Mem. Fac. Fish., Kagoshima Univ.
Vol. 34, No. 2, pp. 183~244 (1985)

Mechanism of Manganese Uptake by a Green Alga, *Dunaliella tertiolecta* Butcher [*1]

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Abstract

Although trace metals are present at low concentrations in seawater, algae are capable of accumulating such metals in high concentrations against a gradient. In order to clarify the uptake mechanism of trace metals, manganese (Mn) uptake from a culture medium by a euryhaline alga, *Dunaliella tertiolecta* BUTCHER was studied.

D. tertiolecta was affected physiologically by Mn in the medium. Higher growth rates and evident elongation in cell length were observed from 0.1 to 1.0 ppm Mn in contrast to other concentrations tested. Crude protein and carbohydrate increased with increasing Mn concentrations with an optimum value at 0.05 to 0.1 ppm Mn. At 1.0 to 10 ppm Mn, protein and carbohydrate synthesis was inhibited. Chlorophyll-a content did not diminish in an Mn deficient medium, but apparently decreased when both Mn and Fe are deficient.

Live *D. tertiolecta* cells accumulated Mn from the culture medium subject to environmental factors. Maximum uptake of Mn occured at pH 9.0, 10,000 lux and 20℃ temperature. Dead cells are unable to take up Mn from the medium and, instead released accumulated Mn to the cell exterior. Mn uptake was activated during early exponential growth phase of the batch culture. It also synchronized with cell growth, with Mn content reduced at cell division and increased with cell growth. Mn levels inside the cell elevated sharply with increasing levels of exogenous Mn from 0.0 to 0.1 ppm. The absorption systems was apparently saturated at 0.1 ppm Mn since further increase up to 1.0 ppm external Mn resulted in virtually no further increase in the Mn content of *D. tertiolecta*. This saturation kinetics followed Michaelis Menten equation. Mn concentration factor in *D. tertiolecta* was 10-fold at 0.3 to 5.0 ppm and 100-fold at 0.1 ppm Mn in the medium. Mn uptake was inhibited by DCMU, TPAC, and KCN, and stimulated by respiratory substances, glucose. Zn and Cu ions in the medium also inhibited accumulation, while Na, K, Mg, Ca, P and Fe accelerated uptake of Mn. In *D. tertiolecta*, 72.8% of accumulated Mn was found in the tonoplast, 25.1% in protoplast as free ion or low molecular weight compound, and only 2.1% as firmly bounded to organelles or protoplast.

The presence of Mn-stimulated ATPase was confirmed in whole cell homogenates. Mn-ATPase activity was one-tenth lower than that in Na, K-ATPase, and was strongly temperature dependent with an optimum at 25℃. Optimum pH for the activity was pH 8.0. With

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increasing Mn concentration, Mn-ATPase activity rose and reached a limiting value at 1.0 mM Mn (=55 ppm Mn). At higher concentrations, activity slowly diminished and eventually vanished at 8.0 mM Mn (=440 ppm Mn). Mn-ATPase was inhibited strongly by oligomycin and weakly by ouabine. The activity of Mn-ATPase was detected on cell plasma membranes. A highly significant correlation was noted between Mn-ATPase activity and Mn influx at concentrations of 0.0 to 5.0 ppm Mn ($P<0.025$; $r=0.896$).

Form these results, Mn uptake mechanism of *D. tertiolecta* outlines an active transport process. Channels or pumps in the cell membrane are activated by energy from Mn-ATPase, which then takes up Mn from outside into the cell. Accumulated Mn are stored mostly in the form of ions or low molecular weight compounds near the cell membrane, while minimum amounts are transported to organelles i.e., chloroplast. Trace metal accumulation in other algae are likely to follow a similar mechanism found in *D. tertiolecta*.

Table of contents

I. Introduction

In general, higher green plants take nutrients by their root. However, algae, whether marine or freshwater organisms, benthic or planktonic organisms, absorb many kinds of elements dissolved in the aquatic environment mainly through the surface of plants. This characteristic feature makes the algae favorable material for experiments of the nutrient utilization.

These nutrient elements are usually divided into macro-and micro-nutrients (or trace metals), though there is no clear differentiation between two definitions of nutrients. Most macro-nutrients (e.g. P, N, and C) are needed for the medium of algal culture at concentrations over 1 ppm, while micro-nutrients (e.g. Fe, Al, Zn, Mn and Co) are enough at concentrations less than the above value. The concentration of micro-nutrients or trace metals are quite low in the sea, but algae can highly accumulate them against a gradient in concentration.

The existence of trace element in various forms in the natural water is recently becoming better known by examing nutrient elements which phytoplankton selectively utilized at

certain time of the year or by the presence of elements adsorbed by other colloidal particles. Although such elements may vary in concentration with depth and location, Fe, Al, Mo and Zn are generally estimated to be in the order of 10 ppb, Cu, Mn, Ni and Ti in the order of 1 ppb and Co and Cr in the order of 0.1 ppb. BOWEN (1966) reviewed the data published so far on the mean values of trace elements concentrated by green, brown and red marine algae and showed that many algae could accumulate the elements at 10^3 to 10^4 times or more as much as the sea contained, though their content was quite scanty in the environment.

Of these micro-nutrients, manganese is one of the most interesting elements. Manganese plays the roles of catalytic, activating or diminishing reaction for some enzyme in the following various ways : (1) in relation to the process of respiration, oxidative phosphorylation, decarboxylaton, hydrolysis, citric-acid cycle, glycolysis and the metabolism of carbohydrates in general ; (2) in relation to the process of photosynthesis, chlorophyll synthesis, water photolysis and oxygen liberation ; (3) the reduction of nitrate to ammonia, and other reactions particularly in nitrogen assimilation ; (4) plant hormone metabolism through the activation of oxidase ; (5) synthesis of aminoacid, peptide and protein, and metabolism of nitrogen and phosphorus ; and (6) many other physiological functions (MULDER & GERRETSEN, 1952 ; POSSINGHAM & SPENCER, 1962 ; HEATH & HIND, 1969 ; CHENG & OUELLETTE, 1971 ; AMBERGER, 1973).

At the level of concentration in the sea, manganese is apparently not toxic to algae, but essentially necessary for these organisms as their nutrient in the first part of marine food chain. In fact, the presence of Mn is ecologically significant in the sea with respect to affecting the growth of marine phytoplankton and finally causing the bloom of the red tide organisms such as *Chattonella* and *Exuviaella* (IWASAKI, 1978, 1979).

The nuclear power industry is developing to meet future energy needs, but in the process of treatment of the resultant by-products, radio-active wastes have leaked into the environment. One of these radio-active substances possibly released into aquatic systems is Mn^{54} which is an activation product formed from stable Mn^{55}, and Fe^{54} in the presence of high energy neutrons (NEILSON & YAKABE, 1966). But little is known about the distribution of radioactive manganese (Mn^{54}) in aquatic systems and the uptake of this radioisotope by marine organism.

In addition to except references quoted above, various biologically active roles of Mn in algae and terrestrial plants have been published. However, almost nothing is known of the forms of Mn taken up by algae or the process of incorporation in connection with passive or active uptake. Furthermore, few information is available in relation to other nutrients present in the medium and Mn uptake.

This research was carried out to know what forms of Mn are taken up by algae and whether transport of Mn is active or passive in euryhaline green alga *Dunaliella tertiolecta* BUTCHER.

Ⅱ. State of manganese taken up by *Dunaliella tertiolecta* BUTCHER

Ⅱ-1. Introduction

Manganese is commonly contained in igneous, sedimentary or metamorphic rocks on the earth. Interest in Mn has been aroused by the occurrence of Mn nodules which are widely

distributed as a certain form of pelagic sediments, particularly in the Pacific basin. MASON (1966) estimated Mn concentration in the crust of the earth to be 0.1% (1,000 ppm).

The concentration of manganese dissolved in the sea water generally lies in the range between 0.1 and 3 $\mu g/l$ (=ppb) (BENDER *et al.*, 1977). The element seems to be present in the divalent state, and distributes throughout the water column in the sea as fine particles which are less than 0.5 μm in size but more bigger in surface water (RILEY & ROTH, 1971 ; KREMLING & PETERSEN, 1978). According to HOOD (1966), a chemical form of Mn in the sea water is substantially an organic complex.

On the other hand, concentration gradients and other variations in geochemical conditions are seen in estuaries. This phenomenon has suggested that estuaries are just a transfering site at two states of Manganese : insoluble in the river sediment and dissolved in the sea (MURATA, 1939 ; MORRIS & BALE, 1979).

Ⅱ-2. Materials and methods

Chemicals

All chemicals used in this experiment were of guaranteed grade (Wako Chemicals, Ltd.) and reagents were prepared after dissolving in re-distilled water. The Mn solutions used for the experiments were prepared by the following procedure. One gram of Mn metal (99.99%) was dissolved in 10 m*l* of 12 N HCl and diluted to 1,000 m*l* with H_2O to prepare the Mn stock solution (1,000 ppm Mn^{2+}/0.1 N HCl). The Mn^{54} tracer solution was obtained from Japanese Radioisotope Center, Tokyo, as $MnCl_2$ dissolved in 0.1 N HCl. This radiochemical was diluted to give a concentration of 1.0 μCi/1.0 m*l* HCl and stored at 4℃.

Determination of physical state of Mn in the medium

In order to elucidate the state of the particulate Mn in the medium, 3.0 μCi of Mn^{54} were added to 20.0 m*l* culture medium (Table Ⅳ-1) in flasks to give a total Mn concentration of 0.0, 0.1 and 1.0 ppm. These flasks were autoclaved and shaken using the shaker under continuous illumination of 3,000 lux and at 20℃. One m*l* aliquots were removed and filtered through 0.2 μm pore-sized membrane fillters and the filters were washed with 5.0 m*l* of Mn-free culture medium. The radioactivities of the filters were measured as described below. To know whether the filterable Mn was ionic or soluble Mn, 1 m*l* of the solution was removed periodically from the medium, and passed through a column of cationic exchange resin (Dowex 50). The ionic Mn was eluted with 6 N HCl solution and its radioactivity was determined.

Radioactivity measurement of Mn^{54}

The radioactivity measurements were carried out in a welltype gamma ray NaI scintillation counter at room temperature (Ca. 10° to 20℃). Either the time to total 10,000 counts or the counts per 5 minutes were recorded.

