible, and (2) by increasing small-scale turbulence within the cellular microenvironment through flagellar beating, cell surface/nutrient-ion interactions may be maximized. Flagellates may be capable of rather extensive vertical

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migrations, at maximum rates of 1 to 2 m/hr.[386] Kamykowski and Zentara[387] showed that the ability of various flagellate species to cross a temperature gradient may permit such species to ascend and descend through thermocline barriers. Although the argument that flagellate vertical migration may be a strategy enabling utilization of higher subsurface nutrient concentrations is most often advanced with reference to N uptake and assimilation,[388-390] this suggestion should be considered relevant for P_i uptake as well.

b. Effects of Cell Size and Morphology

On purely geometrical grounds it would be expected that a selective competitive advantage for P_i uptake would be exhibited by small spherical cells, which have maximal surface area/volume (S/V) ratios for nutrient absorption. When the growth kinetics of two tropical neritic species, the diatom *Biddulphia sinenis* and the dinoflagellate *Ceratium furca*, were compared under P_i-depleted conditions, the larger diatom displayed a higher K_t for growth than the dinoflagellate, which was at least partly attributed to slower uptake.[123] Similarly, Eppley et al.[409] were able to show a correlation between K_t values for nitrate and ammonium uptake and cell size. In view of the taxonomic differences between species which have been investigated and the consequent differences in physiological strategies for P utilization, as well as the much greater vacuolar size of many diatoms and large dinoflagellates when compared with smaller flagellates, it would be unwarranted to conclude that cell size is the determining factor in uptake kinetics. Such simplistic assumptions have been contradicted by experiments which failed to show significant dependence of growth rates[672,673] and P_i uptake rates[9] upon cell size. Nevertheless, although the rule may not be invariably applicable, it is typical for smaller cells to maintain higher uptake rates per unit cell volume than larger cells of the same approximate morphology.[392] Similarly, Munk and Riley[122] and Smith and Kalff[658] suggested an inverse correlation between uptake per unit mass and cell size. In support of these hypotheses, Correll et al.[8] found that although dinoflagellates represented around 90% of the total phytoplankton biomass during an estuarine dinoflagellate bloom, the total nanoplankton P_i uptake rate exceeded that for the dinoflagellates, since nanoplankton uptake rates per unit cell volume were 300 to 500 times greater. On the basis of evidence from $^{33}P_i$ microautoradiography for an estuarine phytoplankton community, Friebele et al.[392] maintained that uptake velocity per unit biomass is a direct function of the S/V ratio. However, one cautionary point must be made: all 10 species studied in the size fraction >5 μm were flagellates, and eight of the 10 species were dinoflagellates, whereas the smaller nanoplankton fraction consisted primarily of unidentified microflagellates, chrysophytes, chlorophytes, and small diatoms. Nutrient stress may have been responsible for the small size of the diatoms, resulting in enhanced uptake rates which were not a direct function of cell size, but rather cell nutrient status. Furthermore, the major fractions examined may be discriminated not only on the basis of cell size or biomass, but may be described as representing two relatively distinct taxonomic groupings, with potentially different P-requirements.

The bewildering array of cell sizes and morphologies exhibited by phytoplankton cells suggests that there are compensatory mechanisms for remaining competitive while deviating from a small spherical form. Consider the following evidence from P_i-limited diatom cultures:[9] although the much larger *Thalassiosira weissflogii* would seem to be consistently outcompeted for P_i uptake by the smaller *T. pseudonana* on the basis of its higher K_t for uptake (1.72 vs. 0.58 μM) and lower S/V ratio, its higher V_m per unit surface area (7.26×10^{-9} vs. 2.0×10^{-9} μmol μm^{-2} day^{-1}), permitted it to survive with equivalent P requirements. In this case, the higher transport rate per unit surface area may be related to the production of more carrier protein or more rapid carrier turnover, although this

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was not directly demonstrated. Recent calculations for a large number of species tend to suggest a relative constancy of P_i uptake rate per unit surface area with changing size.[658]

Ultrastructural observations have shown that many species possess considerable surface complexity, with abundant convolutions, invaginations, and surface processes which increase S/V ratios for transport. As a general observation,[99] larger-celled species tend to sink faster than smaller ones. Increased sinking velocity would thus tend to outweigh the comparative disadvantage of lower S/V ratio for larger cells. Although accelerated sinking rates may be advantageous in P-depleted areas, in terms of maximizing nutrient exposure, high sinking rates result ultimately in the loss of cells from the euphotic zone. Some of the selective advantage of spherical shape with regard to nutrient uptake must be balanced against increased sinking rate loss. Grazing pressure may also impinge, with small spherical cells eaten in preference to long cylindrical and plate-like configurations.[122]

The nutritional status of cells may induce morphometric changes which ultimately affect cell size, uptake kinetics, and sinking rate. For some species in P_i-limited continuous culture, including *Ankistrodesmus falcatus,*[100] *Scenedesmus,*[93] and *Euglena gracilis,*[561] cell volume varied inversely with growth rate. Upon entry into P depletion, cell size of the chlorophyte *Pediastrum duplex* increased, with an increase in V_m for P_i uptake correlated with an expanded cell surface area.[558] Such an increase in cell size induced by P exhaustion is often attributed to the accumulation of storage products, particularly carbohydrates,[558] and the inability of cells to complete mitosis and/or cytokinesis.[93,109,558] However, the trend towards increased cell size noted above is not universal, since for three other species cell volume remained approximately constant under P_i-limited conditions.[100] When grown in a medium with glycerophosphate as the sole P source, *S. obtusiusculus* became predominantly colonial, rather than the solitary form typical of growth on P_i.[124] Under P_i limitation, many freshwater planktonic species secrete a mucous-like substance resulting in cellular aggregation and increased sinking velocity.[99] However, for *Asterionella formosa,* the number of cells per colony decreased linearly with declining growth rate, under P_i-limiting conditions, to a minimum of two cells per colony.[125] Therefore, it is not necessarily valid to consider changes in cell or colony size and morphology as specific adaptive responses for increasing sinking velocity in nutrient-limited situations.

c. *Diffusion Transport Limitation and Sinking Rate*

Theoretically, stationary cells in a quiescent low P environment could be limited by the diffusion rate of the nutrient into the cellular boundary layer. The conventional approach to nutrient uptake kinetics, for example, the Michaelis-Menten model (Equation 2) makes the assumption that the external P_i concentration of the medium also represents the P_i concentration at the cell surface. However, if the cellular uptake rate were to exceed the rate of molecular diffusion to the cell membrane, transport limitation would occur. A transport limitation component of P_i uptake may be incorporated into the Michaelis-Menten uptake model, where the P_i concentration is considered to be that at the cell surface rather than the integrated ambient concentration.[126] In spite of the potential importance of such theoretical considerations, the experimental determination of nutrient concentrations at the cell boundary layer remains problematic.

The transport limitation phenomenon has been demonstrated in quiescent cultures of the diatom *Ditylum brightwellii* in low nitrate and nitrite medium,[127] and, from theoretical considerations, is suspected to occur with certain other potentially N-limited species in stationary medium.[126] However, comparable diffusion limitation of P_i uptake has not been adequately investigated. In dense laboratory cultures, cells with overlapping zones of microdepletion could produce a similar effect. Since transport limitation is minimized by dynamic systems and completely eliminated at high shear, it is

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unlikely that diffusion limitiation of P_i uptake has any substantial impact in aquatic ecosystems, except possibly in poorly mixed oligotrophic waters.

