PHOSPHORUS EXCHANGE IN MARINE PHYTOPLANKTON

BY THEODORE R. RICE

FISHERY BULLETIN 80

UNITED STATES DEPARTMENT OF THE INTERIOR, Douglas McKay, *Secretary*

FISH AND WILDLIFE SERVICE, John L. Farley, *Director*

ABSTRACT

Phosphorus exchange in *Nitzschia closterium*, isolated and grown in pure culture, was demonstrated by using radioactive phosphorus and was shown to vary with changes in the phosphorus concentration of the medium and with the physiological conditions of the cells. A modification of Miquel's nutrient solutions was made to prevent the formation of precipitates when autoclaved, since it was necessary that the radioactive phosphorus be either in solution or within the cells. Complete recovery of cells for radioactive assay was accomplished by using a barium-sulfate filter pad in a detachable filtering apparatus. The greatest exchange occurred when cells grown in high concentrations of phosphorus were filter-washed. Also exchange between cells and medium was determined while the cells were photosynthesizing, and when they were kept in the dark. A redistribution of intracellular phosphorus between the inorganic and organic fractions occurred when cells were placed in the light in medium containing only a trace of phosphorus. Little phosphorus was converted into the organic state by cells kept in the dark.

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By Theodore R. Rice, *Fishery Research Biologist*

The labeling of planktonic algae with radioactive phosphorus was investigated as one phase of a study of the food of filter-feeding invertebrates. The phytoplankton first had to be grown under conditions that would result in the cells absorbing and retaining large quantities of active phosphorus before it could be used as labeled food. This required that the processes of phosphorus exchange in phytoplankton be analyzed.

The relative abundance of phosphorus has been known to be a factor limiting the growth of phytoplankton in the sea since the time of Brandt (1899, 1902). Studies in the laboratory with pure cultures, as well as correlations resulting from work in the field, have proved phosphorus to be of critical importance. Its abundance in natural bodies of water previous to rapid increases in population sizes and its seasonal depletion with an increase of phytoplankton have been shown many times.

A decrease in the phosphorus concentration of the medium following an increase in the phytoplankton population has been relatively easy to demonstrate in the laboratory. Detection of phosphorus exchange was not possible until tracer techniques for active phosphorus were developed. By using a culture medium containing a mixture of radioactive and nonradioactive phosphorus, a measurement of phosphorus exchange is possible. The normal movement of phosphorus can be traced by following the movement of radioactive phosphorus, since it is generally accepted that cells cannot distinguish between the two isotopes of phosphorus and that isotopic effects can be disregarded. Furthermore, the addition of small amounts of radioactive phosphorus, in these studies, has not changed the physiology of the cells through radiation, nor the rate of uptake of phosphorus by them.

Using radioactive phosphorus, Gest and Kamen (1948) and Goldberg, Walker, and Whisenand (1951) have demonstrated that phosphorus is exchanged by planktonic algae. In both of these investigations, exchange was tested for only short intervals of time by either centrifuging or filter-washing the cells containing radioactive phosphorus. The appearance of radioactive phosphorus in the washings demonstrated exchange. Although filter-washing also has been used in this investigation to determine factors influencing exchange, emphasis has been placed on measuring exchange over longer periods of time while the cells were maintained under various environmental conditions.

NOTE.—This study was part of a project carried on under a cooperative agreement between the United States Fish and Wildlife Service and the United States Atomic Energy Commission.

MATERIALS AND METHODS

CULTURE MEDIUM

The addition of nutrients to natural sea water is still apparently the best method of preparing culture medium for marine planktonic algae. One disadvantage of this method is that precipitates often form when the enriched sea water is autoclaved. It was important that no precipitates form in the medium used in these experiments, since the radioactive phosphorus must be contained either in the cells or in the medium. Sea water enriched with Miquel's solutions, as modified by Ketchum and Redfield (1938), was used since *Nitzschia closterium* grows well in this medium. It was found that their solutions A and B caused precipitates to form when added to sea water and autoclaved. By adding iron as $Fe(NH_4)(SO_4)_2$ instead of as $FeCl_3$ in solution B, and by removing the phosphate compound from solution B and adding it as solution C, the formation of precipitates was avoided. The same concentration of phosphorus, 56 μgAP/L added as KH_2PO_4 instead of as $Na_2HPO_4 \cdot 12H_2O$, was used to prepare solution C. The important change is that solution C was not added until after the sea water containing solution A and the modified solution B had been autoclaved and allowed to cool. If solution C was added to the

autoclaved sea water before it had cooled completely, precipitates still formed.

The three solutions used in the preparation of this culture medium were—

Solution A--	KNO_3----------	20.2 grams.
	Distilled water----	To make 100 cubic centimeters of solution.
Solution B--	$MgSO_4$----------	4 grams.
	$CaCl_2$----------	4 grams.
	$Fe(NH_4)(SO_4)_2$----	1 gram.
	HCl (conc.)-------	2 cc.
	Distilled water----	To make 100 cc. of solution.
Solution C--	KH_2PO_4----------	1.53 grams.
	Distilled water----	To make 100 cc. of solution.

Particulate matter was first removed from the sea water by filtering it through cotton. Then, 0.55 cc. of solution A and 0.50 cc. of solution B were added to each liter of sea water. This medium was autoclaved and, after cooling completely, 0.50 cc. of solution C, which had been autoclaved separately, was added aseptically.

CULTURE PROCEDURE

Nitzschia closterium was isolated into pure culture and used in these experiments. It was maintained in pure culture on agar containing 2 percent glucose and 0.5 percent peptone in addition to Miquel's inorganic nutrients. Tests for purity were not made on cultures during or after the experiments since sterile techniques and inorganic media were used and since most experiments were completed within a short period of time.

The cultures were grown in a refrigerated cabinet (fig. 1) at a temperature of 20°±2° C. The cabinet was equipped with adjustable wooden shelves to each of which six 40-watt daylight fluorescent lights, spaced 2 inches apart, were attached. Approximately 2 inches above the lights were glass shelves upon which the cultures were placed.

PHOSPHORUS MEASUREMENTS

The radioactive phosphorus used in these experiments came from Oak Ridge National Laboratory.[1] Activity was determined by means of a conventional dip-type Geiger-Müller counting tube connected with a scaler-of-64 circuit. The

[1] Supplied on authorization from the Isotopes Division, United States Atomic Energy Commission.