Ⅱ-3. Results and discussion

Amount of Mn particles retained on 0.2 μm membrane filter after filtration was very low in percentage (Table Ⅱ-1). On analysis, it becomes apparent that Mn dissolved in the medium is present in the remarkably high ratio of 97%, and 99% of the soluble Mn is ionic

Table Ⅱ-1. Percentage of Mn retained on 0.2 μm membrane filter as particles from medium containing different amount of nonactive Mn. Three μCi of Mn^{54} were added to 20 m*l* culture medium in flasks to give a total Mn concentration of 0.0, 0.1 and 1.0 ppm. Test flasks were shaken using the shaker at 20℃. One m*l* of aliquots were removed and filtered through 0.2 μm pore-size membrane filters. After washing, radioactivities of the filter were measured. Less than 4 % of the Mn added to the medium was present as particulate Mn. So that, the manganese taken up by cell was dissolved in the medium as Mn^{2+} ion.

Added Mn (ppm)	$\frac{\text{Particulate Mn}}{\text{Total Mn}}$ (%)			
	0.5	24	48	72 hrs*
0.0	0.9	1.5	0.4	0.9
0.1	0.8	0.5	1.9	1.9
1.0	2.8	3.3	2.9	2.4

* Hours after the addition of Mn.

Table Ⅱ-2. Relationship between pH and ionc Mn^{2+} in the medium. To know whether the filterable Mn was ionic or soluble Mn, 1 m*l* of the solution was removed from the medium described in Table Ⅱ-1, and passed through a column of cationic exchange resin (Dowex 50). The ionic Mn was eluted with 6 N HCl solution. Percentage of Mn^{2+} was calculated from Mn^{54} absorbed by anion exchange resin. More than 99 % of the manganese was ionic in wide range of pH. This result shows the manganese absorbed by *D. tertiolecta* was Mn^{2+} ion.

pH	$\frac{Mn^{2+}}{\text{Total Mn}}$ (%)		
	0.5	4	24 hrs*
pH 6.0	99.8	100.0	99.5
8.0	99.2	99.5	99.3
10.0	99.9	99.2	99.9

* Hours after the addition of Mn.

Mn^{2+} (Table Ⅱ-2).

According to ICHIKUNI (1972), several forms of Mn may be present in aqueous solutions depending upon pH value, Mn concentration, reduction-oxidation potential, ionic strength and pH-dependent forms of Mn can be described.

In general, chemical form of trace element in sea water is classified as follows : (1) soluble (=ionic) ; (2) colloidal ; and (3) particulate form. But it is difficult to distinguish soluble form from colloidal form, because colloidal trace elements can pass through membrane filter, for example. ICHIKUNI(1972) showed the physical composition of Mn dissolved in aqueous solution and considered that Mn seemed to be present in the sea mostly as Mn^{2+}, MnO_2 and $Mn(OH)_2$. It has been known that in the cultural experiments of marine

planktonic algae metals are utilized for their growth not only in ionic form but also in particulated form, e. g. *Asterionella japonica* takes up particulate or colloidal iron (GOLDBERG, 1952) and *Nitzschia closterium*, the particulate cesium (RICE & WILLIS, 1959). However, as shown in Tables Ⅱ-1 and Ⅱ-2, the results obtained in the present experiment show that the manganese added to the medium is present as Mn^{2+} ion.

Ⅲ. Structure of *Dunaliella tertiolecta* BUTCHER in relation to transport of manganese

Ⅲ-1. Introduction

The genus *Dunaliella* TEODORESCO is a naked, unicellular flagellate alga in which 12 species are enumerated (BUTCHER, 1959) and is a member of the family Polyblepharidaceae belonging to the division Chlorophycophyta (BOLD & WYNNE, 1978). This genus is widely distributed in the sea and often found in hypersaline environments and/or in environments subjected periodically or frequently to drastic salinity fluctuation.

Dunaliella tertiolecta BUTCHER is an oceanic euryhaline species and used in the present research by the reason why it is not considered typical of oceanic phytoplankton but profitable in (1) the ease in handling and (2) the tolerance to highly concentrated toxic heavy metals than most of other marine planktonic algae (DAVIES, 1976). The properties mentioned above made this alga more useful test organism than other marine phytoplankton with respect to the investigation of the heavy metals uptake (OVERNELL, 1975 ; SARAIVA & FRAIZIER, 1975 ; DAVIES, 1976 ; BETZ, 1977 ; JENNINGS, 1979 ; JENNINGS & RAINBOW, 1979 ; WHEELER *et al.*, 1982).

Prior to studying the Mn uptake mechanisms in *D. tertiolecta*, the ultrastructure of the organism was described in this chapter.

Ⅲ-2. Materials and methods

Organism and culture conditions

The culture of *Dunaliella tertiolecta* * was obtained through the kind offers of Dr. Y. NISHIHAMA, Hokkaido Institute of Mariculture. The culture was cleaned of contaminated bacteria using capillary and was grown axenically. The culture medium used was modified from JOHNSON *et al.* (1968) by NORO (1978) as shown in Table Ⅳ-1. Cells were added to 5 m*l* of medium in a 15 m*l* test tube with screw and incubated at 20℃ under a light intensity of approximately 3,000 lux from fluorescent lamps, 12 : 12 h LD.

Electron microscopy

For electron microscopy, the cells were harvested at a stationary phase by centrifugation and fixed with 2.5% (V/V) glutaraldehyde in 0.1 M γ-collidine buffer, pH 7.4, for 1.5 hr at 4℃. The osmolarity of the fixative was adjusted by the addition of 0.25 M sucrose. Samples were subsequently embedded in 2% noble agar and washed 3 times with 0.1 M γ-collidine buffer at 5 min interval at 4℃. Small agar blocks (Ca. 1mm^3) containing cells were

* The origin of this strain is from Dr. DROOP, Plymouth Oceanographic Institute, England.

then postfixed in 1.0% OsO_4 buffered with 0.2 M γ-collidine for 30 min at 4℃. The blocks were washed, dehydrated in an increasing ethanol series (25—100%), placed in propylene oxide (QY—1) and finally embedded in pure EPON. The EPON embedded cells were polymerized at 65℃. Blocks were sectioned on an MT-1 ultramicrotome and sections were post-stained with saturated aqueous uranyl acetate for 30 min followed by Reynold's lead citrate for 5 min. Grides were examined under Hitachi H-300 electron microscope.

Ⅲ-3. Results and discussion

Fig Ⅲ-1. is a longitudinal section of the organism showing profiles of the organelles. The cell is found only by a cytoplasmic membrane, and lacks a cell wall or scales. Two flagella extrude through the apex. The axonemes appear to have the typical 9+2 microtubular arrangement. Below the flagellar bases lies the Golgibody, consisting of one or more dictyosomes. In the centre of the anterior portion of the cell lies the nucleus with a porous nuclear envelope. The nucleolus is nearly central, surrounded by clumped heterochromatin. Although typical of both logarithmic and stationary phase cells, vacuoles and vacuolar inclusions are somewhat more abundant in the latter (Figs. Ⅲ-3, & Ⅲ-4). Vacuoles contain portions of membrane and vesicles. The campanulate chloroplast occupies the entire posterior portion of the cell and extends anterioly along the sides, almost completely enclosing the central region of cytoplasmic matrix and nucleus (Fig. Ⅲ-2). The lamellae runs approximately parallell to the longitudinal axis of the chloroplasts, each band consisting of 1—4 tylakoids. The chloroplast stroma contains small electron dense materials which are assumed to be lipid droplets. The pyrenoid is immersed in chloroplast matrix located at the posterior end of the cell and is usually penetrated by lamellae on all sides. These lamellae normally consist of pairs of thylakoids and they terminate before reaching the centre of the pyrenoid matrix. Between the pyrenoid matrix and chloroplast proper is found the starch sheath which may either consist of a few large curved plates or a number of smaller discoidal grains.

The use of giant algal cells has been very important in recent-advances in our understanding of mechanism of solute and water transport in plants. On the other hand, studies with unicellular micro algae have contributed to our knowledge of Na^+/K^+ regulation (BARBER, 1986) and the transport of metabolized nutrients such as phosphate, nitrate and sugars (NISSEN, 1974).

In these micro algae, *D. tertiolecta* is one of the most useful alga for nutritional study. This species grows actively in the artificial medium and is a widely used organism in the study of the effect of nutrient on the growth of algae. Nutritional study of heavy metals also studied in *D. tertiolecta*. But, uptake mechanisms of heavy metals is not clear.

In the analysis of the uptake mechanism of *D. tertiolecta*, adsorption by cell wall is neglected (omitted) because this flagellate alga is a wall-less or naked cell organism. The present study of *D. tertiolecta* was almost the same as that of HOSHAW & MALUF (1981) of *D. tertiolecta*. The relatively large fraction of the cytoplasm occupied by the chloroplast and vacuoles, makes it important in considering the distribution of solutes and the significance of electrical measurements in the cytoplasm. Quantitative electron microscopy shows that