For stationary single spherical cells, Pasciak and Gavis[127] suggested the use of a unitless parameter, P, to establish a relationship between cell size, nutrient concentration, and the Michaelis-Menten constants, where:

$$P = 14.4 \pi R D K_s/V_m, \quad (6)$$

and R = the cell radius (cm), K_s = the half-saturation constant for uptake of the limiting nutrient (μM), V = the maximal uptake rate (μmol cell^{-1} h^{-1}), and D = the nutrient diffusivity. For this model, small P values would be indicative of diffusional transport limitation of nutrient uptake, when the nutrient concentration is low. For a spherical cell, the diffusion rate into the cellular boundary layer may be assumed to be essentially unidimensional, where:

$$dN/dt = -kA\ dN/dx, \quad (7)$$

with N = the ambient nutrient concentration, k = the molecular diffusion coefficient, A = the spherical surface area ($4\pi r^2$), and x = the width of the cellular boundary layer. Although the above equations are directly applicable only to spherical cells, modifications may be made to account for "shape factors". However, in such cases simplifying assumptions may yield only low accuracy numerical solutions.

In his critical review of the earlier work of Munk and Riley,[122] Gavis[128] contended that the enhancement of uptake through increased sinking is substantial only for larger cells which have a small value of the parameter P. For cell movement due to sinking, floating, or swimming, a modified parameter, P′, which takes into account the velocity of the cell relative to the medium, was proposed.[126] For *Gymnodinium splendens* in a nonmotile state, low calculated values of the P parameter for nitrate uptake were indicative of potential diffusion transport limitation. When motility was considered, the higher P′ value for this species suggested a reduction, but not elimination, of the transport limitation effect. Nevertheless, swimming is apparently more effective at eliminating such an effect than sinking. Cannelli and Fuhs[129] showed that unless cells move at high velocities, they are rarely exposed to the integrated ambient P_i concentration, particularly when that concentration is very low.

P_i-limited cultures of the diatoms *Asterionella formosa, Melosira agassizii, Cyclotella meneghiniana*, and the chlorophyte *Scenedesmus* showed increased sinking velocity as P_i was depleted from the medium.[99] Stationary phase cells exhibited a fourfold increase in sinking rate over exponentially growing cells. In natural ecosystems, the enhanced sinking rate of P_i-limited cells would enable them to more efficiently utilize subsurface maximal nutrient concentrations above the pycnocline. Upon replenishment of P reserves, decreased sinking velocity would tend to minimize cell loss from the euphotic zone.

The best available data relating sinking rate effects to P_i uptake were obtained by passing $^{32}P_i$-labeled medium at defined flow rates across cells adhering to membrane filters.[129] In this way the "sinking" of two different sized species of *Thalassiosira* was simulated. However, this technique must be viewed critically since, unlike the natural free-floating condition, attachment to the filters did not permit uniform medium access to the entire cell surface. The filter-attached condition more closely approximates uptake by benthic forms subject to abundant water movement. As might be expected, for P_i concentrations ranging from 1 to 100 μM, the P_i uptake velocity for both species increased with the flow velocity. As "sinking rate" increased, the P_i concentration within the cell boundary layer hyperbolically approached the integrated ambient P_i

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concentration in the medium for the larger-celled *Thalassiosira weissflogii;* for the smaller-celled *T. pseudonana,* cell boundary layer concentrations never exceeded 80% of the integrated ambient P_i concentration, even at the highest flow ("sinking") rate. The above results should be compared with the work of Fuhs et al.[9] who established that the effects of the more favorable S/V ratio for the smaller species were partially offset by the higher transport velocity per unit surface area for the larger species.

The experiments of Canelli and Fuhs[129] seem to support the earlier hypothesis of Munk and Riley[122] that P_i-uptake rate is partially a function of the cell velocity in the medium. However, it did not seem likely to these authors,[129] that stationary cells of the two *Thalassiosira* species studied would be subject to growth limitation by P, since the P_i uptake rates were consistently greater than those required to supply P at growth rate limiting concentrations, even at zero flow rate. According to a recent view of transport limitation,[128] since the half-saturation constant for P_i uptake greatly exceeds that for growth, growth rate is less susceptible to transport diffusion limitation than uptake rate.

d. Microfibrils and Phosphate Availability

Along with the complement of physiological strategies for dealing with P limitation, certain species of microalgae have surface ultrastructural modifications which may serve as binding sites for P_i and other ions. However, the mere possession of ion-exchange capabilities by these fibrils does not sufficiently substantiate their putative role in ion uptake. A variety of alternative hypothetical functions have been proposed, including locomotion,[130] reduction of sinking velocity,[131] binding of heavy metals and other cations,[35] heterotrophy,[132] extracellular storage,[20] attachment during sexuality,[133] and facilitation of cell adhesion.[35,560] These functions are not mutually exclusive, and it is likely that microfibril production represents a multipurpose adaptation.

Early ultrastructural observations of diatoms by electron microscopy[130] revealed the existence of minute fibrils extending from the raphe fissures. More recent examination of the diatom species *Cyclotella cryptica, C. meneghiniana, Thalassiosira pseudonana,* and *T. weissflogii* indicated that these fibrils originate from special invaginations of the plasma membrane.[131,134] Chemical analysis of fibrils isolated from *T. weissflogii*[132] showed that they were composed primarily of chitan (poly-N-acetyl glucosamine), which possesses substantial ion-binding ability.

Leppard and Massalski[35,559,560] have examined fibrils produced by freshwater prokaryotes and eukaryotic microalgae. While they do not exactly resemble the chitan fibrils of marine diatom species,[131,134] they are, nevertheless, rigid enough to suggest a possible analogy. These microfibrils often appear as ribbon-like strands on the cell surface of freshwater microalgae and bacteria.[559,560] The fibrillar strands are typically 3 to 10 nm in diameter,[35] and are, thus, within the colloidal size range. In addition to the ribbon-like fibrils found at the cell surface, electron microscopic analysis of freshwater samples has revealed a rather morphologically diverse array of external fibrillar material produced by cells (see Massalski and Leppard[560] and references therein for details). Although fibrillar material is often found directly attached to cell surfaces, it may also occur as free aggregates in the water or be associated with sediments.[35,559] Analysis of the fibrillar fraction from lake water indicates a 20 to 30% uronic acid component, sufficiently rich in carboxyl ligands to serve as an organic carrier.[35] Such microfibrils can be readily dislodged from the cells by filtration and it has been suggested[25] that fibril-bound phosphate can be directly assimilated into cells. Microfibrils may also serve as an external reservoir of colloidal phosphate. By ooserving the rapid surface binding of $^{33}P_i$ label to assemblages of lake water microorganisms, including the cyanophyte *Anabaena* and bacteria, using microautoradiography, and by scanning electron microscopic examination of the fibrillar colloidal-size matrices attached to or loosely associated with planktonic cells, Paerl and Lean[20] established an essential link between excreted

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phosphorus and colloid-bound phosphorus. Cells labeled with $^{32}P_i$ returned a substantial quantity of labeled compounds to fibrillar colloidal fractions within 2 hr.[20] After 4 hr a high level of activity was detected in the fibrillar and amorphous cell components. Excretion of labeled organic phosphate into an extracellular fibrillar component tends to support a storage role for this material. In terms of metabolic P requirements, it is somewhat difficult to explain the role of microfibrils as an external P reservoir subject to reuptake, especially since production is apparently maximal when cells are already P deficient.[20] Energetically, it would seem to be more favorable to retain residual P metabolites intracellularly as polyphosphate (see Section V), rather than to expose the limiting resource to competitive P uptake demands made by other organisms. However, if excretion of P metabolites is to occur, production of a colloidal-bound form, as opposed to excretion of free P_i, must be seen as advantageous in reducing loss of P nutrients to sediments through ion complexation.