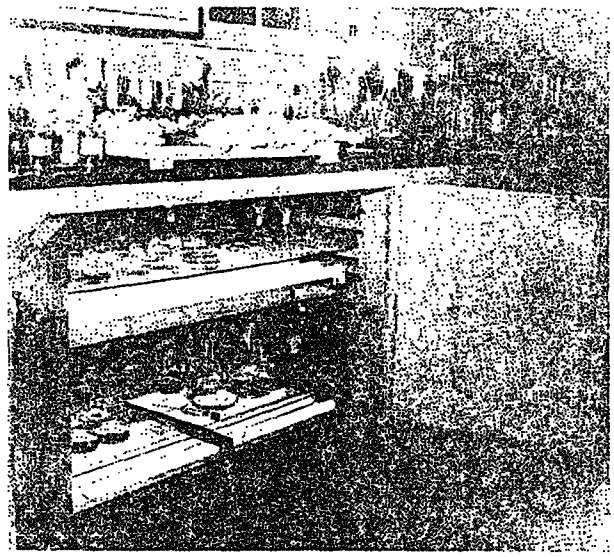

FIGURE 1.—Culture cabinet showing illumination of cultures.

geometry was constant, and corrections for decay and coincidence loss were made. The radioactive phosphorus was standardized by comparison with a simulated standard of known microcurie strength. Inorganic-phosphorus concentrations were determined by the Atkins-Deniges molybdate method as modified by Wattenberg (1937). Salt-error corrections were made and algal cells were removed by filtering before making determinations. The intensity of the blue color was measured with a Klett-Summerson photoelectric colorimeter which had been standardized with solutions of known phosphorus content. Klett filter No. 66 and a 4.5 x 2.5 centimeter cell were used. Since the intensity of the blue color changes with time, determinations were made approximately 10 minutes after the addition of reagents. The concentration of phosphorus is expressed as microgram atoms of phosphate phosphorus per liter (μgAP/L) as recommended by the International Association of Physical Oceanography (Sverdrup, et al. 1942, table 34).

DETERMINATION OF POPULATION SIZE

The number of cells per liter was determined by removing approximately 5 cc. of medium after vigorously shaking the culture to distribute the cells evenly throughout the medium. The average of two cell counts, made with an improved Neubauer haemocytometer, was taken as the population size. A careful check has shown that

this method is very accurate for determining unialgal population sizes, the accuracy agreeing favorably with that found by other investigators (Pearsall and Loose 1937, Pratt 1940, and Winokur 1948).

PREPARATION OF FILTERS

A stainless-steel filter (fig. 2), which could be taken apart for the insertion of specially cut Whatman No. 42 filter paper, was used with a vacuum pump. The barium-sulfate precipitate was formed by heating 15 cc. of one-fifth normal H_2SO_4 and adding 0.6 cc. of normal $BaCl_2$. The precipitate was filtered onto the filter paper and rinsed with distilled water before filtrations were made.

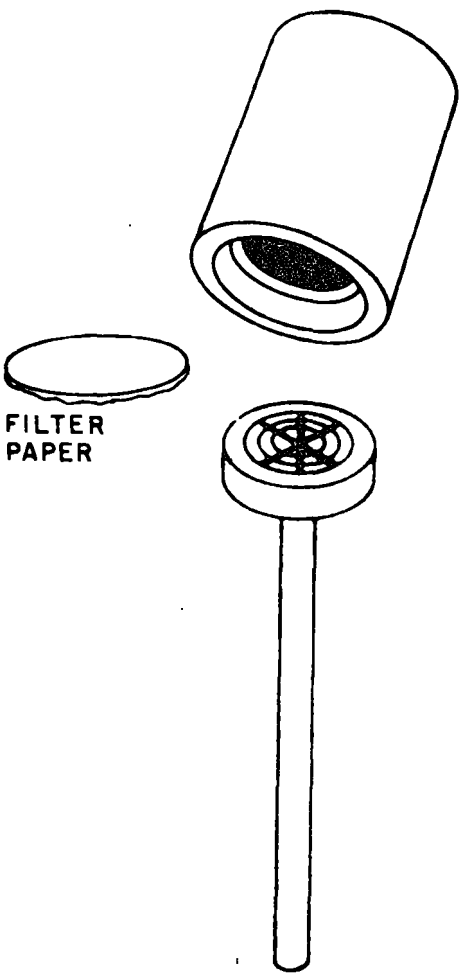

FIGURE 2.—Stainless-steel filter.

FRACTIONATION OF CELLS

The cells were centrifuged from the culture medium and extracted in the cold with 10 cc. of cold 10-percent trichloracetic acid (TCA) for about 1 hour. After centrifuging, extraction was repeated with a new volume of TCA. The TCA-insoluble residue was dissolved in HNO_3 and diluted with distilled water for counting. The TCA-soluble fraction was neutralized to phenolphthalein with NaOH and the inorganic phosphorus fraction was precipitated with 10-percent $CaCl_2$ in saturated $CaOH_2$. The precipitate, after centrifuging, was dissolved in a small amount of dilute HCl, made to volume with distilled water, and the radioactive disintegrations were counted. The supernatent was also made to volume with distilled water and counted. Thus the radioactive-phosphorus content of the inorganic phosphorus, the TCA-insoluble phosphorus (considered as protein-bound phosphorus), and the TCA-soluble organic phosphorus (ester phosphorus) were measured. The protein-bound phosphorus and the ester phosphorus were always combined and reported as the organic fraction.

EXPERIMENTS

PHOSPHORUS ABSORPTION

Phosphorus in culture medium containing dividing cells can be reduced below a concentration detectable with the colorimetric method of analysis if no other factor limits cell division. Consequently, it has been assumed that phosphorus completely disappears from the medium. By using radioactive phosphorus, the reduction of phosphorus in the culture medium can also be determined, provided no exchange of phosphorus occurs between the cells and the medium. To accomplish this requires only that a known amount of radioactive phosphorus be added to medium containing nonradioactive phosphorus prior to the addition of cells. Since the cells cannot distinguish between these two isotopes, the same percentage of each should be absorbed in any period of time. Thus, measurements of the radioactive phosphorus absorbed can be converted to represent inorganic phosphorus absorbed, since the initial ratio of the two is known.