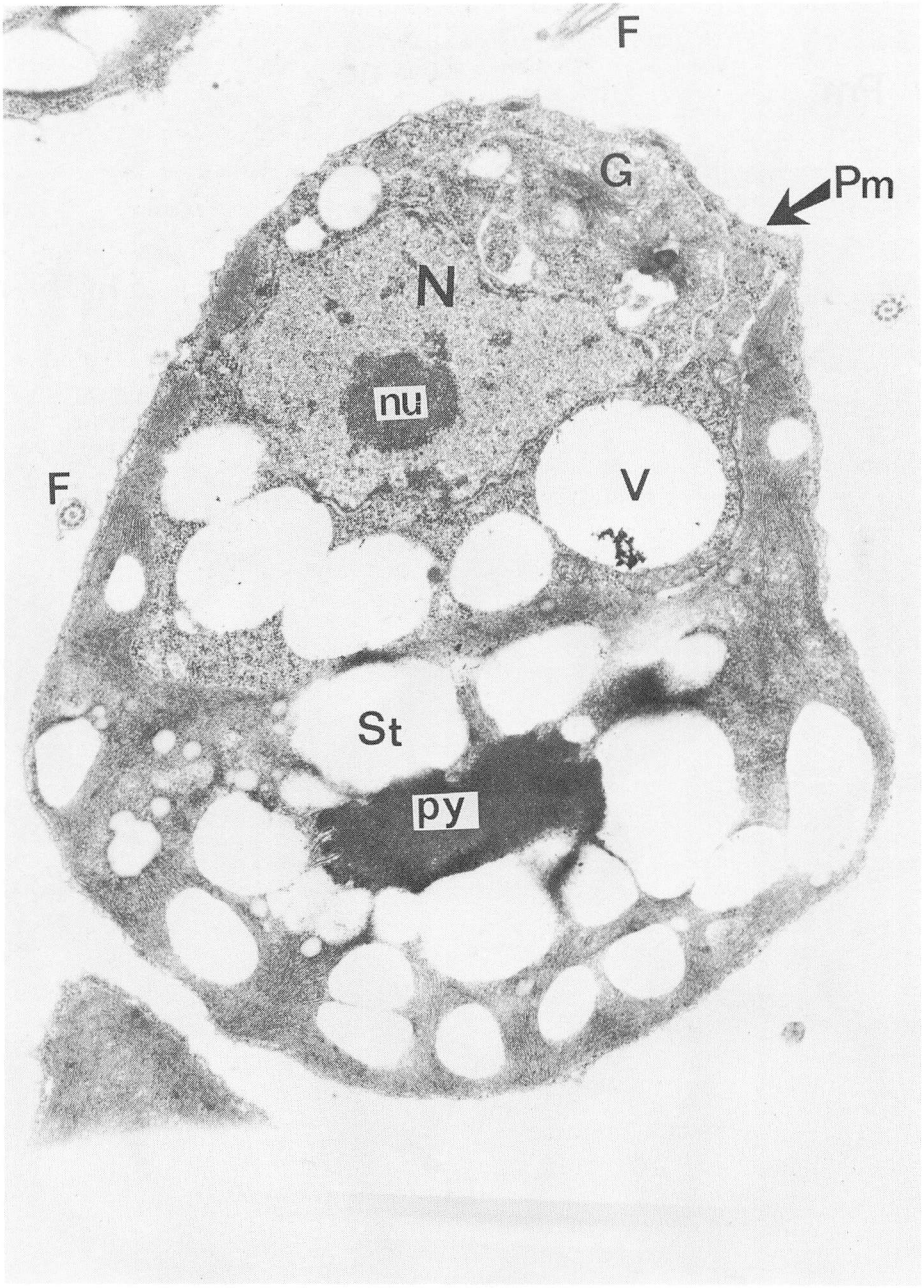

Fig. Ⅲ-1. Electron micrograph of *D. tertiolecta*. Longitudinal section showing flagella (F), nucleus (N), nucleous (nu), chloroplasts (ch), vacuoles (V) and Golgi bodies (G). The plasmalemma (Pm)surrounds the cell. Starch (St) surrounds pyrenooid (py). Scale=1 μm.

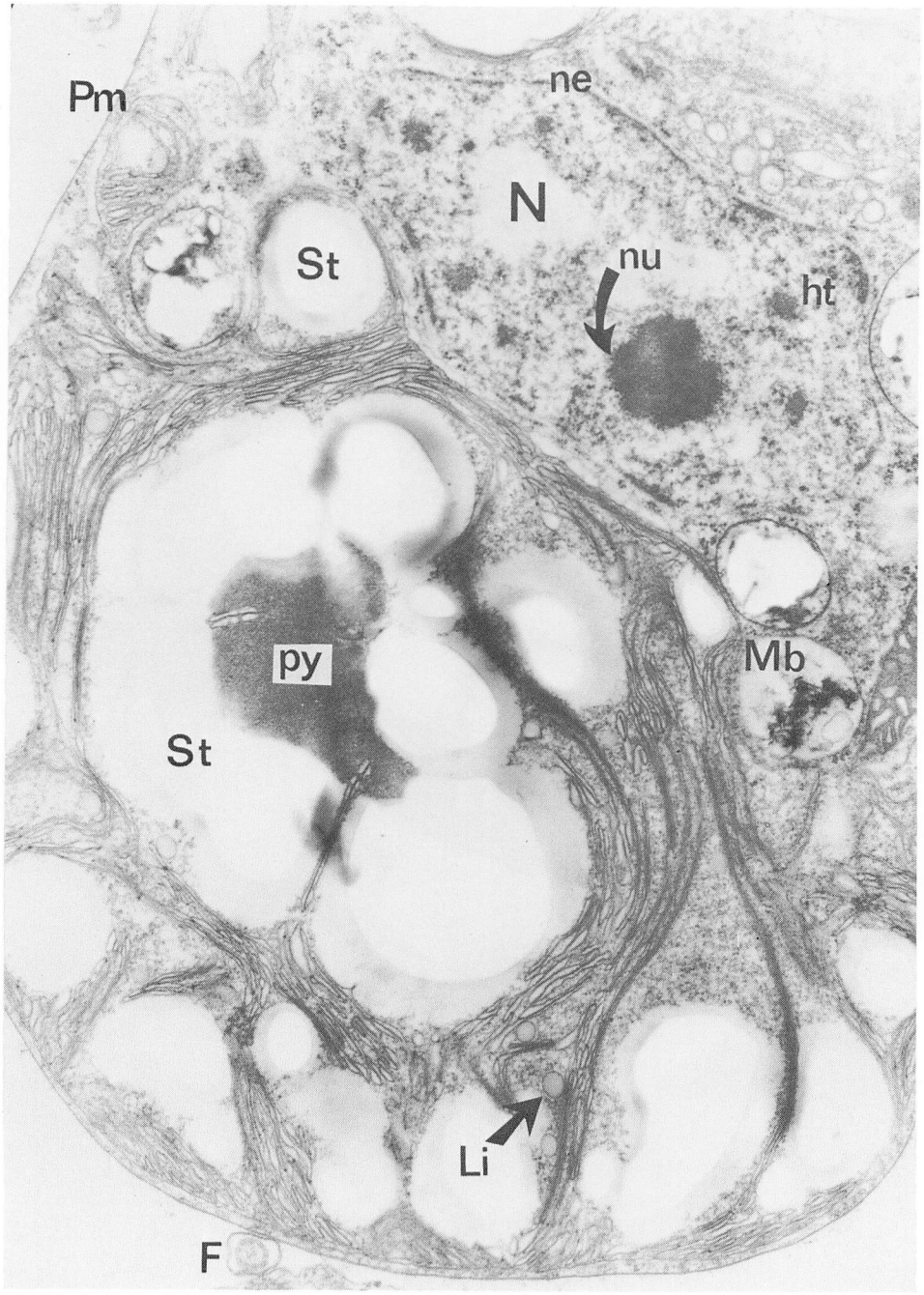

Fig. Ⅲ-2. Electron micrograph of *D. tertiolecta*. Numerous chloroplast lamellae penetrate the pyrenoid and starch sheath consisting of grains. Scale=1 μm.

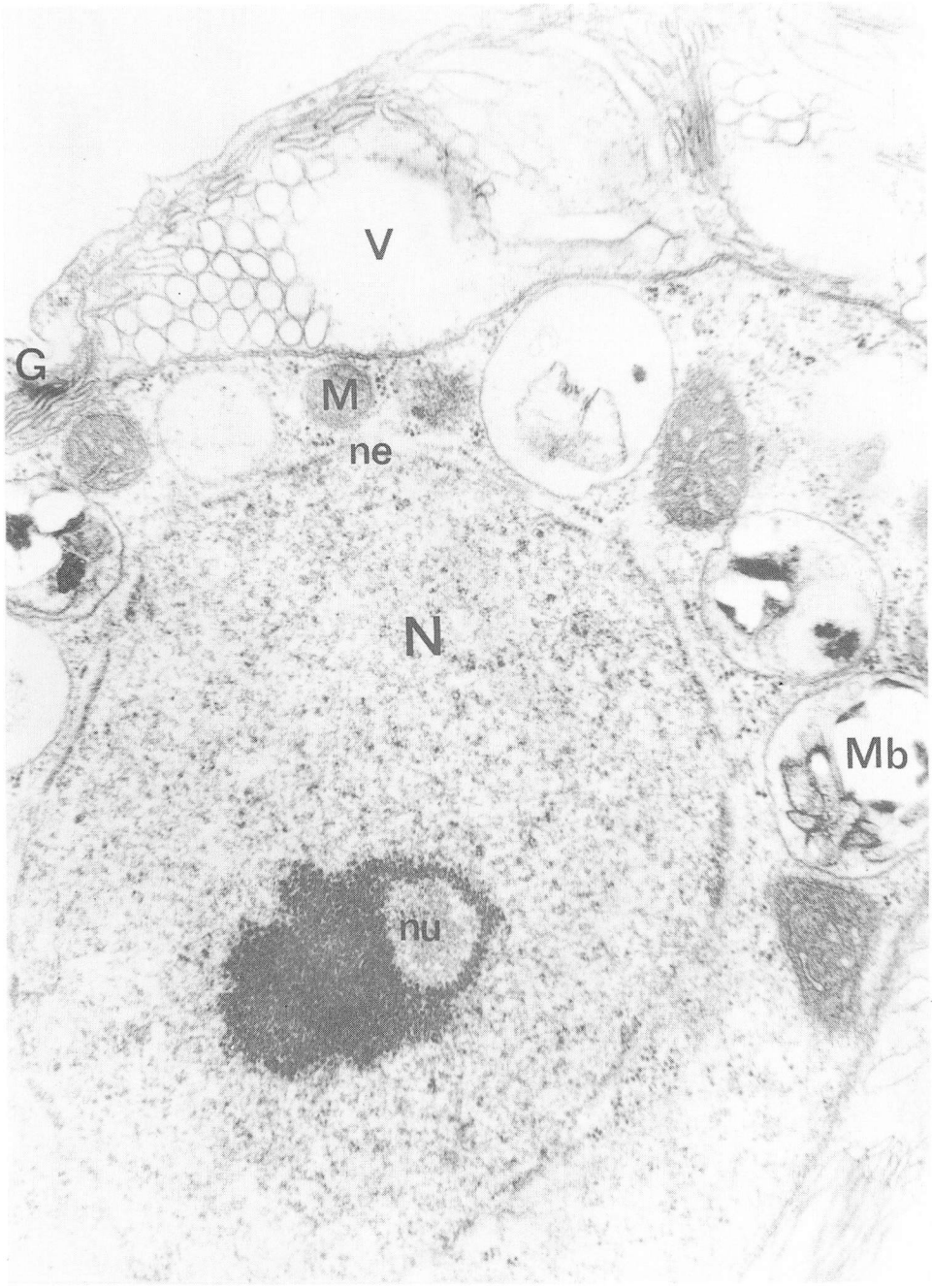

Fig. Ⅲ-3. Electron micrograph of *D. tertiolecta*. The nuclear envelop (ne) is characterized by many nuclear pores. Heterochromatin scatters within the nucleus. Mitochondria (M) and Golgi boby (G) are seen near Vacuoles (V). Scale=1 μm.