6. Uptake Rhythmicity

Since many investigators have noted the stimulative effect of light on P_i uptake (see Section III.D.1), it would not be unreasonable to expect a diel rhythm for uptake in natural phytoplankton assemblages. Indeed, phytoplankton off South California did exhibit a diel periodicity in P_i uptake.[117] Similarly, in another experiment shipboard incubations of natural assemblages from the California coast also revealed a periodic maximal uptake rate during the day, with a corresponding minimum between 1800 to 2400 hr.[135] For lake phytoplankton, the P_i uptake pattern at ambient concentrations was temporally phased such that maximal uptake by the total assemblage occurred in the morning, whereas selected large diatoms exhibited maximal uptake in the evening.[434]

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However, in contrast to the above observations, natural populations from the oligotrophic central North Pacific showed neither light stimulation nor periodicity in P_i uptake.[57,368] The lack of detectable diel rhythmicity in P_i uptake for these populations may be a reflection of the nutritional status of the cells, as nitrogen levels were apparently limiting, and P concentrations also approached zero, suggesting the possibility of P_i limitation as well. Thus, the most plausible interpretation of the discrepancies between uptake patterns in this oligotrophic area and those observed in other studies is that the stimulative effect of light is most significant when phosphorus is obviously not a limiting nutrient. Evidence that light stimulation of P_i uptake is more pronounced when P_i is saturating[5,86] supports this contention.

Under illumination, and when the external P_i concentration no longer limits uptake, excess photosynthetic energy may be diverted towards increased uptake, thereby satisfying the high demands for phosphometabolite synthesis, while swamping the contributions of respiratory energetic pathways to P_i uptake. On the other hand, since nutrient-stressed cells tend to have higher respiration rates,[136] energy production for P_i uptake may shift towards respiratory transduction and away from photoperiod-dependent reactions under P_i limitation. When intracellular P levels are depleted, the constant cellular demands for maintenance P_i levels may be better satisfied by relatively nonperiodic respiratory activity.

This is undoubtedly a simplistic view of the light-stimulated periodicity effect. Sournia[45] has reviewed a number of phytoplankton physiological characteristics which exhibit diel periodicity, including cell division, sinking and migratory movements, photosynthetic capacity, respiration, and nutrient uptake kinetics. Since these characteristics are mutually interdependent, periodicity in P_i uptake is best understood as the result of integrated interplay between a number of rhythmic physiological processes. Thus, the ability of P_i addition to stimulate an increase in photosynthetic capacity to a maximal value within several hours after enrichment[436,437] must be superimposed upon

the endogenous diel rhythmicity in photosynthetic capacity (Sournia[45] and references therein).[436,437] For laboratory cultures of *Chlorella pyrenoidosa*[495] and another chlorophyte, *Pediastrum duplex*,[558] photosynthetic rates at saturating irradiance were dependent upon the cell P quota, thereby indicating the indirect mutual interdependence of P_i uptake and photosynthesis. High photosynthetic capacity results in increased P demand for synthetic reactions, which may be turn induce greater activity of the P_i uptake mechanisms.

Controversy has arisen as to whether observed rhythms in P_i uptake are tightly coordinated with sequential events in the cell division cycle, or represent an endogenous response. Eppley et al.[135] suggested that the apparent increase in uptake rate, noted just before dawn for natural marine phytoplankton assemblages, may be partially attributed to high metabolic demands for phosphometabolites specific to a cell cycle event. In a P_i-limited cyclostat, maximal cell division of *Euglena* occurred during the dark period.[137] For such phased populations, P_i uptake was rhythmic, and, while K_s remained constant, maximal uptake rates were expressed toward the end of the light period.[86] In P_i-sufficient batch cultures, oscillations in both V_m and K_s were observed with an apparent shift in the highest V_m values to the middle of the light period.[97] Uptake maxima were therefore temporally coordinated with peaks in photosynthetic capacity. This periodicity was maintained even in nondividing stationary phase cells[97] and in the cyclostat in which less than one third of the cells underwent division per light/dark cycle.[86] Although cell division may be more tightly phased in laboratory cyclostats than in nature, due to the rigid regulation of the photoperiod and temperature, such experiments serve to illustrate that uptake oscillations are probably not tightly coupled to the cell division cycle, and may represent an endogenous mechanism.

IV. UTILIZATION OF DISSOLVED ORGANIC PHOSPHATES BY MEANS OF PHOSPHATASES

A. Enzyme Definition and Substrate Specificity

The availability of organic phosphates (specifically phosphate monoesters) as a potential nutrient source for microalgae is generally contingent upon the ability of the cells to enzymatically cleave the ester linkage joining the P_i group to the organic moiety. For most species, such hydrolysis is achieved by the activity of broad-spectrum phosphomonoesterases (commonly called phosphatases) at the cell surface. These enzymes are commonly classified as either alkaline or acid, according to their pH optima, and generally exhibit a high degree of specificity for the phosphate moiety of the P-O-C bond of phosphate monoesters (see Figure 2), but little specificity for the carbon moiety.[237,238] Thus, P_i may be released from a wide variety of phosphate monoesters such as polyol and sugar phosphates, nucleoside monophosphates, phenyl and *p*-nitrophenyl phosphates, phosphoenol pyruvate, phosphocholine, phosphoethanolamine, phosphoserine, etc., and this broad spectrum is true, albeit with some differences, of both alkaline[138] and acid[139,175,183] phosphatases. Furthermore, this relative lack of specificity for the organic moiety may extend beyond phosphate monoesters in some microbial and plant phosphatases, where phosphate diesters (e.g., bis[*p*-nitrophenyl]phosphate)[140,175] or phosphoanhydrides, such as inorganic pyrophosphate,[138,175,183] ADP, and ATP,[141,175,183] can also serve as substrates. Nevertheless, both acid and alkaline phosphatases showed no lipoid phosphodiesterase activity (implicit in phospholipases C and D; see Section V.E) towards lecithin and acted only on phospholipids with a phosphate monoester group on the C_3 position of glycerol.[142] Similarly, the acid phosphatases of the chrysophyte *Ochromonas danica* could not hydrolyze lecithin or DNA.[141] Interestingly, bacterial alkaline phosphatase was also found to catalyze the hydrolysis of P-N bonds in

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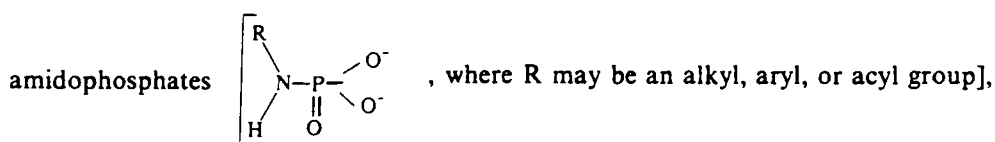

indicating a much greater range of substrates available to the so-called nonspecific phosphatases than is generally believed.[143]

However, we must point out that most of the reported research on eukaryotic algal phosphatases has been grossly negligent in not testing even the minimal range of utilizable substrates (apart from the standard substrate of assay methods, viz. *p*-nitrophenyl phosphate) and many investigators have hastily tended to draw vague *nonspecific* conclusions from results previously obtained with bacteria, fungi, or higher plants. Since highly specific phosphatases are also known for certain monoesters (or phosphoanhydrides), such as phosphoglycolate,[144,145] 3-phosphoglycerate,[146] fructose-1,6-diphosphate,[147,148] sedoheptulose-1,7-diphosphate,[149] and inorganic pyrophosphate,[49,150,189] this tendency to ignore or overlook phosphatase specificity for the organic C-moiety is considered undesirable and misleading. Perhaps the sternest warning against this tendency arises from the surprising finding of an alkaline phosphatase in yeast, which showed little or no action on all the common phosphate esters tested, but proved entirely specific toward *p*-nitrophenyl phosphate,[151] and yet this latter substrate is frequently used as the only substrate in recommended methods[152] for identifying supposedly *nonspecific* phosphatase activity in unicellular algae (e.g., see references[32,153,154,156]). This substrate specificity problem is further complicated by the occurrence of two or more nonspecific phosphatases in the same organism, where these multiple enzymes may show great differences in preference for one or more substrates,[155,176] and the 'total' phosphatase activity assayed with a single standard substrate may, at best, reflect a poor average of the actual enzyme potential of the organism.