To compare the absorption curves of phosphorus as determined with radioactivity measurements and with colorimetric analysis, culture medium was prepared with enough radioactive phosphorus to give 11,000 counts per minute per 25 cc. of medium and an inorganic phosphorus concentra-

tion of 44 μgAP/L. *Nitzschia* cells were added to this medium in sufficient quantity to give a population of 18.5×10^7 cells per liter and the culture was placed in the culture cabinet in the light. Counts for the radioactive phosphorus content of cells and colorimetric analysis for the inorganic-phosphorus content of the medium were made periodically. This colorimetric analysis also included the radioactive phosphorus in the medium, but there was such a small number of radioactive atoms that little, if any, change was caused by their presence or absence.

In figure 3, the amounts of radioactive and nonradioactive, or inorganic, phosphorus absorbed by the cells are shown. Since both radioactive and inorganic phosphorus are plotted on the vertical scale so that a certain percentage of one is equal to the same percentage of the other, the determinations for radioactive phosphorus also represent microgram-atoms of phosphorus. The cells continued to absorb phosphorus from the medium until it was reduced below a concentration detectable with the colorimetric method. However, radioactive determinations for phosphorus made at the end of this experiment showed that a trace of phosphorus still remained in the medium. This may be due to radioactive determinations being many times more sensitive than colorimetric determinations. It is believed that contamination can be ruled out. The author suggests that this trace of radioactive phosphorus in the medium is due to exchange from the cells.

From the two curves in figure 3 it can be seen that at first the absorption of radioactive phosphorus by the cells was greater than absorption determined by the decrease of inorganic phosphorus in the medium. This difference must be the result of exchange or of errors in measurement

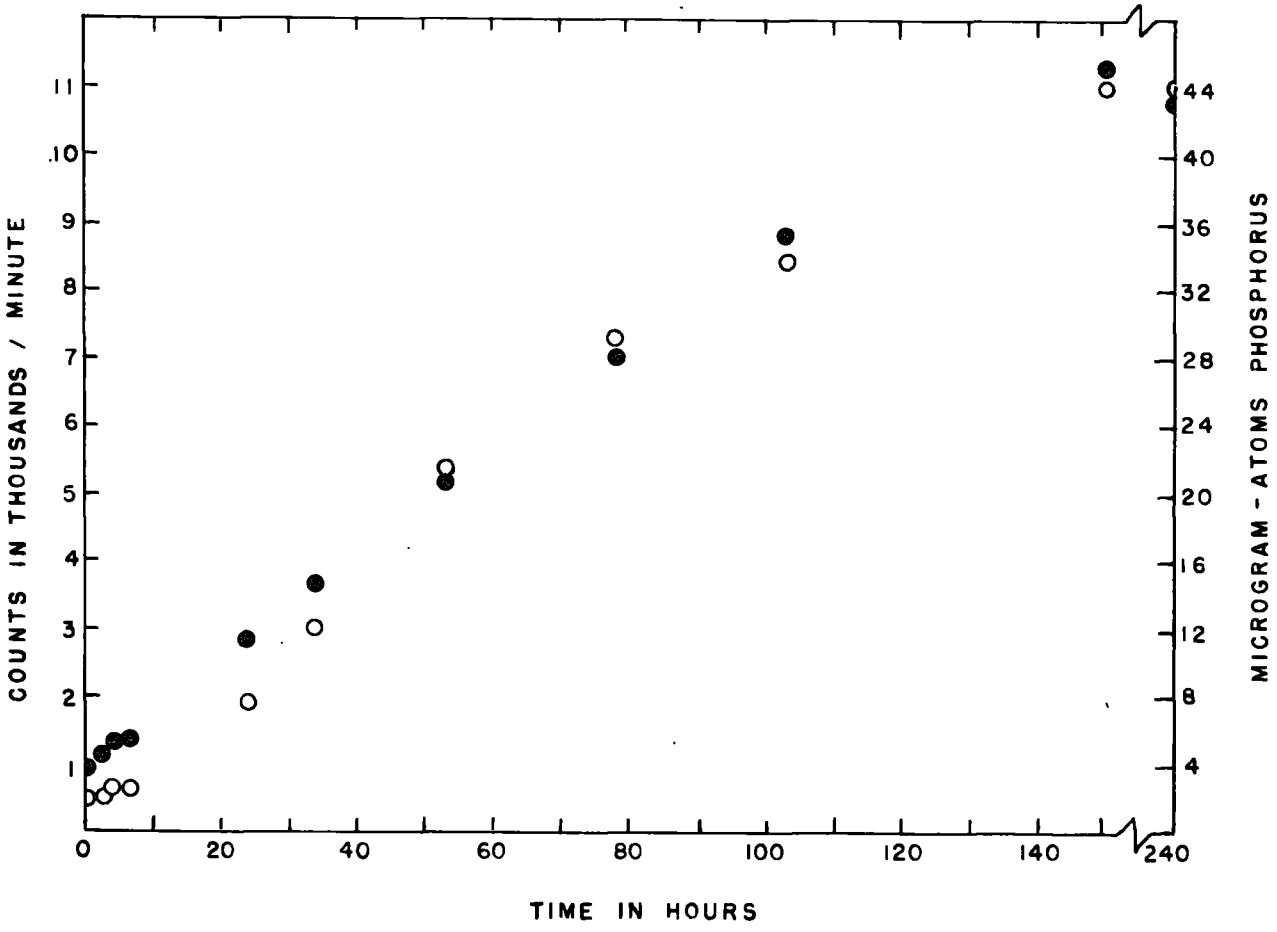

FIGURE 3.—Phosphorus absorption by *Nitzschia* cells as determined by both colorimetric and radioactivity measurements. Circles show amounts of inorganic phosphorus measured colorimetrically; dots show amounts of inorganic phosphorus from radioactivity observations.

or of a combination of the two. When exchange occurs, the specific activity, or ratio of radioactive to total phosphorus atoms, of the medium is changed. In this experiment only nonradioactive phosphorus could initially be exchanged from the cells since the cells had previously grown in medium containing only nonradioactive phosphorus. That the observed differences in the two curves could have been caused by exchange seems improbable, when the accuracy of the colorimetric method is considered. With this method small changes cannot accurately be determined. This is especially so when the medium contains large amounts of phosphorus requiring dilution to obtain satisfactory color intensities for measurement.