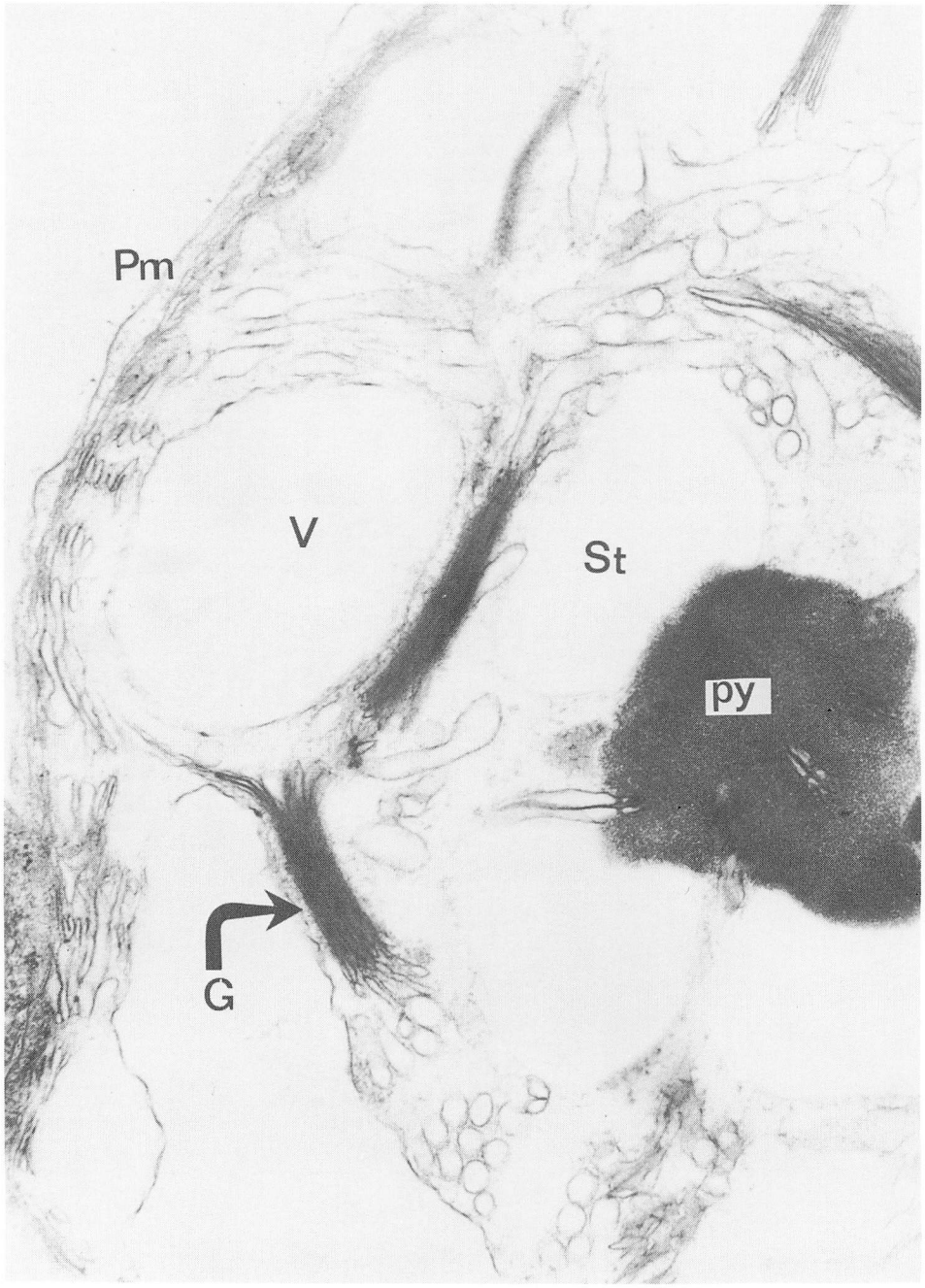

Fig. Ⅲ-4. Electron micrograph of *D. tertiolecta*. Vacuoles (V) are seen near Golgi bodies (G). Scale=1 μm.

D. tertiolecta has about 50% of their cell volume occupied by vacuoles. Chloroplast, nucleus, starch and pyrenoid occupied about 20, 15, 10 and 5% of the cell, respectively.

This chapter showed how *D. tertiolecta* can make good experimental material for a number of transport problems as well as for the biophysical approaches to which giant algal cells are so well suited.

Ⅳ. Effect of manganese on the growth and the metabolism in *Dunaliella tertiolecta* BUTCHER

Ⅳ-1. Introduction

The recent realisation of the significant effects of dissolved heavy metals on the growth of marine phytoplanktons has been reflected in several literature (MANDELLI, 1969 ; STEEMANN NIELSEN & WIUM-ANDERSON, 1970 ; HANNAN & PATOUILLET, 1972 ; Jensen *et al.*, 1974, 1976 ; OVERNELL, 1975 ; BRAEK *et al.*, 1976 ; BERLAND *et al.*, 1977). The position of the phytoplankton at the base of many marine food chains means that any inhibition or promotion of their growth may cause changes in the population levels of herbivores and carnivores of the food chain. Additionally, many phytoplankton species accumulate heavy metals, (SARAIVA & FRAIZIER, 1975 ; COSSA, 1976 ; SCHULZ-BALDES & LEWIN, 1976 ; STYRON *et al.*, 1976 ; GERHARDS & WELLER, 1977 ; JENNINGS & RAINBOW, 1979) and such heavy metals are further accumulated at higher food chain levels.

The initial report on the manganese requirements for algae was made by HOPKINS (1930 a, b). HARVEY (1947) reported the manganese starvation in natural inshore waters. BARBER *et al.* (1973) also demonstrated that poor growth of phytoplankton in fresh upwelling seawater was reversed by addition of Fe or Mn. In these situations, it is apparent that the growth of phytoplankton is limited when manganese is at low concentration. The effects and functions of manganese on algal metabolism has been reviewed by many phycologists including BOWEN (1966), IWASAKI (1967), O'KELLEY (1974) and SANDERS (1978). The most studied effect of manganese on algal metabolism is the role of Mn in the O_2-evolving system of photosynthesis. This micronutrient activates a number of phosphate transferases and decarboxylases, notably those used in the Krebs cycle.

The marine alga, *D. tertiolecta*, has been used in the physiological experiments of nutrients. HARVEY (1947) investigated the effects of added Mn on the growth of *D. tertiolecta* (as *Chlamydomonas* sp.). In the report of PIRSON & BERGMANN (1955) and REISNER & THOMPSON (1956), Mn-requirement for trophical growth and no growth carried out by Mn deficiency in *Chlorella* under heterotrophic carbon nutrition was conspicuous. Manganese concentration of *D. tertiolecta* in culture was analyzed by RILEY & ROTH (1971). However, the function of Mn on the physical condition of *D. tertiolecta* is obscure.

This chapter treated with the growth rate and the variation of three cell contents, protein, carbohydrate and chlorophylls, at various level of Mn concentration in the medium.

Ⅳ-2. Materials and methods

D. tertiolecta was freed of bacteria by washing. The bacteria-free isolates were grown in

Table Ⅳ-1. Composition of culture medium *)

NaCl	2.4 g
$MgCl_2 . 6H_2O$	1.5 g
$MgSO_4 . 7H_2O$	0.5 g
KCl	0.2 g
$CaCl_2$	0.2 g
KNO_3	1.0 g
$NaHCO_3$	0.043 g
TRIS	2.45 g**)
K_2HPO_4	0.045 g
Fe-EDTA	3.64 mg
EDTA-2Na	1.89 mg
$ZnSO_4 . 7H_2O$	0.087mg
H_3BO_3	0.61 mg
$CoCl_2 . 6H_2O$	0.015mg
$CuSO_4 . 5H_2O$	0.06 mg
$MnCl_2$	0.23 mg***)
$(NH_4)_6Mo_7O_{24} . 4H_2O$	0.38 mg
H_2O	1,000 ml

*) Modified from medium used by JOHNSON *et al.* (1968).
**) Adjusted to pH 8.0 with HCl.
***) Stock manganese solution: dissolve 1.000 g of manganese metal (99.99%) in the minimum volume of HNO_3 (40%). After evaporating to dryness on a water bath, again dissolve in 10 ml of conc. HCl and dilute to 1,000 ml with H_2O.

modified JOHNSON'S medium (Table Ⅳ-1) at 20℃ under 12 : 12 hrs LD cycle (cool white fluorescent lamps giving an illumination of ca. 5,000 lux). Manganese solution was prepared by dissolving metal Mn (p. 187). Before the experiment, *D. tertiolecta* cells were Mn-starved in Mn-free medium with aeration.

The number of cells of *D. tertiolecta* was determined using a haemocytometer. Chlorophyll content was determined according to the procedure of JEFFREY & HUMPHREY (1975). Crude protein extraction using 1.0 N NaOH took 3 hrs at room temperature, was determined by the modified LOWRY'S method (HARTREE, 1972). The protein value was converted to albumine. Crude hydrocarbon analyzed by the method of DUBOIS *et al.* (1956) was converted to glucose value.

Ⅳ-3. Results

(1) The relationship between the growth and the manganese content of the medium

The growth in cell number responses differetly in proportion to the concentration of Mn in the medium as illustrated in Fig. Ⅳ-1. Fig. Ⅳ-2 and Table Ⅳ-2 also show the cell density on the 10th day after inoculation and the growth rate constant (K) in logarithmic phase, respectively. At the lowest concentration (0.05 ppm Mn), the growth continued exponentially after the addition of Mn, though the growth ceased up at 16 ppm Mn concentrations. Higher growth rate and a slight enlargement of cell length was observed in

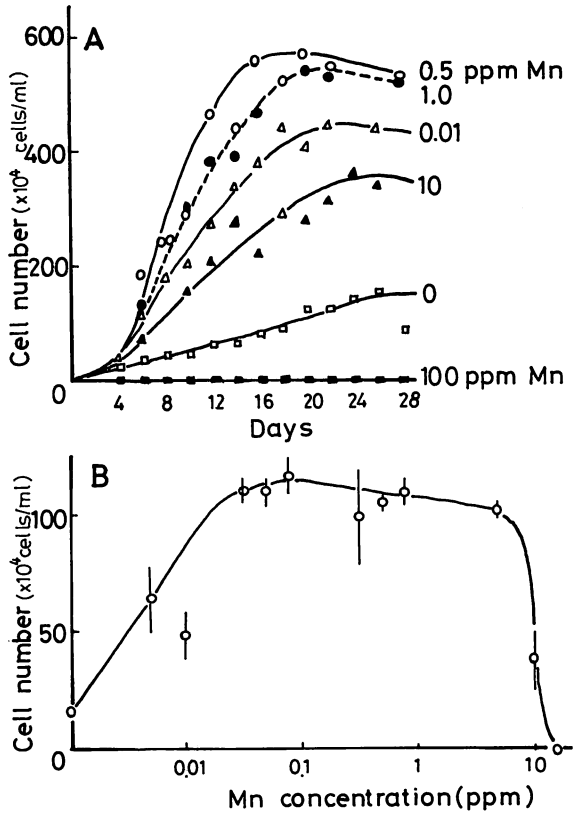

Fig. Ⅳ-1. Effect of Mn at various concentrations on the growth of *Dunaliella tertiolecta* in batch culture. Growth curve is expressed as a function of time (A) and shown on the end of 10 days culture (B). The results shown in (A) and (B) come from different experiments, so that the cell density attaiend is different between (A) and (B). Cells were incubated at 20℃, 12:12 hrs LD, 5,000 lux.