B. Dissolved Phosphatases in Natural Waters

Using sensitive assay methods, various levels of dissolved phosphatase activity have been detected in neritic[30,194] and oceanic waters,[56] neritic and oceanic sediments,[159] and lake waters.[160–162] The production of these enzymes in the aquatic environment has been generally attributed to P_i-limited algae,[53,163] but evidence has also been obtained for additional production by zooplankton[164] and bacteria.[165] Substrate specificity problems, such as those discussed above, were encountered at least in the case of one mesotrophic and one eutrophic lake, where about 50% of the DOP was found to be resistant to commercial preparations of alkaline phosphatase and phosphodiesterase but hydrolyzable by a wheat-bran (nonspecific) phosphatase capable of additional action on phytic acid (myoinositol hexaphosphate).[166] More recently, a phytic-acid active alkaline phosphatase was identified in the sheath material and surface hairs of a P_i-starved blue-green alga,[649] some of which is likely to be dispersed through lake water in a colloidal or "dissolved" state. Dissolved alkaline phosphatases were reported to be capable of directly degrading particulate matter in the water column of lakes, resulting in rapid P_i release.[157] In natural systems, the role played by such phosphatases may be highly significant, perhaps determining the outcome of interspecific competition when P_i is in short supply. Kuenzler[54] showed that algal species, which did not produce cell-surface phosphatases, were incapable of assimilating phosphate from glucose-6-phosphate, and soon became P deficient, leading to cessation of growth.

It should also be noted that dissolved or extracellular phosphohydrolytic enzymes are not always nonspecific, since specific deoxyribonuclease activity has been identified in natural seawater.[168] Interestingly, extracellular cyclic-AMP was recently shown to be

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released into lakes by several algae,[201] both eukaryotic[198-200] and prokaryotic,[200,202] but its function appears to be complex, and it is not known if it is hydrolyzed by specific or nonspecific extracellular phosphatases, nor if it is reutilized intact by the producer or other algae.

C. Occurrence of Multiple Phosphatases in Algae

Like those of bacteria, fungi, and higher plants, the phosphatases of algae appear to be *constitutive P_i-irrepressible enzymes* or *phosphate ester-induced P_i-repressible enzymes,* and both types often occur simultaneously in the same organism. Consequently, much effort has been expended in attempts to establish the nature of these phosphatase components in algae. One of the earliest efforts was due to Antia and Watt,[153] who found considerable constitutive acid phosphatase but no alkaline phosphatase activity in cell-free extracts of three marine diatoms, two chrysomonads, and one chlorophyte, cultured in phosphate-enriched seawater medium and harvested towards the end of exponential growth. Likewise, Lasman and Kahan[156] found only acid phosphatase in log-phase cells of five species of *Ochromonas*. On the other hand, Patni et al.[174] observed both acid and alkaline phosphatase activities (stated to be P_i-irrepressible) in log-phase cell homogenates of *O. danica*. Surprisingly, *Euglena* produced only acid phosphatases (two constitutive, one P_i-repressible) even under conditions of P_i-limitation in the stationary phase of growth.[183] Perhaps the most extensive survey of phosphatases in marine microalgae was carried out by Kuenzler and Perras,[55] who examined 27 species (including 12 diatoms, 4 chlorophytes, 4 chrysomonads, 3 cryptomonads, 2 dinoflagellates, 1 eustigmatophyte, and 1 cyanophyte) and found both acid and alkaline phosphatases in 13 species, alkaline phosphatase only in 11 species, and acid phosphatase only in 3 species; virtually all the alkaline phosphatases appeared to be P_i-repressible. Interestingly, *Scenedesmus quadricauda* could not utilize exogenously supplied phosphomonoesters or pyrophosphate although it contained at least three intracellular phosphatases unable to reach the cell surface;[186] however, the alga appeared to overcome this handicap by obtaining utilizable P_i through the phosphatases produced by associated bacteria.[187] We have noted some major differences (or discrepancies) between certain investigations dealing with the same algal species or related clones in the same genus. Thus, *Dunaliella tertiolecta* showed significant acid phosphatase production and no alkaline enzyme under the culture conditions of Antia and Watt,[153] but no production of either enzyme under those of Kuenzler and Perras.[55] Two estuarine clones of *Chlamydomonas* produced no acid phosphatase and one to two P_i-repressible alkaline phosphatases,[55] while the freshwater species *C. reinhardi* produced two constitutive acid phosphatases and three P_i-repressible (one neutral, two alkaline) phosphatases.[188] In the latter case, the separation of the numerous multiple enzymes required sophisticated protein resolution by polyacrylamide gel electrophoresis of crude algal extracts. Using similar techniques, Wynne[158] succeeded in separating at least five intracellular (three constitutive acid and two P_i-repressible alkaline) phosphatases from a *Peridinium* bloom in Lake Kinneret (Israel).

D. Characteristics of Multiple Phosphatases

The cell-surface P_i-repressible enzymes responsible for extracellular orthophosphate cleavage are generally distinct from the intracellular phosphatases of intermediary energy-deriving metabolism. Although the surface (or extracellular) phosphatases are generally operative at alkaline pH, the formation of similar phosphatases with pH optima in the acidic range is known for certain species of both marine and freshwater algae. Thus, Kuenzler and Perras[55] demonstrated the formation of both acid and alkaline

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P_i-repressible phosphatases by an estuarine dinoflagellate, Price[169] found only the acid phosphatase in *Euglena gracilis*, Patni et al.[167] noted the secretion of either the acid only or both acid and alkaline phosphatases by *Chlamydomonas reinhardi* depending upon the growth medium used, while Patni and Aaronson[141] reported the formation of an extracellular acid phosphatase by *Ochromonas danica*. Intracellular acid phosphatases are known to play crucial and multifarious roles by participating in cleavage and phosphate transfer reactions of both biosynthesis and catabolism of cell structural and dynamic components, involving phosphate monoesters as well as diesters, pyrophosphates, and polyphosphates. Depending on their location inside the cell, intracellular acid phosphatases may be of two types: (1) allosteric regulatory enzymes specific for particular phosphate esters (e.g., fructose-1-6-diphosphatase)[147,170] involved in essential metabolic pathways (e.g., gluconeogenesis) and localized in the appropriate organelles (e.g., chloroplast); and (2) nonspecific enzymes active on many phosphate esters and localized in lysosome-like vacuoles,[171-173,185,210] where the phosphatases may serve a general catabolic function in autophagic reactions securing release of P_i. The extent of the fine line dividing nonspecific intra- and extracellular acid phosphatases is not known, but at least in the case of log-phase *O. danica* sufficient differences in properties were observed to suggest that they were different enzymes,[141] although both appeared to be partially P_i-repressible.[184]

Apart from pH optima and P_i-repressibility, other properties have been used to distinguish the acid from alkaline phosphatases. The former enzymes generally show no divalent-metal ion requirement for activity and no inhibition from chelators (EDTA, citrate),[139,174,175,177,183] but appear to be specifically inhibited by fluoride.[141,158,175-177] The latter enzymes are generally stimulated by (or require) specific divalent-metal ions, in particular Mg^{2+},[165,174,178,557] or Zn^{2+},[179] or both,[180] and are inhibited by chelators,[158,174,178] but not by fluoride.[158,179] In fact, the alkaline phosphatases of *Escherichia coli* and *Neurospora crassa* have been shown to be zinc metalloenzymes.[180,181] Both acid and alkaline phosphatases show competitive inhibition from arsenate.[141,175,176,179,182,183]