Even if all the difference between the two absorption curves could be attributed to exchange, which it cannot, the total amount of phosphorus exchanged would still be very small. From this experiment it can be concluded that, since the metabolic need for phosphorus by dividing cells is so great and the errors of the colorimetric method for measuring phosphorus are so large, any movement of phosphorus due to exchange cannot be detected with a reasonable degree of accuracy.

CONDITIONS AFFECTING PHOSPHORUS EXCHANGE BY CELLS WHEN FILTER-WASHED

Another way to detect exchange is to incorporate radioactive phosphorus into the cells before placing them in a medium which is continually renewed and which contains only nonradioactive phosphorus. If an exchange of phosphorus occurs, the radioactive phosphorus leaving the cells is removed before it can be reabsorbed by the cells. The appearance of radioactive phosphorus in the medium would indicate that phosphorus was being exchanged between cells and medium. Since the washing medium in these experiments contained the same concentration of nonradioactive phosphorus as the medium in which the cells had been growing, no differentiation could have been made between phosphorus originally in the washing medium or in the cells without the use of radioactive phosphorus.

In experiments conducted by Gest and Kamen (1948) on exchange by fresh-water algae, washings were made by centrifuging the cells containing radioactive phosphorus from the medium, decanting the medium, resuspending the cells in new medium, and repeating the process several times. This method was first used as a means of washing in these investigations, but it was later found that filter-washing was more suitable. Goldberg, et al. (1951) found that 50 percent of the radioactive phosphorus in the diatom *Asterionella japonica* is lost when the organism is washed while suspended on a filter. However, it will be shown that several factors may influence the amount of phosphorus exchanged from phytoplankton when filter-washed.

In the following experiments, *Nitzschia* cells in two different physiological states, nondeficient and phosphate-deficient, were used to follow phosphorus exchange. The nondeficient cells were grown in a north window in culture medium containing phosphorus, while the phosphate-deficient cells were grown for several days in the culture cabinet in medium containing no added phosphorus, following methods developed by Ketchum (1939b). The deficient cells continue to divide in the absence of phosphorus in the medium until the phosphorus in each cell is greatly reduced and a condition is reached where further division without phosphorus is impossible.

Exchange by nondeficient cells

The required number of cells were centrifuged from stock culture medium containing phosphorus. These cells were then resuspended in new culture medium and divided into two equal cultures. To one culture enough radioactive and nonactive phosphorus was added to give a concentration of 45.5 μgAP/L, and enough to give 1,137.5 μgAP/L was added to the remaining culture. To each culture, radioactive phosphorus was added in sufficient amounts to give a specific activity of 0.1624 μc/μgAP. Both cultures were placed in the culture cabinet in a constant source of light for 20 hours. During this time the cells took up phosphorus and became radioactive. At the end of 20 hours the cells from 50 cc. of each of these cultures were filtered by means of a $BaSO_4$ filter, and a radioactive count was made on them to determine the total radioactive phosphorus absorbed. To measure the loss of phosphorus, cells from another 50-cc. portion were then filtered and washed five times with 50-cc. portions of culture medium containing the same concentration of phosphorus as the medium in which these cells

had been growing but to which no radioactive phosphorus had been added.

The 45.5 and 1,137.5 μgAP/L in these experiments will be referred to as low and high phosphorus concentrations. It is realized that the 45.5 μgAP/L is not a low concentration when compared with concentrations occurring in nature. However, the quantity of phosphorus available per cell is more important than the concentration per unit volume of medium. Since some populations of cells used are often many times greater than those ordinarily occurring in nature, the conditions in these cultures with the low concentrations may after all be very similar to natural conditions.

In all filter-washing experiments, the activity in the washings plus the activity still remaining in the cells after washing always amounted to at least 90 percent of the total activity contained in the same quantity of cells before washing. Thus, never more than 10 percent of the radioactive phosphorus was unaccounted for even though six different activity readings were made in the washing procedure.

Although the medium with the high phosphorus concentration contained 25 times more phosphorus than that of the low concentration, it also contained 25 times more radioactive phosphorus, specific activity being the same in both cultures. Like amounts of radioactive phosphorus absorbed or exchanged in either culture would therefore represent the same amount of phosphorus.

Loss of radioactive phosphorus to the medium showed that nondeficient cells grown in both the low and high phosphorus concentrations exchanged phosphorus with the medium when filter-washed (table 1). The cells grown in the high concentration not only absorbed more phosphorus from the medium in which they were grown but also exchanged more phosphorus with the medium when filter-washed. Ketchum (1939a) has previously shown that *Nitzschia* cells absorb more phosphorus when grown in medium containing high phosphorus concentrations. Since the amount of phosphorus entering the cells is proportional to the concentration in the medium, it necessarily follows that any phosphorus entering the cells in excess of that which the cells can convert into the organic state, will remain in the inorganic state. This may explain the observation that, when other conditions are similar, the concentration of phosphorus in the medium in which cells are grown controls the amount of phosphorus in the cells available for exchange.

TABLE 1.—*Exchange by nondeficient cells grown in low and high phosphorus concentrations for 20 hours before filter-washing*

[Initial cell count was 46×10^7 cells per liter. Specific activity was 0.1624 μc/μgAP in both media]

Medium	Activity in cells from 50 cc. of medium in counts per minute	Activity lost by cells from 50 cc. of medium in counts per minute	Percentage exchanged
Low concentration (45.5 μgAP/L)	8,730	2,672	30.6
High concentration (1,137.5 μgAP/L)	185,100	171,670	92.7

Exchange by phosphate-deficient cells

Ketchum (1939b) has shown that phosphate-deficient cells contain only a small fraction of the phosphorus found in nondeficient cells, and that more phosphorus is absorbed by deficient cells when again placed in medium containing phosphorus. Since the physiology of phosphate-deficient cells must be different from that of nondeficient cells, the amount of phosphorus exchanged by this type of cell when filter-washed was tested. The effects of low and high phosphorus concentrations on the amount of phosphorus exchanged was also determined.