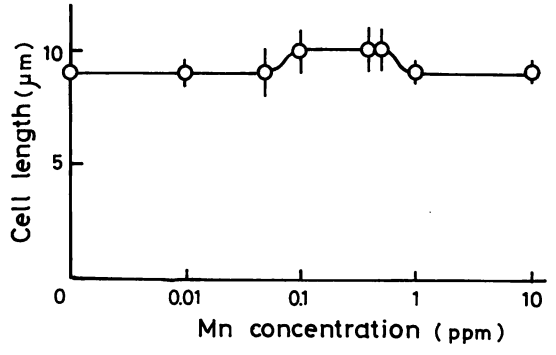

Fig. Ⅳ-2. Effect of Mn at various concentration on the cell enlargement of *Dunaliella tertiolecta* maintained in batch culture for ten days. Cells were incubated at 20℃, 12:12 hrs LD, 5,000 lux. Slight enlargement of cell length was observed in Mn concentration from 0.1 to 1.0 ppm.

Table Ⅳ-2. Mn effects on the growth rate constant (K) of *Dunaliella tertiolecta* incubated at 7 concentrations from 0 to 16 ppm Mn at 20℃, 12:12 hrs LD, 5,000 lux. K is calculated using the equation:

$$K=\frac{1}{T_2-T_1}\log\frac{N_2}{N_1}$$

where N_1 and N_2 are the cell number at 3-day (T_1) and 5-day (T_2) growth respectively. Higher growth rate constant (K) in logarithmic phase is observed from 0.1 to 0.5ppm Mn concentration.

Concentration of Mn in medium (ppm)	K
0.00	0.08
0.01	0.22
0.10	0.38
0.50	0.38
1.0	0.30
10.0	0.26
16.0	0.00

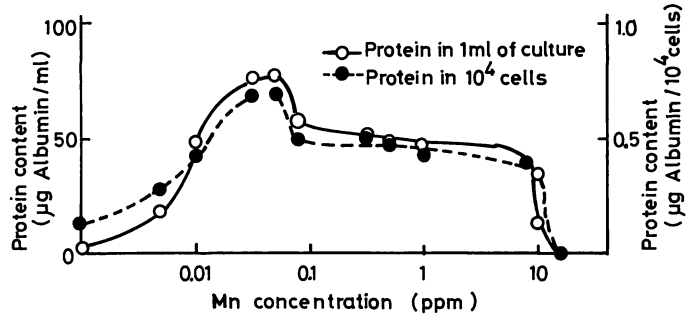

Fig. Ⅳ-3. Protein content of *Dunaliella tertiolecta* grown at various levels of Mn in batch culture for ten days. Cells were incubated at 20℃, 12:12 hrs LD, 5,000 lux. Protein was analysed using the method of HARTREE (1972). Protein content attained a maximum value when 0.05-0.1ppm Mn was added to medium.

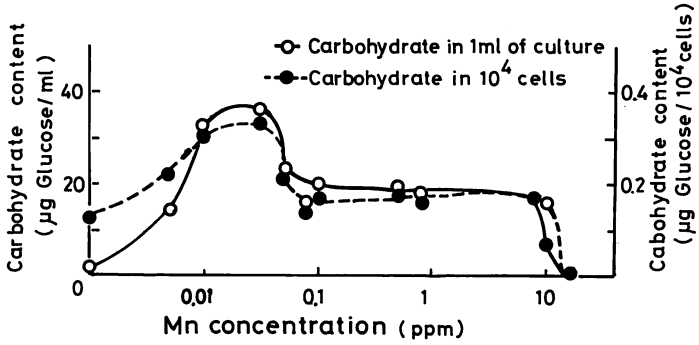

Fig. Ⅳ-4. Carbohydrate content of *Dunaliella tertiolecta* grown at various levels of Mn in batch culture for ten days. Cells were incubated at 20℃, 12:12 hrs LD, 5,000 lux. Carbohydrate was analysed using the method of DUBOIS *et al.* (1956). Carbohydrate content, as well as protein content, attained a maximum value when 0.05-0.1ppm Mn was added to medium.

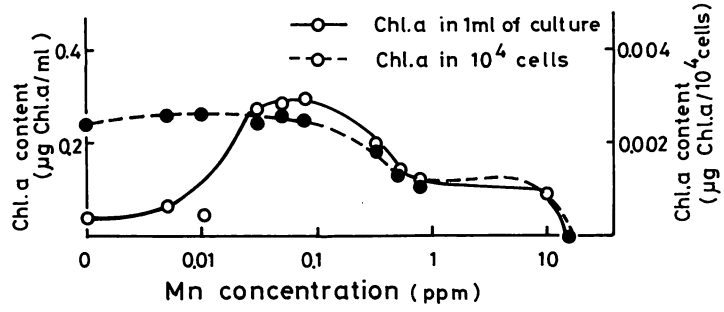

Fig. Ⅳ-5. Chlorophyll-a content of *Dunaliella tertiolecta* grown at various levels of Mn in batch culture for ten days. Cells were incubated at 20℃, 12:12 hrs LD, 5,000 lux. Chlorophyll-a was analysed using the method of JEFFREY *et al.* (1975). Chlorophyll-a in cells did not decrease in Mn deficient medium.

Table Ⅳ - 3. Chlorophyll-a and -b of *Dunaliella tertiolecta* grown in batch culture system with and without Mn and Fe for ten days. Cells were incubated at 20℃ , 12:12 hrs LD, 5,000lux. JOHNSON'S medium (JM) was shown in Table Ⅲ − 1. JM-Mn:Mn deficient JOHNSON'S medium, JM-Fe:Fe deficient JOHNSON'S medium, JM-Mn, Fe:Mn and Fe deficient JOHNSON'S medium. Chlorophyll-a in cells did not decrease so much in Mn deficient medium but decreased remarkably under Mn and Fe deficient condition.

Medium	(1) Cell No/m*l* ($x10^4$cells/m*l*)	(2) Chl.a/m*l* (μg/m*l*)	(3) Chla /cell ($x10^{-3}$μg/10^4cells)	(4) Chl. b/m*l* (μg/m*l*)	(5) Chl.b/cell ($x10^{-3}$μg/10^4cells)	(6) Chl.a/Chl.b
JM	452±63	0.63±0.08	1.39	0.15±0.02	0.33	4.21
JM-Mn	337±26	0.49±0.03	1.45	0.13±0.01	0.39	3.71
JM-Fe	349±35	0.37±0.03	1.06	0.09±0.01	0.26	4.08
JM-Mn, Fe	353±27	0.30±0.02	0.85	0.07±0.01	0.20	4.25

(3)=(2)/(1), (5)=(4)/(1), (6)=(3)/(5), ±S. D.

Mn concentration from 0.1 to 1.0 ppm.

(2) Effect of manganese on protein and carbohydrate contents

Crude protein and carbohydrate composition of *D. tertiolecta* are shown in Figs. Ⅳ-3 and Ⅳ-4, respectively. As expected from the growth data shown above, some relationship exists between the protein and carbohydrate content of cells and Mn concentrations in the medium. These cell components increased with the increase in Mn concentration in medium and attained a maximum value when 0.05−0.1 ppm Mn was added to medium. From 0.1 to 10 ppm Mn, protein and carbohydrate contents were constant but decreased above 10 ppm Mn.

(3) Effect of manganese on chlorophyll contents

The variations of chlorophyll-a content per cell in each culture at increasing concentration of Mn were little different from those of protein and carbohydrate (Fig. Ⅳ-5). Chlorophyll-a in cells did not decrease so much in Mn deficient medium (Fig. Ⅳ-5 & Table

Ⅳ-3), but decreased remarkably under Mn and Fe deficient condition (Table Ⅳ-3, see column (3)). This chlorosis was apparent in deficiency of Mn and Fe, but not obvious in Fe free culture.

Ⅳ-4. Discussion

As mentioned above, it has been well known that trace amount of Mn is essential for algal growth. In *Dunaliella tertiolecta*, HARVEY (1947) reported that the growth was activated by the addition of 0.5—2.0 mg Mn/m³(ppb). However, in the present batch culture experiment the organism in problem grew actively in the presence of 0.1 ppm Mn and this fact was supported by the results that the growth rate and cell components became to the highest value at the level of Mn in the medium. This value of Mn concentration is almost the same level as Mn component in several artificial sea water culture medium for algal growth.

There are many reports on the effect of culture condition on the chemical composition of algae. However, the effects of Mn on the metabolism of protein and carbohydrate was obscure. In the present study, Mn sensitivity and relationship with metabolism are re-proved.

According to CONSTANTOPOULOS (1970), the presence of Mn affects the growth of *Euglena gracilis* but do not affect chlorophyll contents. This phenomenon was observed also in *D. tertiolecta*. Algae without hydrogenase particularly show chlorosis if deprived of Mn, but the chlorophyll content of hydrogenase-containing algae is more stable (KESSLER, 1968). Iron is known as an activator of hydrogenase (WARING & WERKMAN, 1944). It is interesting to know that *D. tertiolecta* has become chlorosis in the present study when cultivated in the Fe and Mn deficient medium.

Ⅴ. Manganese uptake and release in *Dunaliella tertiolecta* BUTCHER

Ⅴ-1. Introduction

Algae, like other organisms, accumulate a number of trace metals and require these elements for growth. The accumulation of trace metals and their radioisotopes by algae in culture has been the object of numerous studies. In the case of essential trace metals, JENSEN *et al.* (1976) and SAKAGUCHI *et al.* (1977) have investigated the uptake of Cu by three diatoms and *Chlorella*, and BERNHARD & ZATTERA (1969) and PARRY & HAYWARD (1973) have studied the uptake of Zn by *Phaeodactylum tricornutum* and *D. tertiolecta*, respectively. Some emphasis has also been placed on the uptake of non-essential metals such as Cd (SARAIVA & FRAIZIER, 1975 ; COSSA, 1976 ; GERHARDS & WELLER, 1977 ; JENNINGS & RAINBOW 1979), Pb (SCHULZ-BALDES & LEWIN, 1976), Hg (GERHARDS & WELLER, 1977) and Ni (GERHARDS & WELLER, 1977 ; SKAAR *et al.*, 1974).

Previous studies on Mn uptake by algae have been conducted by TSUKIDATE (1974), who found that the amount of Mn absorbed by *Porphyra* in cultures varied with the illumination and temperature.

A number of workers have studied the effects of trace metals on algal growth, but the mechanisms of uptake are not fully understood. GUTKNECHT (1961) and FUJITA & HASHIZUME

(1975) concluded that Zn and Hg uptake by *Ulva lactuca* and *Synedra ulna* was a passive process since it was affected neither by metabolic inhibitors nor by anaerobic conditions. However, PATON & BUDD (1972) have concluded that Zn uptake by *Neocosmosphora vasinfecta* occurs both by a rapid passive process or iox-exchange and also by a slower, metabolically dependent process.