E. Pyrophosphatase Function

The suspected role of extracellular or surface nonspecific phosphatases in algal utilization of exogenous inorganic pyrophosphate as a phosphorus source[557] remains ambiguous, but, at least in four cases, so-called pyrophosphatases have been identified in this connection. In *Chlorella*, a cell-wall pyrophosphatase was induced from exposure to pyrophosphate and lost after the adaptation when the substrate was removed,[51] while in *Prymnesium parvum* the pyrophosphate appeared to be taken up intact and hydrolyzed intracellularly by a specific pyrophosphatase which was readily solubilized and hence believed to be cytoplasmic or membrane-loose in some subcellular organelle.[49] Since the enzyme from *Chlorella* was not tested on any organic phosphate, it is not unlikely that it may have been a P_i-repressible nonspecific phosphatase rather than a specific pyrophosphatase. However, the *Prymnesium* enzyme was clearly inactive towards the phosphate esters tested and is likely a specific pyrophosphatase. A third case of reported pyrophosphatase was that from the "naked" (cell-wall lacking) chlorophycean alga *Dunaliella tertiolecta*, where a surface-membrane-bound, Mg^{2+}-activated, and presumably constitutive (P_i-irrepressible) alkaline phosphatase was observed,[635] but, like the case of the *Chlorella* enzyme, its specificity was not checked on any organic phosphate and therefore remains doubtful. Interestingly, the rhodophyte *Porphyra* produced a specific pyrophosphatase with additional hydrolytic action on tripolyphosphate and hexametaphosphate;[241] however, the likelihood of multiple enzymes acting individually, although simultaneously, on the polyphosphate and metaphosphate was

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not explored. This was also the case with a report on the hydrolytic utilization of polyphosphates as P source for growth of *Chlorella*, where the identified pyrophosphatase was believed to be responsible.[51]

F. Mechanism of Induction of P_i-Repressible Phosphatases

The essential feature of phosphatases which allows them to participate efficiently in cellular metabolism is the ability to be alternately induced and repressed, depending upon metabolic requirements. The increased hydrolysis of stored polyphosphate by intracellular acid phosphatases, accompanied by a concomitant increase in external alkaline phosphatase activity,[190] is characteristic of this interrelationship. Upon entry into P_i limitation, cells typically exhibit an increase in alkaline phosphatase activity, the magnitude of which is dependent upon a species-specific response, the availability of organic phosphates, and the degree of phosphate limitation experienced by the cells. Such enzyme induction presumably entails *de novo* protein synthesis, since agents known to interfere with protein synthesis, such as cycloheximide, were found to inhibit acid phosphatase formation by *Ochromonas danica*[191] and *Chlamydomonas reinhardi.*[192] Nevertheless, we must caution against overgeneralization of the belief that intracellular stored phosphate must be exhausted along with external P_i in order to induce P_i-repressible phosphatase, since, in the case of a yeast,[197] the enzyme was induced only after external P_i depletion while significant amounts of intracellular P_i and polyphosphate were still present. Furthermore, evidence was recently obtained in the case of the dinoflagellate *Pyrocystis noctiluca*,[557] where the induced alkaline phosphatase activity was not altered by inhibitors of protein synthesis, but instead its P_i repression was largely eliminated, suggesting that a P_i-induced protein, rather than P_i itself, inhibited the phosphatase synthesis.

The inverse relationship between low external P_i concentration and alkaline phosphatase activity is well documented. Fitzgerald and Nelson[53] reported that for freshwater green algae and diatoms, alkaline phosphatase activity was 5 to 20 times greater when the cells became P_i limited. The detection of significant alkaline phosphatase activity in Lake Kinneret was associated with a declining bloom population of *Peridinium cinctum* at a time when P_i was no longer mixed into the epilimnion.[158] In an earlier Lake Kinneret study,[160] a reciprocal relationship was discovered between alkaline phosphatase activity per unit biomass and total P_i concentration, which varied seasonally. Using a sensitive fluorometric technique for phosphatase detection in central North Pacific waters, Perry[56] substantiated that maximum phosphatase activity was typically present when P_i levels were undetectable. When deep-water phytoplankton-containing samples with detectable P_i, but low alkaline phosphatase activity, were incubated at *in situ* temperatures without further addition of P_i, alkaline phosphatase activity increased markedly. The difference in specific enzyme activity, when comparing low P_i surface waters with higher P_i deep water, tends to support the idea that induced phosphatase activity is an adaptation to utilize the substantial DOP reserves in the upper layers. This induction of high phosphatase activity is a specific cellular response to only phosphate depletion, and not to depletion of other nutrients. Thus, P_i-repressible alkaline phosphatase activity in P_i-limited cultures achieved levels in excess of 30-fold greater than for cultures subjected to equivalent nitrogen, silicate, vitamin, or trace-metal limitation.[55]

Under conditions of sufficient assimilable P_i, the demands upon DOP supplies are reduced, typically resulting in the repression of alkaline phosphatase activity. For the bacterium *Micrococcus sodonensis*, active repression of extracellular alkaline phosphatase production was observed upon P_i addition to the culture medium.[193] Similarly, the extracellular diesterase and 5'-nucleotidase production were reduced under this

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condition, but in their case the repression was partly reversed by the addition of supplementary organic nutrients. In laboratory cultures of the dinoflagellate *Pyrocystis noctiluca*[32] isolated from tropical waters, alkaline phosphatase activity was depressed at ambient P_i concentrations in excess of $0.05 \mu M$, while, at concentrations approaching 3.0 μM, new enzyme synthesis was completely suppressed, apparently through the action of a P_i-induced protein.[557]

The concentration of external inorganic phosphate at which repression occurs varies widely from species to species; however, the repression phenomenon is undoubtedly widespread, if not ubiquitous.[32,56,92,169,192,595] For *Ochromonas*, subjected to P_i limitation,[184] acid and alkaline and phosphatase activities were only partially repressed upon addition of exogenous P_i. In eutrophic Tokyo Bay, there was a positive correlation between alkaline phosphatase activity and P_i concentration, which suggested that, at least at the measured ambient P_i levels, enzyme activity was not significantly inhibited.[194] It is not unlikely that the presence of high concentrations of DOP, expected to enhance the normal induction of phosphatase activity, may serve to counteract the normally suppressive response of significant ambient P_i concentrations.

There is considerable variation between some algal species subject to comparable phosphate limitation stress, with respect to the induction of phosphatases. In comparing two marine diatoms, Møller et al.[154] found that the alkaline phosphatase induction of *Chaetoceros affinis* was consistently much higher than that of *Skeletonema costatum*. In other algal species, such alkaline phosphatase levels may be either below the level of detectability or noninducible by P_i deficiency.[110,186] Although it is tempting to speculate otherwise, Kuenzler and Perras[55] were unable to define any dependable relationship between the natural habitat of selected species and the inducible enzyme activity of their alkaline phosphatases. This may also be true of taxonomically related species.[54]

The metabolic regulation of phosphatase synthesis in terms of induction/repression mechanisms is crucial to the understanding of cellular utilization of organic phosphates and the detection of phosphorus limitation. Since the presence of organic phosphates, such as β-glycerophosphate[195] and glucose-1- or -6-phosphate,[54,184] stimulate alkaline phosphatase activity, it is possible that they directly induce the genes responsible for the synthesis of such enzymes. Healey[110] points out that, since alkaline phosphatase is normally induced shortly after the depletion of P_i from the medium and before the growth rate declines, the inductive effect may be more reasonably attributed to internal phosphorus compound(s), rather than a change in response to growth rate. Nevertheless, present evidence indicates that the repressive effect on *de novo* phosphatase synthesis is exerted primarily upon the mRNA transcriptional mechanism, but there is also a secondary means of enzyme regulation, with the internal pool products functioning as end-product modulators.[158,192,595]

Using differential labeling with $^{14}C/^{3}H$ and enzyme separation by electrophoresis, the *de novo* synthesis of acid phosphatase was clearly demonstrated in synchronous *Chlamydomonas* cultures.[192] The same authors employed ^{32}P labeling to show that when the intracellular P_i pool decreased, with a consequent rise in organic phosphate constituents, derepression of phosphatase resulted. Although the authors attributed the derepression effect to low internal P_i levels, the possibility is not precluded that the trigger may be an intracellular organic phosphate compound. Further proof that algal synthesis of inducible acid phosphatase may be considered *de novo* is indicated by the suppressions observed for the *Euglena* enzyme by actinomycin D, known to inhibit mRNA synthesis, and by *p*-fluorophenylalanine, known to interfere with phenylalanine incorporation into new protein.[196] Since, after P_i enrichment of the medium, the normal decrease in the induced activity could not be further suppressed by actinomycin D, it was inferred that new mRNA was not required for P_i repression.

