gested an absence of measurable nitrogenous nutrient, shore-based analyses of stored portions from the same samples were found to contain measurable concentrations (*9, 23*). Data from a recent study (*24*) on a freshwater green alga, *Selanastrum capricornutum*, grown under nitrogen limitation were related to earlier data for oceanic nutrient concentrations; the conclusion was drawn that nitrogen should not be considered as a primary limiting nutrient in oceanic waters. Based on our comments above, we suspect that the nutrient values used in this comparison contained artifacts (*23*).

In light of our inability to measure nitrogenous nutrients in oceanic surface waters and our experimental evidence for the rapid short-term nitrogen uptake characteristics of marine phytoplankton, we offer a hypothesis on how primary producers obtain their nutrition in oceanic waters that appear to be devoid of nitrogenous nutrient. Bacterial degradation of particulate and dissolved organic nitrogen plus animal excretion products, mainly NH_4^+ and urea, are significant sources of nitrogenous nutrients supporting phytoplankton growth in estuarine, coastal, and oceanic waters (*3, 16, 25*). A single phytoplankton cell with a history of nitrogen deprivation that has the same capacity for enhanced nitrogen uptake displayed by the two clones of *T. pseudonana* in this study could rapidly and efficiently utilize nitrogenous excreta before they are physically dispersed to the surrounding media. Thus the microenvironment surrounding a phytoplankton cell probably determines the magnitude of the cell's supply of nitrogen. Such minute patches, perhaps only nanoliters, with elevated nitrogen content cannot be observed with present-day techniques. The impact of a single zooplankter in supplying nitrogen on this spatial scale is considerable. For example, calculations indicate that herbivorous oceanic copepod such as *Oithona* or *Clausocalanus* can excrete enough NH_4^+ in 5 seconds to increase the ambient concentration of this nutrient by about 5 μg-atom/liter in a volume of water approximating the displacement volume of the animal (*26*).

If our results with *T. pseudonana* typify the nitrogen uptake capabilities of other marine phytoplankton, then conceivably nitrogen that is either excreted by zooplankton or remineralized by bacteria is continually being utilized by adjacent phytoplankton cells at rates that can keep the momentary average concentration for larger water volumes at less than the limits of analytical detection. Extension of these ideas and observations to achieve a more complete understanding of the small-scale temporal and spatial coupling between the nutrient supply and utilization components of near-surface oceanic nitrogen cycling will require a consideration of dispersion processes such as diffusion, shear, and even the physical mixing resulting from phytoplankton and zooplankton motility.

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18. In Fig. 1 both V'_{max} and μ are normalized to plant nitrogen in order to permit direct comparison of uptake and growth rates in dimensions of time^{-1}. Eppley and Renger (*11*) grew *T. pseudonana* (clone 13-1) on a light-dark cycle and in a different manner determined maximum uptake rates at three growth rates. Multiplying their hourly uptake value measured at a growth rate of 19 percent μ_{max} by 12 also yields an uptake rate adequate to meet the nitrogen demand for one doubling in 3 percent of the doubling period. The 20-minute experiments of Conway and Harrison (*12*) yielded values of 14 to 25 percent of the doubling period at 20 percent of the maximum growth rate.
19. Data from (*11*) were shown to fall on a curve of similar shape (*13*).
20. At $\mu = 1.6$ cellular doublings per day, Q would be reduced by 14 percent during the 120-minute delay had cellular doubling continued at its steady-state rate, and V'_{max} would have increased by 23 percent (Figs. 1 and 2).
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27. We thank R. R. L. Guillard for providing the culture inocula and J. Nevins for technical assistance. Supported by NSF grants OCE75-14781 and OCE77-26401 and Energy Research and Development Administration contract E(11-1)-2532.

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Cytidylate Cyclase: Possible Artifacts in the Methodology

Cech and Ignarro (*1*) have reported the enzymatic synthesis of cytidine 3′,5′-monophosphate (cyclic CMP) from [α-^{32}P]CTP (cytidine triphosphate) in mouse liver homogenates and other tissues. The method used by the authors to isolate cyclic CMP, however, appears to be unreliable.

Using the assay of cytidylate cyclase in mouse liver as reported by Cech and Ignarro (*1*), we were able to obtain similar values for the formation of apparent cyclic CMP (100 to 120 pmole per milligram of protein per minute) during 1 to 5 minutes of incubation. But the product did not behave as cyclic CMP in various chromatographic systems. On rechromatography on alumina column, more than 99 percent of the apparent ^{32}P-labeled cyclic CMP was retained by the column, while more than 85 percent of authentic [5-^{3}H]cyclic CMP was eluted from the column. This clearly indicated that more than 99 percent of the ^{32}P radioactivity obtained in the assay of Cech and Ignarro (*1*) was not associated with cyclic CMP. Moreover, when the alumina eluate was rechromatographed on a Dowex 50-H$^+$ (0.5 by 4 cm) column (*2*) about 75 to 80 percent of the apparent ^{32}P-labeled cyclic CMP was recovered of which more than 50 percent was eluted in the first 2 ml, and the rest was eluted in the 3- to 10-ml fractions. By contrast more than 85 percent of the recovered authentic ^{3}H-labeled cyclic CMP present