ROTHSTEIN *et al.* (1958) and JENNINGS *et al.* (1958) showed that Mn uptake by yeast cell was stimulated by phosphate and they concluded that the uptake was a metabolically active process. While, PARRY & HAYWARS (1973) demonstrated that the uptake of Zn by *D. tertiolecta* was temperature and pH-dependent but not directly linked with metabolism. The relationship between iron uptake and various environmental factors (e.g. light, pH and temperature) which influence cell metabolism has been studies also in *Porphyra* (TSUKIDATE, 1974). However, little is known as to how such factors affect the Mn uptake by *D. tertiolecta*. In this chapter the existence of an energy-coupled transport system of Mn in *D. tertiolecta* was proved.

Table V-1. Capture of *Dunaliella tertiolecta* by filtration (Whattman GF/C) and centrifugation (2,000 rpm, 2 min.). Stationary growing *D. tertiolecta* was captured by above both methods and the biomass was evaluated by chlorophyll-a concentration in one m*l* of culture. The cells captured by filtration is as by centrifugation. The destruction of cells by filtration is negligible.

	Chlorophyll-a ($\times 10^{-3}$mg Chl. a/m*l*-culture)
Centrifugation	6.80±0.37
Filtration	7.06±0.28

±95% C. I.

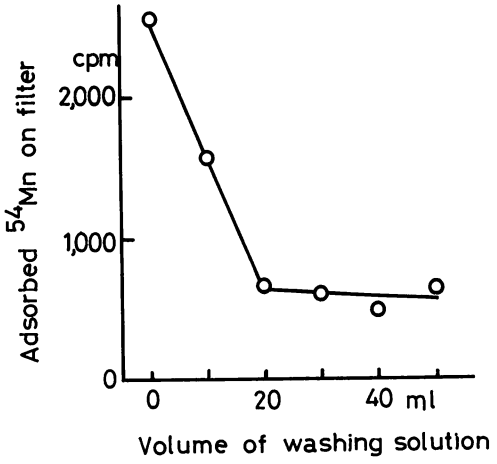

Fig. V-1. Residue of adsorded Mn^{54} on filter paper after repeated washing. Mn deprived culture medium was used for washing.

V-2. Materials and methods

Organism and culture

The experimental techniques employed for culturing *D. tertiolecta* were basically identical as described in chapters Ⅲ and Ⅳ(see p. 189, 196).

Manganese uptake studies

After the medium was inoculated with *D. tertiolecta* ($1-3\times10^4$ cells/m*l*) and the culture was grown to an appropriate number of cells ($200-400\times10^4$ cells/m*l*), 0.05 μ Ci of Mn^{54} was added to the 5.0 m*l* of the culture suspension, giving a total Mn concentration of 0.1 ppm. Before the experiment, *D. tertiolecta* was Mn-starved in Mn-free medium with aeration. After shaking the culture by means of mini-shaker (Shaker Mini SS-80, Tokyo Rikakikai Co. Ltd.) under the light, a sample of the culture was periodically filtered through Whattman GF/C glass fiber filter to separate the cells. Filtration was carried out using vacuum pump. Controls were also prepared with the same amount of radio-active Mn^{54}, but containing no cells. Similarly, the controls were filtered. The filtrate were terminated by rinsing with 30 m*l* each in cold Mn-free culture medium (Table V-1, Fig. V-1). The filter plus cells were dried under a lamp. The radioactivities of filtrate were measured by a well-type gamma ray NaI scintillation counter. Further procedures are described in detail in result of respective part.

V-3. Results

(1) Mn uptake by living and dead cells as a function of time

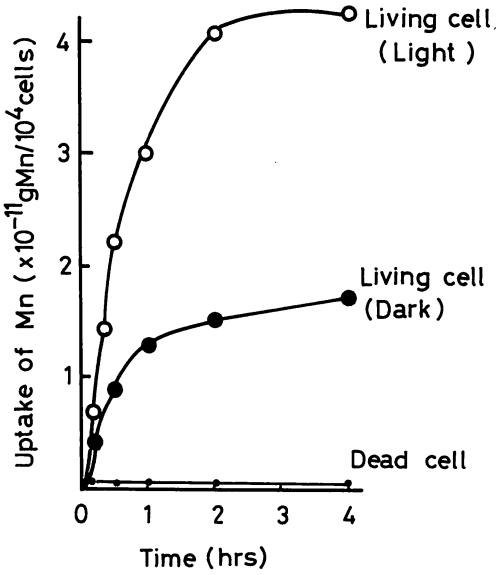

Fig. V-2. Uptake of Mn by living and dead cells of *D. tertiolecta*. Cells were incubated in 50 m*l* flasks using a shaker at 20℃, 5,000 lux for 4 hrs. Living cell showed a rapid rate of uptake during the first hour, followed by a slower uptake. The amount of Mn accumulated by *D. tertiolecta* was stimulated by light. Dead cells initially took up slight amount of Mn but this uptake ceased within 10 min.

One method of trying to understand how cells concentrate Mn is to compare the uptake by living and dead cells. The uptake of Mn^{54} by living cells and by cells killed with formaldehyde was compared (Fig. V-2). Cells, preincubated for 12 hrs in Mn-free medium in darkness, were incubated in 50 m*l* flasks using a shaker at about 20℃, 5,000 lux for 4 hrs. Living cells showed a rapid rate of uptake during the first hour, followed by a slower uptake. The amount of Mn accumulated by the *D. tertiolecta* reached a maximum value after incubation period of 2—4 hours. Fig. V-2 also shows that in this experiment Mn^{54} uptake was stimulated more in the light than in the dark. Dead cells initially took up slight amount of Mn^{54} but this uptake ceased within 10 min. Mn^{54} was also rapidly bound on the living cells within the initial 10 min after the addition of Mn, even in the absence of an energy source. This Mn adhesive phenomenon was also observed in dead cells. This kind of uptake of Mn^{54} seems to be due to the physical process of adsorption to the cell surface.

Cells of *D. tertiolecta*, which had been exposed to Mn^{54} for 3 hrs, were transferred to Mn-free medium and killed by heating. So that, Mn^{54} was lost from the dead cells, rapidly at first but then more slowly. Living cells transferred to Mn^{54}-free medium did not appear to lose Mn to the medium but little for the duration of the experiment (Fig. V-3).

(2) Mn uptake as a function of cell density

The amount of manganese taken up by *D. tertiolecta* was measured at the different population sizes. In this experiment, Mn-starved cells harvested in logarithmetic phase were incubated for 3 hrs after the cell density in the culture medium was regulated from 0.5 to 6.0 x 10^6 cells/m*l*. As shown in Fig. V-4, Mn uptake was activated under the condition

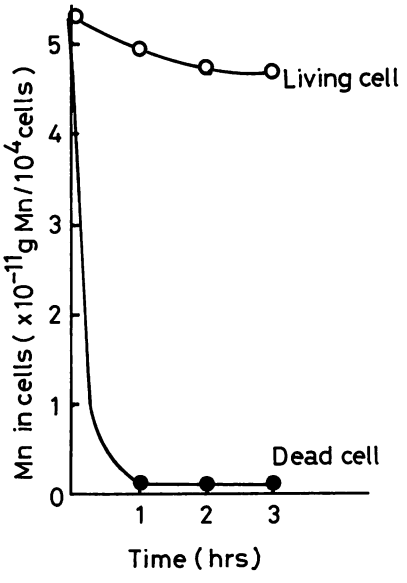

Fig. V-3. Retention of Mn by living cells and loss of Mn from dead cells of *D. tertiolecta*. The measurement was done after cells was labelled with Mn^{54} and suspended in Mn^{54} free medium.

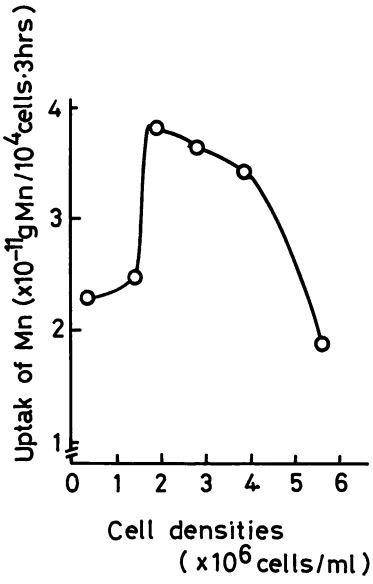

Fig. V-4. Uptake of Mn by *Dunaliella tertiolecta* under condition of various cell densities. Cells were incubated at 20℃, 5,000 lux for 3 hrs. Mn uptake was activated under the density of 2-4 x 10^6 cells/m*l*. The depression of Mn accumulation occurred under low and high cell desities.

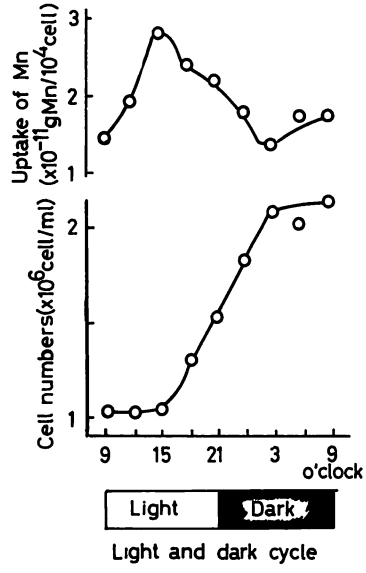

Fig. V-5. Changes in Mn content and numbers during the synchronized cycle of *Dunaliella tertiolecta*. Synchronization was tried under the conditions of 3,000 lux, 12:12 hrs LD and 20℃ with aeration. The cell division mostly occurred in the early part of the dark period. And Mn content was reduced by the divisions and increased with the cell growth.

of 2—4 x 10^6 cells/m*l*. The depression of Mn accumulation occurred under high cell density.

(3) Mn uptake in synchronous culture

Synchronization of *D. tertiolecta* was tried under the conditions of 3,000 lux, 12 : 12 hrs LD. and 20℃ with aeration (EPPLEY & COATSWORTH, 1966 ; WEGMANN & METZNER, 1971) and was successfully established. The degree of synchrony was rather high up to 3 x 10^6 cells/m*l*. For uptake experiment in the synchronized culture, Mn starved cells were preincubated with aeration. When the cell density reached 1 x 10^6 cells/m*l*, Mn^{54} and non-active Mn were added to the culture medium, giving to the concentration of 0.1 ppm Mn, and the number of cell and uptake of Mn^{54} was determined every 3 hrs.