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Too little is known about the influence of environmental variables on algal phosphatase induction, but there are several lines of evidence to indicate the dependence of alkaline phosphatase activity on irradiance and photoperiod. Recently, Rivkin and Swift[32] studied diel and vertical variations in alkaline phosphatase activity for the dinoflagellate *Pyrocystis noctiluca* from the Caribbean and Sargasso Seas, and discovered a diel pattern of highest activity during the day with the lowest occurring several hours before sunrise. A similar pattern was observed by Berman[160] in Lake Kinneret, which was attributed to increased phosphatase induction following rapid P_i exhaustion as a result of high photosynthetic production in the sunlit portion of the day. Laboratory studies by Rivkin and Swift[32] indicated again that enzyme activity was proportional to light intensity, with a positive relationship between activity and preconditioning irradiance rather than the irradiance supplied during the incubation period. As might be expected on the basis of phosphate concentrations of surface water, standing stock of plankton, and irradiance, alkaline phosphatase activity was highest in the subsurface mixed layer and lowest at the bottom of the euphotic zone.[32] The results of photosynthesis and respiration inhibitor studies also point to the relationship between light and phosphatase activity. When cells were exposed to prolonged darkness, DCMU, or CCCP, alkaline phosphatase activity was markedly reduced.[32] Similar results were obtained with CN^- and DNP, which suppressed activity completely within an hour.[32] Although this is not conclusive, the fact that in vitro alkaline phosphatase is not affected by these metabolic inhibitors suggests an interference with enzyme synthesis or transport in vivo from blockage of energy-yielding pathways rather than denaturation or inhibition of the enzyme itself. Presumably energy for enzyme synthesis is derived from photosynthetic or respiratory reactions, with illuminated conditions facilitating greater synthesis from surplus photochemical energy. However, the light-effected stimulation of phosphatase induction/release may not be always true, since there was little difference between the presence or absence of light on phosphatase secretion by the chlorophycean macroalga *Cladophora glomerata.*[190]

G. Cellular Location of P_i-Repressible Phosphatases

Although P_i-repressible phosphatases are generally believed to be cell-surface enzymes, their precise location has generally been difficult to pinpoint at the cellular level, and observations are easily confused by the production of artifacts and the limitations of the localization techniques used. There is considerable evidence to suggest that at least some phosphatases are truly "extracellular", i.e., excreted (or secreted) into the medium to exert their effect.[140,141,167,179,184,203] In determining the activity of alkaline phosphatase, diesterase, 5′-nucleotidase, and alkaline proteinase from the bacterium *Micrococcus sodonensis,* Mills and Campbell[193] indicated that in excess of 95% of the total activity was found in the cell-free culture supernatant. These investigators concluded that such enzymes are not periplasmic, as treatment of cultures with EDTA, expected to release such enzymes from cellular binding, did not yield significantly different results from the untreated controls. However, it must be pointed out that wherever maximal enzyme activity is identified with "mature", senescent, or "fragile-cell" cultures and the harvesting of samples involves relatively harsh treatment such as filtration or centrifugation, it may be difficult (if not impossible) to distinguish between excretions and the contributions from cell lysis. Such criticisms have been expressed by certain purists[204,205] and adequately countered by the strict criteria used by the secretion protagonists[206,207] for ascertaining in vivo excretion.

From the standpoint of molecular economy and cellular energetics, it would seem more efficient to retain such active enzymes at or just under the cell surface, and there is

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both cytological and biochemical support for this view. A histochemical study of *Chlorella vulgaris* by enzymatic staining methods localized alkaline phosphatase at the cell wall.[208] Surprisingly, cytological observations of *Chlamydomonas reinhardi*[192] indicated the presence of alkaline phosphatase on both sides of the plasma membrane rather than the cell wall. Using sucrose density-gradient centrifugation of cell homogenates, Patni et al.[209] succeeded in purifying a plasma membrane fraction enriched in both alkaline and acid phosphatases, along with (Na^+/K^+)-ATPase, from *Ochromonas danica*; since this alga lacks a cell wall, the association of the phosphatase sites with the plasma membrane is not surprising. However, a judgment needs to be made as to whether such phosphatase localizations represent the site of synthesis prior to export, the transient passage of enzyme through the membrane, or the active site itself. For *Peridinium cinctum*, with a cellulose-like cell wall, the P_i-repressible alkaline phosphatase appeared to be localized within the cellular interior,[158] possibly in association with the plasma membrane, and the evidence for its extracellular release was equivocal. Possibly the internal membrane is merely the site of synthesis, from which the enzyme is transported to the cell wall and subsequently secreted through its many pores known from ultrastructural and molecular studies.[211,212]

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It appears that for most bacteria and algae with cell walls, the P_i-repressible phosphatases lie in the periplasmic region between the cytoplasmic membrane and the cell wall. In Gram-negative bacteria such as *Escherichia coli*,[213] a marine pseudomonad,[214] and *Pseudomonas aeruginosa*,[215,216] the cell-bound alkaline phosphatase was found in the periplasmic space or sometimes attached to the appropriate periplasmic layer of the four-layered cell wall. Similarly, the alkaline phosphatase of a blue-green alga, with a Gram-negative type of cell wall, was found within the periplasmic space of the cell wall.[217] In the case of another blue-green alga, such cell-wall location of the enzyme was said to extend to the sheath material and surface hairs apparently developed under conditions of P_i deficiency.[649] The bacteriological evidence so far obtained has also indicated that Gram-negative bacteria with multilayered cell walls seldom release the periplasmic enzymes into the growth medium, while Gram-positive bacteria and cell-wall defective mutants of the Gram-negative species, lacking one or more intact cell-wall layers, are more prone to release or leakage of these enzymes.[214,218] In the case of eukaryotic algae, such cell-wall defective mutants have been reported for *Chlamydomonas reinhardi*,[219,220] where the cell-wall lesions caused strong leakage of the P_i-repressible alkaline and neutral phosphatases into the culture medium, and the overall evidence suggested that in the wild-type alga these enzymes were located in the space between the cell wall and plasmalemma, but not on or attached to the latter.[188] However, these investigators have pointed out that even the normal cell wall is not completely leak-proof, since a part of the P_i-repressible enzymes are normally released into the culture medium.[188] This has been explained by hypothesizing that the algal cell wall, at least in *Chlamydomonas*, may play the role of a molecular sieve by partly retaining the phosphatases during their transit to the outside of the cell;[188] presumably such intracellular retention may form part of a mechanism controlling extracellular release of the enzyme. In this connection, it is interesting to note that further evidence has been obtained from bacteria to suggest a mechanism in which the inactive monomer subunits of the enzyme, synthesized on the cytoplasmic polyribosomes, diffuse through the plasma membrane to a periplasmic region where dimerization occurs to form the active enzyme.[203] Nevertheless, it is not unlikely that part of the monomer subunits may be further released through the cell wall followed by extracellular dimerization, since such subunits have been identified in the culture filtrate of a pseudomonad.[203]

In the case of a dinoflagellate, several diatoms, and chrysomonads, it appears that the

P_i-repressible alkaline phosphatases are not loose or in solution in the periplasmic region, but firmly bound to the plasmalemma or a related membrane.[55,154,557] Thus, for the prymnesiophyte *Emiliania huxleyi* and the diatom *Phaeodactylum tricornutum*, repeated treatments involving sonication, explosive decompression, osmotic shock, freezing and thawing, heating, or exposure to lipase, cellulase, isobutanol, and various salt and buffer solutions failed to solubilize the enzyme.[55] The sonication of cultured cells of nine algal species, followed by centrifugation, showed little enzyne activity in solution either before or after sonication, while virtually all the activity known from whole cells was recovered in the centrifuged pellet.[55,557] Some of the solubilization methods used by these investigators may have been too drastic for retention of the "active" enzyme conformation in solution or may have caused protein denaturation. Nevertheless, it appears from the reported evidence that membrane binding may be critical for the optimal conformation and orientation of the enzyme complex required for maximal activity. Similar evidence for membrane binding of the P_i-repressible alkaline phosphatases was obtained for another marine diatom in another investigation,[154] where cell rupture by a French-press followed by centrifugation produced a soluble cytoplasmic fraction rich in a constitutive acid phosphatase and an insoluble particulate fraction rich in the alkaline phosphatase.