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in the same samples was eluted in 3- to 5-ml fractions. Neither of the two major peaks of ^{32}P radioactivity coincided with the peak of authentic ^{3}H-labeled cyclic CMP. Although it still seemed possible that a part of the second peak may represent the cyclic nucleotide, chromatography of the Dowex 50 fractions on PEI (polyethyleneimine) cellulose sheets developed with 1*M* LiCl as the solvent, however, excluded this possibility. The ^{32}P-labeled reaction product had the same R_F value (0.45) as 5′-CMP and was well separated from ^{3}H-labeled cyclic CMP (R_F 0.75) present in the samples. The analysis of the first Dowex 50 peak by thin-layer chromatography on PEI cellulose sheets developed either with 1*M* LiCl or with a solution of 1*N* NH_4COOH and 2*N* HCl (68:32) showed that this ^{32}P-labeled reaction product was CDP (cytidine diphosphate). Even though Cech and Ignarro have shown coincidence of the ^{32}P-labeled product with ^{3}H-labeled cyclic CMP on Dowex 1 chromatography (*1*), we find that 5′-CMP and cyclic CMP are eluted together by 0.5*N* HCOOH employed by Cech and Ignarro. We conclude, therefore, that the product obtained by using the method of Cech and Ignarro is not cyclic CMP.

Since the time Bloch reported the isolation of cyclic CMP from extracts of leukemia L-1210 cells and regenerating liver (*3*), we have also attempted to demonstrate the synthesis of cyclic CMP. The method we used for the assay of cytidylate cyclase consisted of incubating the enzyme with [α-^{32}P]CTP and Mn^{2+}, in the presence of ^{3}H-labeled cyclic CMP and separating cyclic CMP from the other products by chromatography on Dowex 50-H^+ followed by alumina (*2*). By this procedure the recovery of ^{3}H-labeled cyclic CMP added either before or after incubation ranged 70 to 75 percent, and the blank value of the assay was less than 0.001 percent of the added [α-^{32}P]CTP. Even though this method measures the formation of as little as 2 pmole of cyclic CMP (which is equal to five times the nonenzymatic blank value), we were unable to find any significant cytidylate cyclase activity in L-1210 cells, normal and regenerating liver, lung, kidney, heart, or rod outer segments. Whether cyclic CMP is synthesized from CTP in mammalian systems thus remains debatable. Even though it has been shown that a specific cyclic CMP phosphodiesterase occurs in various tissues (*4*), we cannot presume the existence of an enzyme for the synthesis of this cyclic nucleotide without direct evidence. It has been more than 20 years since the discovery of an enzymatic system for the hydrolysis of cyclic 2′,3′-CMP without any evidence for its occurrence in tissues or its synthesis (*5*).

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Gaion and Krishna (*1*) present a valid criticism of our original demonstration of cytidylate cyclase activity in mammalian tissues (*2*). This criticism is based on the observation that the ^{32}P-labeled product, using our assay procedure, does not display a similar elution profile on Dowex 50-H^+ to that of authentic cyclic cytidine monophosphate (CMP). We have been aware of this problem since 1976. The identification of cyclic CMP as the reaction product (*2*) was based on the findings that the reaction product and authentic cyclic CMP showed remarkably similar properties in the following six analytical procedures: (i) column chromatography on neutral alumina; (ii) column chromatography on Dowex 1-formate; (iii) column chromatography on PEI (polyethyleneimine) cellulose; (iv) thin-layer chromatography on PEI cellulose; (v) recrystallization to constant specific activity in two solvents; and (vi) specific radioimmunoassay. Other investigators have also attempted to identify the discrepancy associated with the failure to identify by cation exchange chromatography (Dowex 50-H^+) the reaction product as cyclic CMP. Some data (*3*) already indicate that the cyclic CMP formed from cytidine triphosphate (CTP) by cytidylate cyclase, but not added cyclic CMP, binds by noncovalent linkage to protein, and this complex cochromatographs with authentic cyclic CMP on anionic but not cationic exchange resins. Proteolytic digestion (Pronase or chymotrypsin plus trypsin, followed by precipitation with trichloroacetic acid and extraction with ether) of reaction mixtures liberates 50 to 80 percent of the cyclic CMP formed, which then cochromatographs with authentic compound on Dowex 50-H^+. In addition, repetitive chromatography of undigested reaction mixtures on neutral alumina columns (0.5 g) yields poor recovery of cyclic CMP, whereas, the same procedure applied to protease-treated samples yields consistently good (80 percent) recovery of cyclic CMP. Some of our published data (*2*) are not in agreement with those reported (*1*). First, we cannot detect, by any of our analytical systems, formation of either 5′-CMP or 5′-CDP (cytidine diphosphate). These nucleotides do not elute from properly aged and washed neutral alumina columns (*4*). Second, I cannot detect any ^{32}P-labeled reaction product after Dowex 50-H^+ chromatography, unless reaction mixtures are first treated with proteases. It is, therefore, understandable that Gaion and Krishna (*1*) were unable to identify the reaction product by thin-layer chromatography of eluates obtained from Dowex 50-H^+ column chromatography.

Thus, I agree completely with Gaion and Krishna that cyclic CMP cannot be detected as the cytidylate cyclase reaction product by employing conventional chromatographic techniques with the cationic exchange resin Dowex 50-H^+. Although preliminary data suggest that this technical problem can be overcome, we suggest strongly for the time being that neither our published assay procedure nor that of Gaion and Krishna (*1*) be used to determine cytidylate cyclase activity until the complex technical problems discussed above are clearly solved and a more reliable assay is made available.

Note added in proof: A report appeared recently (*5*) which confirms, by specific radioimmunoassay, the formation of cyclic CMP by mammalian tissues and which indicates that tissue cyclic CMP can be easily isolated and separated by Dowex 1-formate column chromatography. The latter method is one that I used to verify cyclic CMP formation in tissues.

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