Microscopic controls showed that the cell division mostly occurred in the early part of the dark period. Fig. V-5 gives the Mn content and cell number during the synchronized cycle. The data shows that Mn content was reduced by the cell divisions and increased with the cell growth.

(4) Mn uptake in batch culture

Mn starved cells were inoculated in 100 m*l* of JOHNSON'S medium (5 μCiMn^{54}, 0.1 ppm non-active Mn). The culture was aerated and maintained at about 20℃, 5,000 lux and 12 : 12 hrs LD. Every 3 : 00 p. m., 5 m*l* of aliquots were removed and Mn^{54} concentration in cells and number of cells were determined.

The time course of Mn uptake by cells of *Dunaliella tertiolecta* in batch culture for 10 days is

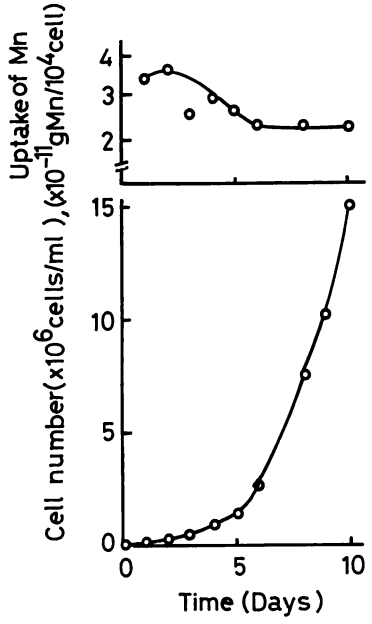

Fig. V-6. Changes in Mn content and cell numbers in the batch culture of *Dunaliella tertiolecta*, maintained at 20℃, 5,000 lux. During the first 2 days after the incubation, Mn was taken up rapidly.

shown in Fig. V-6. During the first 2 days after incubation, Mn was taken up rapidly. The actual quantity of Mn per cell continued to be diluted. Sixth day after the initiation of culture, the Mn concentration in the cells remained constant.

It is well known that physiological activities of phytoplankton, e. g. photosynthetic rate, growth rate etc., are high at early exponential phase (DALEY & BROWN, 1973). Similar phenomenon was seen in this result.

(5) Mn uptake as a function of Mn concentration in the medium

The uptake of Mn by *D. tertiolecta* was measured as a function of the external Mn concentrations (Fig. V-7). *D. tertiolecta* was incubated in JOHNSON'S medium containing different concentrations of Mn from 0.0 to 0.1 ppm for 4 hrs. The Mn was labelled with Mn^{54}.

The Mn levels in *D. tertiolecta* sharply increased as exgenous Mn increse from 0.0 to 1.0 ppm. The absorption system was apparently saturated at 0.1 ppm since further increases in external concentrations up to 1.0 ppm accompanied with no additional increases in the Mn content of *D. tertiolecta*. Such saturation kinetics follow the Michaelis-Menten equation for enzymic catalysis. This saturation of the absorption mechanism of an ion has been taken as evidence for active ion transport (EPSTEIN, 1973). The graphical methods of Lineweaver and Burk was used for the estimation of the kinetic constants Ks of 0.07 ppm and Vmax of 0.6×10^{-11} g Mn/10^4 cells·4 hr for the absorption process.

Fig. V-8 is the effect of Mn concentration on the concentration factor (as wet weight).

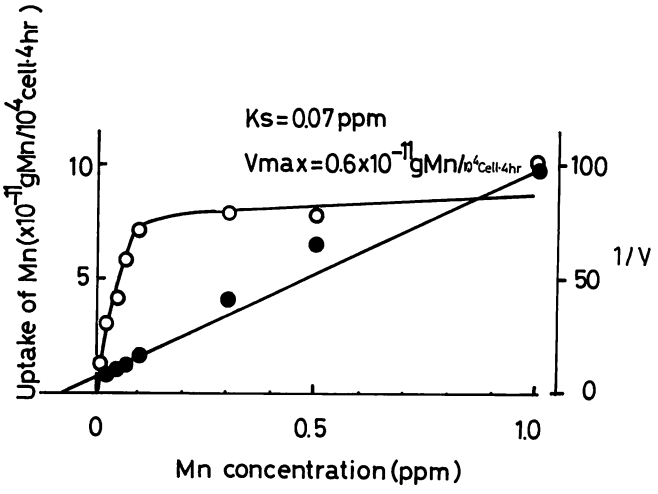

Fig. V-7. Uptake of Mn by *Dunaliella tertiolecta* incubated for 4 hrs in JOHNSON'S medium containing various amounts of available Mn (—○—). The line is a plot of the Michaelis-Menten equation;

$$v = \frac{VS}{Ks+S}$$

(v: uptake rate of Mn, S: concentration of Mn in medium, V: rate of absorption, Vmax: maximum rate of absorption, Ks: Michaelis constant). The graphical methods of Lineweaver and Burk (—●—) was used for the estimation of kinetic constants, Ks and Vmax.

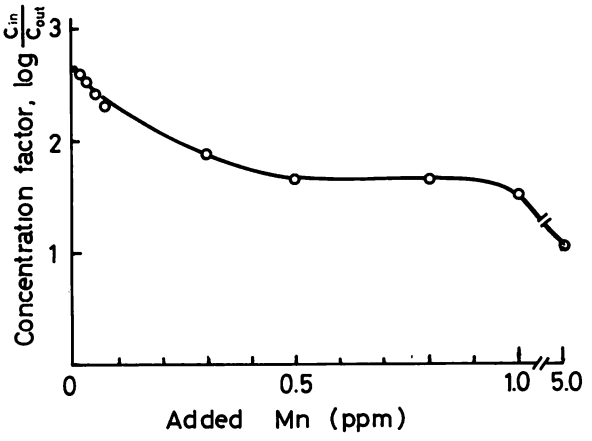

Fig. V-8. Effect of added Mn on the Mn concentration factor of *Dunaliella tertiolecta*. Concentration factor is logarithmically expressed. The date used is the same as those in Fig. V-7. Manganese concentration factor (as wet weight) is the order of 10-fold in 0.3-0.5ppm Mn, but 100-fold in <0.1 ppm Mn.

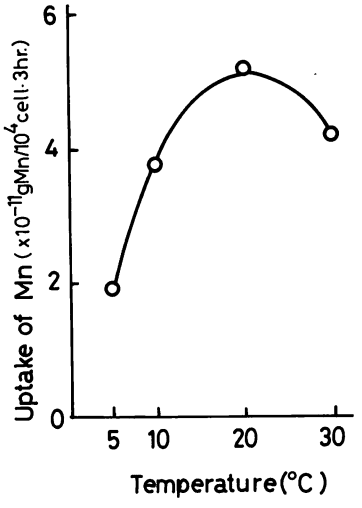

Fig. V-9. Effect of temperature on Mn uptake by *Dunaliella tertiolecta*. Manganese uptake was observed in JOHNSON'S medium at different temperature from 5℃ to 30℃ under a light intensity of 3,000 lux for 3 hrs.

The data used in Fig. V-8 is the same as those in Fig. V-7. Manganese concentration factor is the order of 10- fold in 0.3-5.0 ppm added Mn, but 10^2-fold in 0.1 ppm Mn.

(6) Effect of environmental factors on Mn uptake

Temperature : The manganese uptake by *D. tertiolecta* was observed at differenat temperatures from 5℃ to 30℃ under a light intensity of 3,000 lux for 3 hrs. As shown in Fig. V-9, Mn uptake was strongly temperature-dependent. Maximum Mn was taken up at about 20℃.

These data indicate the mediation of metabolism in the absorption process.

Light intensity: The accumulation of Mn by *D. tertiolecta* was observed at different light intensities from 0 to 60,000 lux at 20℃ for 4 hrs. The intensity of illuminations were obtained to regulate the natural light using tracing papers. The action of light was to stimulate the influx of Mn and this sensitivity is clearly shown in Fig. V-10. The cells used were carbonstarved by being kept in the dark for 12 hrs before the experiment. It can be seen that this process saturated at about 8,000 lux and inhibited at high light intensity. REYTHER (1956) showed photosynthesis-light curve of *Dunaliella*. And the present result concerning Mn uptake-light curve was also of the same pattern.

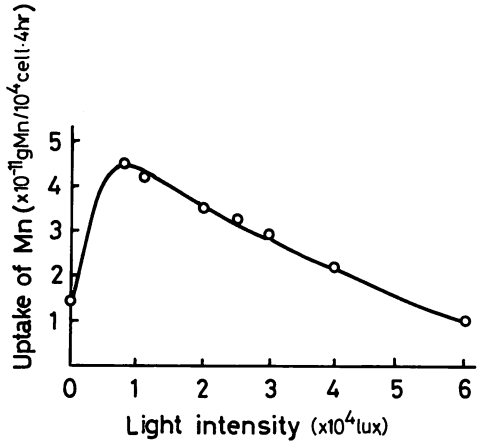

Fig. V-10. Effect of light intensities on Mn uptake by *Dunaliella tertiolecta*. Mn uptake was observed in JOHNSON'S medium at different light intensities from 0 to 6,000 lux at 20℃, 3,000 lux for 4 hrs.

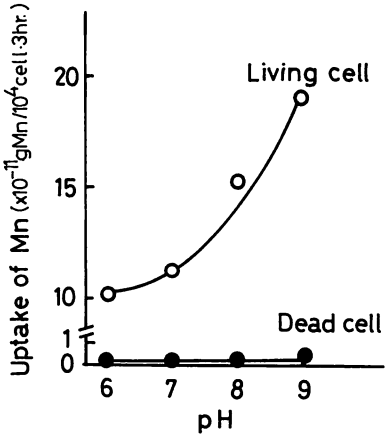

Fig. V-11. Effect of pH on Mn uptake by living and dead cells of *Dunaliella tertiolecta*. Mn uptake was observed in JOHNSON'S medium at different pH from pH 6.0 to pH 9.0 at 20℃, 3,000 lux for 3 hrs.

pH : Mn absorption was investigated in a series of pH value from 6.0 to 9.0 using living and dead *D. tertiolecta*. In this series the media were all buffered with TRIS at a concentration of 2.45 g/*l*, then adjusted to the desired pH with HCl. Fig. V-11 shows the uptake of Mn by living and dead *D. tertiolecta* at different pH's. A pronounced pH dependency of Mn absorption by living cell is indicated. Absorption of Mn by living cell was 10^2-fold as high as formaldehyde treated cell. The amount of Mn found in living cell increased with the increase of pH. At pH values greater than 8.0, Mn uptake was markedly increased. While *D. tertiolecta* could not grow actively at pH 9.0, the organism took up more Mn at the same pH.