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H. Cellular Location of Constitutive Nonspecific Phosphatases

As already pointed out, these constitutive nonspecific phosphatases are generally acid phosphatases functioning inside algal cells and they are little influenced by P_i concentration in the culture medium. Too little is known about their exact biological function and why they occur as multiple enzymes or isozymes in some algae. The ready solubilization of these enzymes following cell disruption[139,153,156] and their oft-cited occurrence in cytoplasmic fractions[154,209] lend support to the general belief that they are cytosolic[210] or diffuse in the cytoplasm, or concentrated in lysosome-like vacuoles.[173,185,210] The cytoplasmic diffuse presence has been identified primarily with the Golgi complex, paramylum bodies, and peri-reservoir vesicles in *Euglena gracilis*.[221] A similar presence was shown to be associated with the maturing face of dictyosomes and related vesicles as well as lysosome-like and multivesicular vacuoles in aged cells of *E. granulata*.[222] In the case of *Chlamydomonas reinhardi*, only the soluble one of the two constitutive acid phosphatases was found in the vacuoles, while the other (insoluble) enzyme appeared to be membrane-bound presumably in vesicles somewhere inside the cell.[188] Acid-phosphatase containing lysosome-like vesicles have also been identified in some cryptomonads.[376,377] Interestingly, the wall-less chlorophyte *Asteromonas gracilis* showed two cytologically differentiable acid phosphatases, one in dictyosome-associated vesicles, provacuoles, and vacuoles, the other localized in membranous coils within vacuoles, and the latter enzymatic activity appeared to be directly related to cellular ageing and autophagic digestion of degrading cytoplasm.[185] Nevertheless, the role of acid phosphatase in autophagy may not be so clear-cut since, in the chlorophyte *Dunaliella primolecta*, this enzyme was cytochemically found to be localized in Golgi cisternae and in postcisternal vesicles rather than the known autophagosome-like bodies.[223] Likewise, the prymnesiophyte *Hymenomonas carterae* showed acid phosphatase localized in Golgi cisternae but also in certain digestive vacuoles believed to be responsible for the digestion of unmineralized coccolith scales.[224,225] It is not unlikely that the cytoplasmic and vacuolar constitutive acid phosphatases may be additionally involved in processes other than autophagy. In the case of halophilic *Dunaliella*, at least, it appears that the constitutive enzyme[153] is involved in the production of the intracellular osmoregulatory metabolite, glycerol, from photosynthetic CO_2-fixation, where the phosphatase is

responsible for the intracellular dephosphorylation of glycerophosphate or dihydroxyacetone phosphate,[226,227] and this process is expected to occur outside the chloroplast presumably in a cytoplasmic vesicle.

I. Miscellaneous Cytological Phenomena Associated with Phosphatases

Certain cytological processes, not directly related to P_i-nutrition, have been correlated with intracellular phosphatase changes. Among these processes may be listed those of cell ageing, cell division, and cell differentiation.

Stationary-phase cultures of *Ochromonas danica* showed a several-fold increase of both acid and alkaline phosphatases with ageing, and this increase appeared to be associated with lysosomal activity.[171] Similar increases in lysosomal vacuolar phosphatases have been reported for ageing cells of *Euglena*[222] and the ciliate *Tetrahymena*.[228] This increase in activity for streptomycin-bleached *Euglena* was further enhanced by deprivation of the carbon-source (acetate) for growth, and indicated that autophagic digestion of cytoplasmic macromolecules was promoted to provide internally generated emergency-survival nutrients as compensation for the lack of exogenous supply of carbon compounds.[229] The age-related accumulation of acid phosphatase was also observed in autophagic vacuoles of the phaeophyte *Ectocarpus*, where the intense enzyme activity was simultaneously accompanied by the formation of lipofuscin-like inclusions inside the vacuoles.[230] There is now a general consensus that the phosphatase-associated autophagy of ageing algal cells is a survival strategy for temporarily generating nutrients (both organic and inorganic) by sacrificing some cells or cell parts in order to maintain viability of the others. It must be pointed out, however, that this autophagic process involves a whole host of catabolic hydrolytic enzymes acting in concert with acid phosphatase.[229]

Although not yet reported for any alga, there is at least one known case of the participation of P_i-repressible phosphatase in the cell division of a pseudomonad.[231] Under conditions of exogenous P_i limitation, the normal motile rod-like cells produced periplasm-located alkaline phosphatase, which was absent from the filamentous forms produced at a higher temperature that was restrictive for cell division. The overall evidence suggested that this phosphatase was required for the cell division normally occurring in rod formation but suppressed in filament formation. Apart from its participation, the exact role of the enzyme in pseudomonadal cell division remains yet unknown. Nevertheless, judging from this example, we suspect that algal phosphatases may play related roles for unicellular algae known to form (pseudo) filaments under certain growth conditions (e.g., *Scenedesmus* spp.[232]).

Histochemical and in vitro studies on the differentiating cellular slime mold *Dictyostelium discoideum* have shown that P_i-repressible alkaline phosphatase changes in activity during the developmental cycle of the organism; the activity increases until spore formation but decreases to a minimum in the mature fruiting bodies or sorocarps.[233] In this case, it appears that the enzyme, though apparently nonspecific, functions largely as a 5′-nucleotidase acting on AMP and dAMP, and that not only exogenous P_i but also exogenous adenosine and deoxyadenosine are involved in regulating its activity in the spores and sorocarps.[233] However, this role of the enzyme in cellular differentiation appears to be clouded or complicated by more recent findings of the involvement of cyclic-AMP and cyclic-GMP as chemotactants in aggregation and pseudopod formation of the ameboid stage of the organism.[234] The possibility is not precluded that the originally described phosphatase may cover multiple enzymes, presumably including those active on the cyclic nucleotides. The probability of phosphatase involvement in algal cellular differentiation has not been hitherto explored

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or even suggested, and we recommand that the case history of *D. discoideum* be regarded as an appropriate starting model for future investigations on the molecular ontogeny of algal life cycles.

J. Ecological Implications of P_i-Repressible Phosphatase Production

The detection of significant quantities of phosphatase activity in algal cultures and in natural systems does not *ipso facto* constitute a valid demonstration of their ecological importance in determining P_i availability. However, the presence of such enzyme activity in a wide variety of aquatic habitats, such as Chesapeake Bay,[235] subarctic Swedish lakes,[157] Tokyo Bay,[194] Lake Kinneret,[158,160] Japanese coastal water,[30] the Central North Pacific,[56,57] and the Caribbean and Sargasso Seas,[32] does suggest an underlying ecological role which requires much further investigation. In order to facilitate phytoplankton growth through utilization of DOP, the following conditions must be met: (1) the external P_i concentration must be below the threshold which suppresses synthesis and activity of phosphatases; (2) the quantity or specific activity of the hydrolytic enzymes must be sufficient to form enough P_i for more than a minimal effect on cellular metabolism and growth; (3) there must be adequate organic phosphate substrate available; (4) both the affinity of the organic substrate for enzyme binding and the hydrolysis rate must be sufficiently high; and (5) the algal cells must be able to take up and assimilate the P_i moiety at the concentration generated from enzymatic reactions.