The phosphate-deficient cells used in the experiment were prepared by suspending cells in medium to which no phosphorus had been added. After placing the culture in the cabinet in the light for 3 days, the population had increased from 45×10^7 to 82×10^7 cells per liter. Since the culture medium contained only a trace of phosphorus, the increase in the number of cells probably had reduced the amount of phosphorus within each cell to a point where further division was impossible. From this culture the cells were centrifuged, resuspended in medium containing radioactive phosphorus, and then filter-washed as described for the nondeficient cells.

Phosphate-deficient cells grown in the higher concentration of phosphorus absorbed more phosphorus than those grown in the lower concentration (table 2). Those grown in the higher concentration also lost a larger percentage of phosphorus when filter-washed. Thus, the phosphorus concentration of the medium in which the cells are grown controls the amount of phosphorus

entering and exchanged by the phosphate-deficient cells, the same as for the nondeficient cells.

Comparison with the nondeficient cells of the preceding experiment (table 1) shows that phosphate-deficient cells grown in low concentrations of phosphorus absorbed more phosphorus than nondeficient cells grown in a similar concentration of phosphorus. Since the initial population sizes were approximately the same in this and the previous experiment, it can be assumed that the metabolic rate of the phosphate-deficient cells was higher than that of the nondeficient cells. Also, less phosphorus was exchanged by deficient cells when filter-washed even though they had absorbed more phosphorus. This was probably due to more of the phosphorus taken into the cells during this period of time being converted into the organic state.

TABLE 2.—*Exchange by phosphate-deficient cells grown in low and high phosphorus concentrations for 20 hours before filter-washing*

[Initial cell count was 45×10^7 cells per liter. Specific activity was 0.3538 $\mu c/\mu g$ A P in both media]

Medium	Activity in cells from 50 cc. of medium in counts per minute	Activity lost by cells from 50 cc. of medium in counts per minute	Percentage exchanged
Low concentration (45.5 μg A P/L)	40,850	2,094	5.1
High concentration (1,137.5 μg A P/L)	399,375	101,604	25.4

Effect of exposure time on exchange

The smaller amounts of phosphorus exchanged by phosphate-deficient cells as compared with that exchanged by nondeficient cells in the previous experiment was due to an altered physiological condition. This difference in phosphorus exchange in the two different types of cells was expressed as the percent of the total radioactive phosphorus contained in the cells. Even though exchange between cells in the same physiological condition and the medium remains constant it would appear to decrease with time if expressed as a percent of the total radioactive phosphorus in the cells. This would be due to the amount of labeled phosphorus in the nonexchangeable fraction increasing during a longer growing period. This experiment only demonstrates the effect of time upon the amount of phosphorus exchanged when computed in the above manner and emphasizes the importance of considering the conditions of the experiment when exchange is expressed as a percent of total radioactive phosphorus.

The cells were taken from a culture similar to that used in the previous experiment with nondeficient cells. The cells were suspended in two cultures, the one with a low and the other with a high phosphorus concentration, and grown for 7 days in the culture cabinet in the light. The initial population of cells in these cultures was 10×10^7 cells per liter, a much smaller number of cells than was used in the previous experiments. This smaller initial population was used in order that the cells could continue to divide and convert inorganic phosphorus into organic-phosphorus compounds. After 7 days in the culture cabinet, the cells were filter-washed as in the two previous experiments.

The cells grown in the high concentration of phosphorus absorbed more phosphorus than those grown in the low concentration, as in the previous experiments (table 3). Similarly, the cells grown in the high concentration of phosphorus exchanged more phosphorus with the medium when filter-washed. Since the cells used in this experiment came from the same type of culture as those cells used in the first of these filter-washing experiments, a comparison of the results is possible. As would be expected, more radioactive phosphorus was absorbed by the cells during the 7-day period than by the cells grown for only 20 hours. Since more radioactive phosphorus entered the cells during this longer period, more was converted into organic compounds. Consequently, when filter-washed, a smaller percent of radioactive phosphorus was lost by the cells.

TABLE 3.—*Exchange by nondeficient cells grown in low and high phosphorus concentrations for 7 days prior to filter-washing*

[Exposure to radioactive phosphorus was also for the 7 days. Initial cell count was 10×10^7 cells per liter. Specific activity was 0.2923 $\mu c/\mu g$ A P in both media]

Medium	Activity in cells from 50 cc. of medium in counts per minute	Activity lost by cells from 50 cc. of medium in counts per minute	Percentage exchanged
Low concentration (45.5 μg A P/L)	43,980	986	2.2
High concentration (1,137.5 μg A P/L)	465,700	80,716	17.3

REDISTRIBUTION OF INTRACELLULAR PHOSPHORUS

Phosphorus exchange is difficult to detect in a culture with a growing population; however, in the filter-washing experiments, exchange was shown. It was further found that the quantity of

phosphorus exchanged depended on the amount of phosphorus in the inorganic fraction in the cells. Whether or not the small amount of phosphorus remaining in the medium at the end of the uptake experiment was due to exchange is not known. For this trace of phosphorus to be the result of exchange would require that some phosphorus always remain in an inorganic state in the cells.

To prepare cells with the inorganic fraction reduced to a minimum necessitates growing them in the light in medium containing no phosphorus. This was the method used to prepare phosphate-deficient cells in the previous experiment. Under these conditions the cells reduce the intracellular phosphorus to a point where further cell division is impossible. Theoretically, under these conditions the inorganic fraction has been reduced to as small an amount as possible.

The rearrangement of intracellular phosphorus was traced in *Nitzschia* cells by following the movement of radioactive phosphorus. To medium containing 14.7×10^7 cells per liter were added 8.75 microcuries of radioactive phosphorus. The same amount of radioactive phosphorus was added again the following day. On the second day the cells were centrifuged from this active medium and resuspended in medium with less than 0.5 μgAP/L of nonactive phosphorus. This medium with cells containing radioactive phosphorus was divided into two cultures. One culture was kept in the light while the other was placed in the dark. Ketchum (1939b) has shown that nondeficient cells similar to these will not absorb phosphorus from the medium in the dark, but it was not shown whether changes occur in the distribution of intracellular phosphorus.

The distribution of intracellular radioactive phosphorus between the organic and inorganic fractions was determined at the time the cells were suspended in the new medium and again on the sixth, tenth, and fourteenth days. The cells were fractionated with trichloracetic acid, as described in Materials and Methods, and radioactivity counts were made on each fraction. The results of this experiment are shown in table 4. The cells kept in the light continued to combine inorganic phosphorus into the organic fraction for most of the 14 days. At the end of this time, 14.7 percent of the radioactive phosphorus still remained in the inorganic state. Under the conditions of this experiment, cell division had stopped by the sixth day. It can be seen in table 4 that little inorganic phosphorus was converted into the organic fraction after cell division stopped. The ceasing of cell division could not have been due to the lack of some other nutrient since the medium contained sufficient amounts of all nutrients except phosphorus. The occurrence of phosphorus in the inorganic fraction at the end of 14 days may indicate that some inorganic phosphorus is always present in *Nitzschia* cells and that division is stopped not by a complete disappearance of inorganic phosphorus but rather by the reduction of the inorganic phosphorus below a threshold amount. From these findings it appears that the trace of phosphorus remaining in the medium in the phosphorus-uptake experiment can be attributed to exchange between the cells and medium.

TABLE 4.—*Distribution of intracellular radioactive phosphorus in* Nitzschia *cells suspended in medium containing only a trace of nonradioactive phosphorus*

[The cells had previously grown for 2 days in medium with phosphorus of high specific activity]

Length of time in new medium	Culture placed in light		Culture placed in dark	
	Inorganic fraction	Organic fraction	Inorganic fraction	Organic fraction
	Percent	*Percent*	*Percent*	*Percent*
0 days	[1] 63.0	[1] 37.0	[1] 63.0	[1] 37.0
6 days	19.1	80.9	59.2	40.8
10 days	20.7	79.3	66.0	34.0
14 days	14.7	85.3	53.4	46.6

[1] Determined before dividing cells into two cultures.

There was not as much change in the distribution of phosphorus between the inorganic and organic fractions in the cells kept in the dark as in those grown in the light, yet there was a slight decrease in the inorganic fraction except on the tenth day (table 4). This one determination was undoubtedly in error. From the data for cells grown in the dark, it can now be surmised that a slight metabolic activity is going on which requires the redistribution of intracellular phosphorus.

EXCHANGE BY CELLS SUSPENDED IN CULTURE MEDIUM

Exchange in the light

The difficulty of detecting phosphorus exchange between cells and medium when phosphorus is being absorbed rapidly by the cells has been shown previously. However, if large amounts of radioactive phosphorus can be incorporated into the

cells in a short enough period of time to prevent much from being combined into organic compounds, there is greater possibility of detecting exchange by measuring the loss of radioactive phosphorus from the cells. It must also be considered that, since each cell takes part in the exchange process, the total amount of radioactive phosphorus exchanged from the cells will depend upon the number of cells used. If a large number of dividing cells are used, a high concentration of phosphorus in the medium is needed. Further, if the concentration of phosphorus in the medium is not sufficient, the radioactive phosphorus exchanged from the cells to the medium will be reabsorbed in too large an amount to permit a detection of exchange.

A culture of *Nitzschia* with 22.7×10^7 cells per liter in medium containing 2 microcuries of radioactive phosphorus per 100 cc. and a phosphorus concentration of less than 0.5 μgA/L was prepared. It was found that cells could absorb this amount of phosphorus within 20 hours and yet retain most of the radioactive phosphorus in the inorganic state. The cells were then centrifuged from this medium and resuspended in new medium containing 2,000 μgAP/L of nonradioactive phosphorus. It has been found that very little reduction in the division rate of *Nitzschia* occurred in this concentration. This new culture was placed in the culture cabinet in the light. Aliquot samples of 25 cc. each were removed periodically, and the cells were filtered from the medium so that radioactive measurement could be made on the culture medium. The sampling was continued over a period of 14 days (table 5).

TABLE 5.—*Loss of radioactive phosphorus to medium by photosynthesizing and dividing cells*

[The cells of 25 cc. of medium contained 9,728 counts per minute]

Length of time	Activity in 25 cc. of medium in counts per minute	Length of time	Activity in 25 cc. of medium in counts per minute
0.5 hour	169	50.0 hours	219
1.0 hour	202	80.0 hours	368
4.0 hours	212	150.0 hours	493
7.0 hours	202	198.0 hours	601
14.0 hours	234	246.0 hours	621
25.0 hours	231	342.0 hours	678

Phosphorus exchange did occur, since radioactive phosphorus from the cells appeared in the medium. The increase in radioactivity in the medium was rapid at first and then decreased with time. Thus, it has been shown that cells, while absorbing and converting phosphorus into organic compounds, do exchange phosphorus with the medium. The radioactive phosphorus in the medium continued to increase until 6.97 percent of the total radioactive phosphorus contained in the cells had been exchanged to the medium at the end of 14 days.

Exchange in the dark

Ketchum (1939 b) has demonstrated that phosphate-deficient cells placed in the dark will continue to absorb phosphate only for about 10 hours. He also showed that the amount of phosphate absorbed in the dark by phosphorus-deficient cells depends on the length of time the cells have been grown in the light in medium containing no phosphate. From these findings it can be concluded that any uptake of radioactive phosphorus by cells can be attributed to exchange if radioactive phosphorus is added to the medium after the phosphate deficiency of the cells has been replaced.

Exchange determined with radioactive phosphorus in the medium.—A culture of 45×10^7 cells per liter was prepared in medium containing 46 μgAP/L. The cells used to prepare this culture came from a medium containing phosphorus. However, to be sure that the cells had no phosphate debt, they were placed in the dark for 16 hours in medium containing phosphorus. The cells were then centrifuged from this medium and resuspended in identical medium to which 0.45 microcuries of radioactive phosphorus per 100 cc. had been added. The culture was again placed in the dark and only removed periodically for sampling. The cells were filtered from aliquot samples of medium, and counts were made for radioactivity in the cells. Inorganic-phosphorus determinations were also made on the medium from each sample. Exchange does occur, as shown by radioactive phosphorus appearing in the cells (table 6). Although the uptake of radioactive phosphorus is small, it is believed to be representative of the amount exchanged, since corrections for contamination were made. This was accomplished by filtering the medium containing the same quantity of radioactive phosphorus and determining the activity retained on the filter, in the $BaSO_4$ precipitate, and on the filter paper. The counts appearing in table 6 were then obtained by subtracting the counts

due to contamination from the observed cell-activity count. Of the total radioactive phosphorus in the medium, about 2.5 percent was exchanged with the cells. There was no detectable change in the inorganic-phosphorus concentration of the medium.