There is sufficient evidence to suggest that, under certain circumstances, all of the above conditions are satisfied. The K_m for extracellular alkaline phosphatases of two marine diatoms was typically much lower than for intracellular acid phosphatases,[154] thus, enabling utilization of extremely low extracellular concentrations of DOP substrate. Many investigators have reported that the rate of cleavage of the P_i moiety is adequate to support a substantial growth yield[10,55,158,235] in both laboratory and natural systems. In the aquatic environment, the influence of phosphatase activity upon phytoplankton nutrition would tend to vary according to the seasonal supply of available phosphorus resources. Although phosphatase activity may contribute somewhat to phytoplankton growth throughout the year in temperate waters, high demands placed upon P_i supplies by a well-developed spring bloom would tend to maximize its importance at this time. In tropical waters, which do not undergo such marked seasonal shifts on a regular basis, variations in phosphatase activity may result from short-term catastrophic perturbations in the phosphate supply. When P_i reserves are consistently low, a relatively stable high-level enzyme-activity pattern would be anticipated. In some special cases, such as acid (pH 4 to 5) lakes, high phosphatase activity in lake water was attributed to the high aluminum concentration "blocking" the phosphatase substrates and thereby compelling the planktonic organisms to increase enzyme production in order to overcome competition from the Al^{3+} ion.[675]

The current practice of using assays of alkaline phosphatase as bioindicators of the nutritional status in natural phytoplankton populations is probably reckless and fraught with undesirable complications. First of all, bacteria are known producers of phosphatase,[165] so that attempted correlations between enzyme activity, phytoplankton biomass, and chlorophyll content tend to be frustrating.[236] In Japanese coastal waters, a relationship between bacterial biomass and alkaline phosphatase activity has been described.[30] Furthermore, it is difficult to discern whether the alkaline phosphatase activity is directly attributable to bacterial secretion, or whether bacterial degradation leads to high organic phosphate production, which in turn induces increased phytoplankton or bacterial phosphatase activity. Secondly, it is possible that much of the phosphatase detected from advanced algal blooms may consist of significant enzyme leakage from lysis of senescent or aged cells, and is, therefore, not necessarily a

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specific derepressible response of P_i-limited, healthy cells. Faulty fractionation techniques have also been blamed for overestimating the algal portion of total phosphatase activity of some lakes.[677] The lack of clearly defined relationship between the measured alkaline phosphatase activity and algal biomers or external P_i concentrations persuaded Taft et al.[235] to conclude that ambient P_i concentration has dropped below the algal cell's maximum ability to store phosphate in excess of metabolic requirements and that additional pathways for P uptake are in use.

APPENDIX 1

List of Abbreviations

AMP—adenosine 5'-monophosphate
dAMP—deoxyadenosine 5'-monophosphate
ADP—adenosine 5'-diphosphate
ATP—adenosine 5'-triphosphate
CCCP—carbonyl cyanide *m*-chlorophenylhydrazone
pCMB—*p*-chloromercuribenzoate
CMP—cytidine 5'-monophosphate
CDP—cytidine 5'-diphosphate
CN^-—cyanide
cyclic AMP—adenosine cyclic 3',5'-monophosphate
cyclic-GMP—guanosine cyclic 3',5'-monophosphate
DBMIB—2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCCD—N,N'-dicyclohexyl carbodiimide
DCMU—3-(3',4'-dichlorophenyl)-1,1-dimethylurea
DNA—deoxyribonucleic acid
DNP—2,4-dinitrophenol
DOP—dissolved organic phosphate
1,3-DPGA—D-glyceric acid 1,3-diphosphate
EDTA—ethylenediamine tetraacetic acid
FDP—D-fructose 1,6-diphosphate
F-6-P—D-fructose 6-phosphate
Glycerate-3-P—D-glyceric acid 3-phosphate
GMP—guanosine 5'-monophosphate
G-1-P—D-glucose 1-phosphate
G-6-P—D-glucose 6-phosphate
K_q—cell quota at which the growth rate equals one half of the growth rate at infinite cell quota
K_s—half-saturation constant for nutrient uptake or growth
K_s'—apparent half-saturation constant for nutrient uptake or growth
NAD—nicotinamide adenine dinucleotide
NEM—N-ethylmaleimide
P_i—inorganic orthophosphate
P_i-irrepressible—unaffected by presence/absence of P_i in growth medium
P_i-repressible—inhibited or repressed by presence of P_i in growth medium
PCA—perchloric acid
Poly-P_i—inorganic polyphosphate
PP_i—inorganic pyrophosphate
PE—phosphatidyl ethanolamine
PG—phosphatidyl glycerol
PI—phosphatidyl inositol
PS—phosphatidyl serine
PSC—phosphatidyl sulfocholine
q or Q—cell nutrient quota; the concentration of substrate (nutrient) per cell or per unit biomass
q_f—fraction of total cell quota in a given internal nutrient pool
q_m or Q_m—cell quota at which the substrate ceases to limit growth
q_o, Q_o, k_q, or k_Q—subsistence quota; the minimum concentration of limiting substrate (nutrient) per cell, or per unit biomass, required for further growth
RHMWP—(molybdate) reactive high molecular weight phosphate
RNA—ribonucleic acid

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mRNA—messenger-RNA
SRP—soluble (molybdate)-reactive phosphate
TCA—trichloroacetic acid
μ—specific growth rate
μ_m, μ_{max}, or $\hat{\mu}$—maximum specific growth rate
μ'_m, μ'_{max}, or $\bar{\mu}$—apparent maximal growth rate at infinite cell quota
V—specific nutrient uptake rate
V_m or V_{max}—maximum specific uptake rate
V'_m or V'_{max}—apparent maximum uptake rate

APPENDIX 2

Algal Nomenclature

Since many algae have undergone nomenclatural revision in the last two decades, the authors of this review were faced with the problem of using changing names as cited by the original investigators or the most recent names along with recently accepted (re)classification of such algal species. In the interest of nomenclatural consistency in this review, we have chosen the latter alternative and list below such algal species with the earlier and revised names and corresponding class placement (shown in parentheses by abbreviations).

Present nomenclature	Previous nomenclature
Asterionella gracilis (Bacil)[654]	*Asterionella japonica* (Bacil)
Chattonella akashiwo (Chlorom)[380]	*Heterosigma akashiwo* (Dino)
Chattonella inlandica (Chlorom)[380]	*Heterosigma inlandica* (Dino)
Clone GSB Sticho[a] (Eustig)[370]	*Stichococcus* sp. (Chloro)
Crypthecodinium cohnii (Dino)[642]	*Gyrodinium cohnii* (Dino)
Emiliania huxleyi (Prym)[369]	*Coccolithus huxleyi* (Chryso)
Heterocapsa niei (Dino)[447]	*Cachonina niei* (Dino)
Hymenomonas carterae (Prym)[369]	*Cricosphaera carterae* (Chryso)
Hymenomonas elongata (Prym)[369]	*Syracosphaera elongata* (Chryso)
Katodinium rotundatum (Dino)[636]	*Massartia rotundata* (Dino)
Monodopsis subterranea (Eustig)[323]	*Monodus subterraneus* (Tribo)
Pavlova lutheri (Prym)[369]	*Monochrysis lutheri* (Chryso)
Peridinium cinctum (Dino)[158,641]	*Peridinium westii* (Dino)[211,212]
Phaeodactylum tricornutum (Bacil)[637]	*Nitzschia closterium f. minutissima* (Bacil)
Poteriochromonas stipitata (Chryso)[650,651]	*Ochromonas malhamensis* (Chryso)
Protogonyaulax tamarensis (Dino)[638]	*Gonyaulax tamarensis* (Dino)
Thalassiosira pseudonana (Bacil)[639]	*Cyclotella nana* (Bacil)
Thalassiosira weissflogii (Bacil)[640]	*Thalassiosira fluviatilis* (Bacil)

Note: Abbreviations used: Bacil, Bacillariophyceae (Diatoms); Chloro, Chlorophyceae; Chlorom, Chloromonadophyceae (Rhaphidophyceae); Chryso, Chrysophyceae; Dino, Dinophyceae; Eustig, Eustigmatophyceae; Prym, Prymnesiophyceae (Haptophyceae); Tribo, Tribophyceae (Xanthophyceae).[323,369]

[a] Previously misclassified and misidentified, this algal isolate is awaiting new generic and species nomenclature.

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