TABLE 6.—*Uptake of radioactive phosphorus by cells from medium when kept in the dark*

[The medium contained 9,887 counts per minute]

Length of time	Activity in cells from 25 cc. of medium in counts per minute	Inorganic phosphorus of medium in μgAP/L
0.5 hour	154	46
2.5 hours	178	46
5.0 hours	224	46
22.5 hours	148	46
26.0 hours	245	46

Exchange determined with radioactive phosphorus in the cells.—Since a tendency for the cells to clump in the previous experiment possibly caused one determination to be low, this experiment was devised with the active phosphorus inside the cells so that determination could be made on the medium. To test for the loss of radioactive phosphorus from the cells through exchange, a culture of 40×10^7 cells per liter was prepared with 1 microcurie of radioactive phosphorus per 100 cc. of medium and less than 0.5μgAP/L. After 16 hours the culture was centrifuged, and the cells containing radioactive phosphorus were suspended in new medium containing 56 μgAP/L of nonradioactive phosphorus. This culture was placed in the dark and samples were removed periodically. There was a loss by exchange of radioactive phosphorus from the cells to the medium (table 7). Of the total radioactive phosphorus in the cells, 3.26 percent was exchanged with the medium.

TABLE 7.—*Loss of radioactive phosphorus to medium by cells kept in the dark*

[The cells from 25 cc. of medium contained 22,570 counts per minute]

Length of time	Activity in 25 cc. of medium in counts per minute	Length of time	Activity in 25 cc. of medium in counts per minute
0.5 hour	87	12.0 hours	519
1.2 hours	155	24.5 hours	615
2.0 hours	175	28.0 hours	746
3.5 hours	210	34.0 hours	735
5.5 hours	325		

DISCUSSION

Some investigators have found it necessary to wash the cells before using them for chemical analysis. According to Gest and Kamen (1948), the washing liquid is usually distilled water, 0.85 percent saline, or less frequently, fresh culture medium. Washing with a saline solution is less drastic than with distilled water. Even so, many organisms have been found to lose phosphorus when washed with saline solutions. A young culture of *Bacterium coli*, after two washings with a saline solution, showed a rapid release of inorganic phosphorus in the absence of glucose (Macfarlane 1939). Also an appreciable quantity of the original phosphorus of *Trypanosoma equiperdum* is lost when the organisms are washed with saline solution (Moraczewski and Kelsey 1948). It must be pointed out, however, that this type of experiment does not demonstrate exchange. For phosphorus exchange to occur, there must be a movement of equal numbers of phosphorus atoms both into and out of the cells. This naturally precludes interpreting the above observations as being exchange, since neither distilled water nor saline solutions ordinarily contain phosphorus.

Fresh-water algae grown in high-phosphate media have been shown to store an appreciable quantity of soluble phosphate which is readily lost when the cells are washed with medium (Gest and Kamen 1948). Also marine species of algae exchange phosphate to a nutrient medium when filter-washed, as has been shown by Goldberg, et al. (1951) and in the experiments reported in this paper. Even with the use of radioactive phosphorus which makes possible an altering of the isotopic distribution of phosphorus in either the medium or the cells, exchange is still difficult to demonstrate, especially in rapidly dividing unicellular forms. This is because the amount of phosphorus absorbed by the cells is many times greater than that leaving the cells through exchange.

Goldberg, et al. (1951) stated that as much as 50 percent of the phosphorus is removed from the marine diatom *Asterionella* when it is washed with sea water; but the factors controlling this removal of radioactive phosphorus from the cells were not discussed. In the filter-washing experiments conducted in this investigation, *Nitzschia* cells were always washed with a culture medium containing the same concentration of

nonradioactive phosphorus as that of the medium in which the cells had previously grown. The amount of phosphorus exchanged with the medium when filter-washed in this manner was found to vary with the physiological condition of the cells. Phosphate-deficient cells grown for the same period of time in medium containing radioactive phosphorus not only absorbed more phosphorus than nondeficient cells, but also exchanged a smaller percentage of that absorbed when filter-washed. Regardless of the condition of the cells, the amount exchanged varied with the phosphorus concentration of the medium in which the cells were grown. Those grown in a high phosphorus concentration not only absorbed more phosphorus but also exchanged more phosphorus with the washing medium.

The percent of radioactive phosphorus exchanged by the cells also depends on the length of time the cells have been grown in radioactive phosphorus. In the filter-washing experiments, nondeficient cells grown for 7 days in the presence of radioactive phosphorus exchanged a smaller percent of radioactive phosphorus than the same type of cell grown in a similar medium for 20 hours. This was the result of more radioactive phosphorus being converted into organic or nonexchangeable compounds in cells grown for the longer period of time. Thus, a smaller percent of the radioactive phosphorus in the cells was exchanged with the medium even though the total phosphorus exchanged in both instances possibly was the same.

In cultures of rapidly dividing unicellular forms, the organic matter increases at a fast rate, quickly removing both radioactive and nonradioactive phosphorus from the medium and thus complicating the measurement of exchange. It is less difficult to detect exchange by measuring the movement of radioactive phosphorus from the cells to a medium continually renewed and containing only nonradioactive phosphorus, provided the specific activity of the exchangeable phosphorus of the cell is high. These were the conditions in the filter-washing experiments. Since under most conditions some radioactive phosphorus remains in the inorganic fraction of the cell and the addition of large amounts of phosphorus to the medium causes an increase in the amount of phosphorus exchanged by the cell, exchange of radioactive phosphorus from the cell can also be detected by increasing the phosphorus concentration of the medium instead of continually renewing it. By following this procedure, phosphorus exchange was measured between dividing cells maintained in the light and the culture medium. While exchange is easily demonstrated by filter-washing, the experiments conducted in the light and in the dark may be more representative of normal exchange.

In the exchange experiment in the dark the cells were in equilibrium with phosphorus in the medium before the radioactive phosphorus was added. The actual amount of phosphorus added with the radioactive phosphorus in terms of μgAP/L was insignificant. Since the cells had remained in the culture cabinet long enough for any phosphate deficiency to have been repaid and, therefore, any absorption of phosphorus from the medium to have stopped, the appearance of radioactive phosphorus in the cells indicated that phosphorus exchange had occurred between cells and medium. In some instances it is only possible to measure exchange over short periods of time; however, it should not be concluded that exchange ceases when it is no longer possible to detect it, but rather that both the radioactive and nonradioactive phosphorus of the medium and the cells have reached equilibrium. Thus, neither a further absorption of phosphorus from the medium nor exchange would alter the specific activity, without which exchange cannot be demonstrated.

In the experiments conducted in the light and in the dark the amount of phosphorus exchanged was very small. It may be questioned whether there was not an exchange between the phosphorus of the medium and that adsorbed on the surface of the cells instead of the phosphorus from inside the cells. That some of the phosphorus exchanged in these experiments came from inside the cells is supported by several observations. In relation to the total amounts of phosphorus adsorbed, the amounts exchanged in the filter-washing experiments were too large to have been exchanged only with the phosphorus adsorbed on the cell surface. Also it was possible to measure exchange for as long as 24 hours in some experiments and for a longer period of time in the experiment tested for 14 days. It is possible that exchange between intracellular phosphorus and the medium is complicated by biological or other processes of the cell, resulting in a longer time being required for

an equilibrium to be reached than if the phosphorus were exchanged only from the surface.

The amount of phosphorus on the surface of the cell may also be directly related to the manner in which phosphorus enters the cell. Two ways in which phosphorus may enter a cell are discussed by Kamen and Spiegelman (1948). One method is believed to be diffusion through the cell membrane of phosphorus as inorganic orthophosphate to combine with the intracellular orthophosphate. Inorganic orthophosphate is assumed to be the source of phosphorus for the various organic phosphates in the cell. The other method is the entry of phosphorus into the cell through esterification at the cellular interface. Intracellular inorganic orthophosphate would then arise primarily from the breakdown of organic phosphate. From their experimental data with yeast these investigators concluded that the primary mechanism of the entrance of phosphate is by esterification.

Phosphorus probably enters algae by one or a combination of these two methods. The mechanism of entry is of importance in phosphorus-exchange studies only if it requires that phosphorus remain on the surface of the cells for any period of time. If the entry of phosphorus into the cell is by diffusion instead of by esterification, it might be very difficult for much phosphorus to be adsorbed on the cell surface, since there would be a tendency for any phosphorus coming in contact with the cell surface to be taken into the cell. The process of esterification, on the other hand, would probably require that phosphorus remain on the surface of the cell for a longer time. Since a population of unicellular algae presents a large surface area, exchange can take place between any phosphorus adsorbed on the surface of the cells and the medium. As far as I can determine there has not been sufficient consideration of the part adsorption plays in phosphorus exchange in microorganisms. It is impracticable to distinguish the phosphorus exchanged from the cell surface from that exchanged from inside the cells. Thus, exchange demonstrated in these studies should be considered to include phosphorus both from the cell surface and from inside the cell.

The amount of phosphorus in phytoplankton varies with the concentration of phosphorus in the medium and some investigators have considered that the minimum phosphorus which can exist in the cell is "bound" phosphate, presumably organic phosphorus. Several observations were made in this investigation which possibly show that all of the minimum phosphate in the cell does not necessarily exist as an organic fraction. It was observed in the phosphorus-absorption experiment that a trace of phosphorus remained in the medium. For this phosphorus to remain in the medium would require that some phosphorus, which could be exchanged with the medium, still remain in the cells. Since a sufficient amount of all other nutrients were present to allow the complete utilization of all the phosphorus, it appears that some phosphorus was still present in the inorganic fraction of the cells. It is well known that cells placed in culture medium containing no phosphorus will continue to divide until they become phosphorus deficient. These cells then could contain only a minimum amount of phosphorus. Cells containing radioactive phosphorus and placed in medium containing only a trace of phosphorus continued to divide and reduce the inorganic-phosphorus fraction for several days. However, after all division had stopped some radioactive phosphorus remained in the inorganic fraction. Thus it appears that some of the minimum phosphorus of the cell may remain in the inorganic fraction.

Hutchinson and Bowen (1950) added radioactive phosphorus at the surface of Linsley Pond and studied its movement over a 4-week period in summer. It appeared that practically all the radioactive phosphorus entered the phytoplankton immediately and that the rate of regeneration was rapid. This continual release of phosphorus from decomposing organisms, which could be mistaken for exchange, is one of several factors in operation tending to complicate exchange studies under natural conditions. Of course, phosphorus lost in this manner could only remain in the water and subsequently be mistaken for exchange when the concentration of phosphorus in the water was already greater than the demands of the organisms. If a lack of phosphorus limited cell division, any phosphorus returned to the water through either regeneration or exchange would be absorbed immediately and any possibility of its detection would be eliminated. However, with cultures grown in the laboratory it is possible to reduce decomposition of cells to an insignificant point and thus eliminate the effect of regeneration. Also, it is possible to control the phosphorus concentra-

tion in the medium so that any radioactive phosphorus exchanged from cells to medium can be measured before being reabsorbed by the cells. Thus it can be concluded that while phosphorus absorption and regeneration can be followed in nature, as shown by Hutchinson and Bowen, the measurement of exchange is limited to the laboratory where conditions can be more closely controlled.

SUMMARY

1. Phosphorus exchange between culture medium and marine planktonic algae was demonstrated.
2. The amount of phosphorus exchanged by *Nitzschia* cells when filter-washed varied with the concentration of phosphorus in the medium in which the cells were grown and with the physiological condition of the cells.
3. The percent of radioactive phosphorus exchanged from cells depends upon the length of time the cells are grown in the presence of radioactive phosphorus. It was found that cells grown over longer periods of time converted more radioactive phosphorus into organic or nonexchangeable compounds.
4. A redistribution of intracellular phosphorus between the inorganic and organic fractions occurred when cells were placed in the light in medium containing only a trace of phosphorus. Cells kept in the dark converted very little intracellular inorganic phosphorus into the organic state.
5. Exchange between photosynthesizing and dividing cells and culture medium was demonstrated by placing cells containing radioactive phosphorus in nonactive medium and measuring the radioactive phosphorus exchanged from the cells to the medium.
6. Exchange between cells and medium was shown while the cells were not photosynthesizing and dividing.

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