(7) Effect of glucose on Mn uptake

A respiratory substrate, glucose, was added to observe the evidence of coupling of oxidative phosphorylation in relation to manganese uptake. As shown in Fig. V-12, accumulation of Mn was stimulated 2 times by glucose. With increasing glucose concentration the amount of Mn accumulated by *D. tertiolecta* rose and reached a limiting value at concentration of 0.5 g glucose/*l*. Therefore, the energy for Mn transport, can be provided from glycolysis.

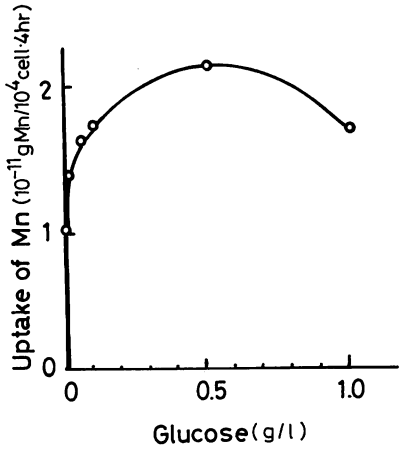

Fig. V-12. Effect of glucose on uptake by *Dunaliella tertiolecta*. *D. tertiolecta* was incubated in JOHNSON'S medium with various concentrations of glucose at 20℃, 3,000 lux. After 4 hrs, *D. tertiolecta* was harvested by filter paper to analyze Mn^{54} in the cells.

(8) Effect of nutrients on Mn uptake

Figs. V-13 to V-16 illustrate the effect of components in the culture medium on Mn^{54} uptake by *D. tertiolecta*. *D. tertiolecta* was harvested by centrifugation, then washed twice with a sodium chloride solution at the same concentration as that of the growth medium.

Na, K : *D. tertiolecta* was incubated in JOHNSON'S medium containing different concentration of NaCl from 10 to 160 mM (=0.58−9.3 %) for 3 hrs to study the effect of NaCl on uptake of Mn^{54}. Manganese accumulation is reduced in 25 mM NaCl and below and reached the maximum value at 50 mM NaCl, although there is slight depression for Mn uptake up to 160

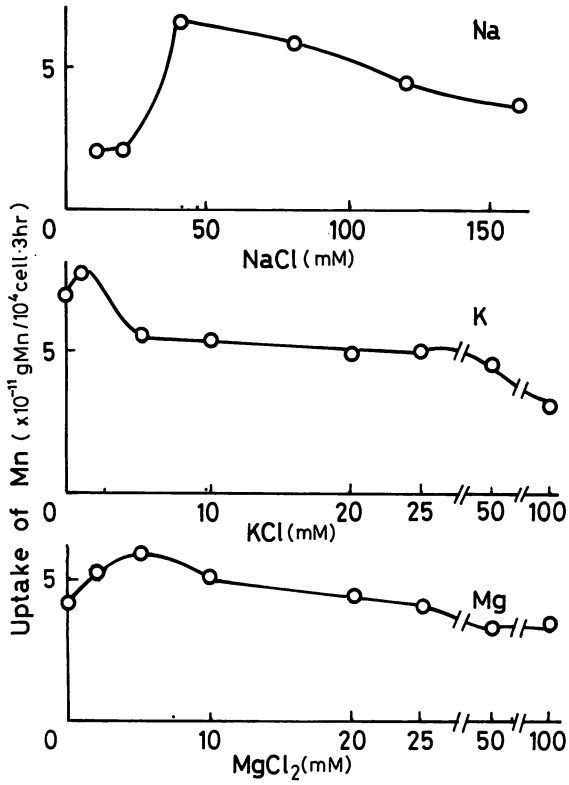

Fig. V-13. Effect of Na, K, and Mg on Mn uptake by *Dunaliella tertiolecta*. Exponential phase of *D. tertiolecta* was harvested and washed with NaCl solution or Tris-HCl buffer. After that cell was incubated in each nutrient solution with Mn^{54} for 3 hrs to analyze Mn which is taken up by cell.

mM NaCl.

JOHNSON *et al.* (1968) studied the effect of NaCl on the growth and enzymatic activity of halophilic *Dunaliella viridis*. The results obtained in the present study were similar to those in *D. viridis* of JOHNSON *et al.*

KCl was added to the culture medium at different concentration from 0 to 100 mM to study the effect on Mn uptake. Mn uptake was stimulated between 0 and 2 mM KCl, although at the concentrations above 5 mM KCl the Mn uptake was reduced (Fig. V-13).

N, P, C: Nitrogen, phosphorus and carbon are important constituents of a variety of organic compounds which are essential to metabolism and the following construction of structure for plants. The effects of N, P and C on Mn absorption were studied using labelled Mn at 0.1 ppm, as same as other experiments. Mn uptake was slightly stimulated in 6-8 x 10^{-4} M K_2HPO_4 and 1-5 x 10^{-4} M $NaHCO_3$ while there was no stimulation of Mn uptake by the addition of $NaNO_3$ (Figs. V-14 & V-16).

These elements were less effective than micronutrients as discussed below. *D. tertiolecta* may consume these major nutrients luxuriously for growth. Therefore, it is difficult to

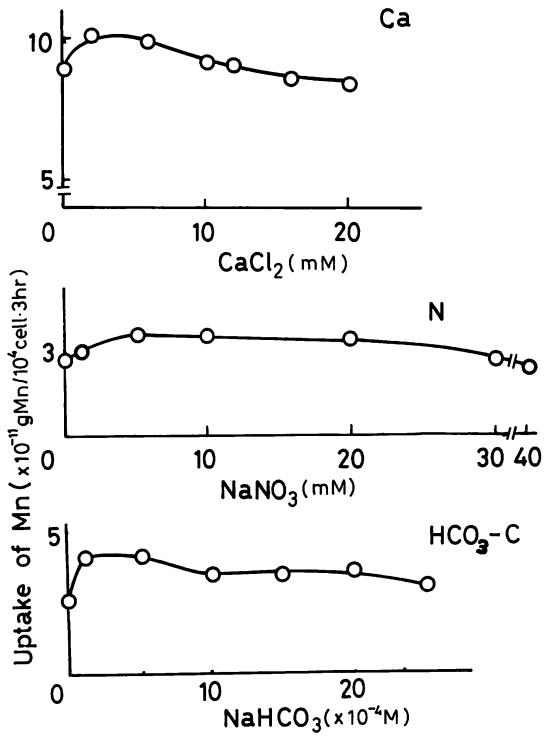

Fig. V-14. Effect of Ca, NO_2-N and HCO_3-C on Mn uptake by *Dunaliella tertiolecta*. Detail method was described previously in Fig. V-13.

confirm distinctive effects on Mn uptake in short expeiment period.

Mg, Ca : Magnesium and calcium ions have a function as activators for a number of enzymes in nature. Calcium salts of phosphatidic acid occur in cell membranes and are essential to the maintenance of their structure. And Mn transport process was expected to be affected by Mg and Ca in the medium while low concentration of these divalent cations slightly accelerated the Mn uptake by *D. tertiolecta* (Figs. V-13 & V-14). The uptakes of Ca and Zn by excised rice roots are known to be inhibited by Mn (RAMANI & KANNAN, 1976).

Co, Cu, Mo : Cobalt is a component of vitamin B_{12}. Copper is also the component of several metalloenzyme including ascorbic acid oxidase, phenolase and cytochrome oxidase. Molybdenium is essential for algal growth.

Mn uptake was a little increased in low concentration of these metals while Mn absorbtion was extremly inhibited in the order of 1 x 10^{-7} M of $CoCl_2$, 1 x 10^{-6} M of $CuCl_2$ and $(NH_4)_6Mo_7O_{24}$. SUNDA *et al.* (1981) showed that Cu competes with Mn for metabolically active site in *Chaetoceros socialis* (Fig. V-15).

Fe : Iron occurs in the prosthetic group of certain proteins, notably the cytochromes which function in electron transport, and in the enzymes, peroxidase and dehydrogenases. It has a specific role in chlorophyll synthesis.

Unlike other nutrients, Fe (as Fe-EDTA) helped the uptake of Mn at the concentration

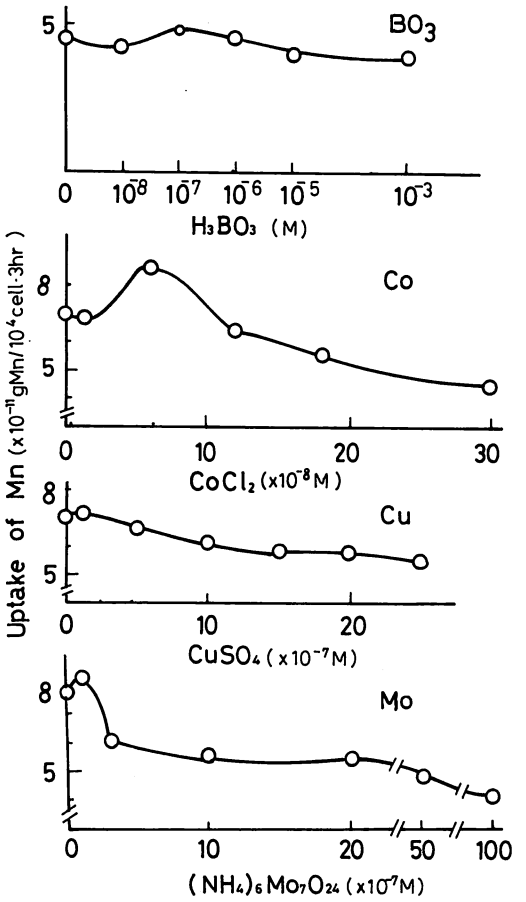

Fig. V-15. Effect of BO_3, Co, and Mo on Mn uptake by *Dunaliella tertiolecta*. Detail method was described previously in Fig. V-13.

of 1-20 x 10^{-5} M Fe, especially in 5 x 10^{-5} M Fe. As described before (p. 199) it is known that Mn physiologically cooperates with Fe. The data obtained here confirm the above Fe—Mn relationship (Fig. V-16).

Zn: Zinc involved in several enzymes as either a constituent or an activator (EPSTEIN, 1973). In this experiment, the absorption of Mn is reduced at all Zn concentration used. Zinc is different from other heavy metals in this respect (Fig. V-16).

B: The role of boron in the translocation of sugars has been much discussed (EPSTEIN, 1973). Boron (as H_3BO_3) is the only trivalent ion used in this experiment. As shown in Fig. V-15, there is no effect on absorption of Mn by BO_3^{3-} addition (Fig. V-15).

(9) Effect of metabolic inhibitors on Mn uptake and release

The experiments already described in articles from (1) to (8) are based on physical and chemical conditions and the data obtained show that the mechanism of the Mn uptake by *D. tertiolecta* is dependent on metabolism. To confirm further above results, the effects of several metabolic inhibitors at the concentrations over 0.1 ppm upon the absorption of Mn

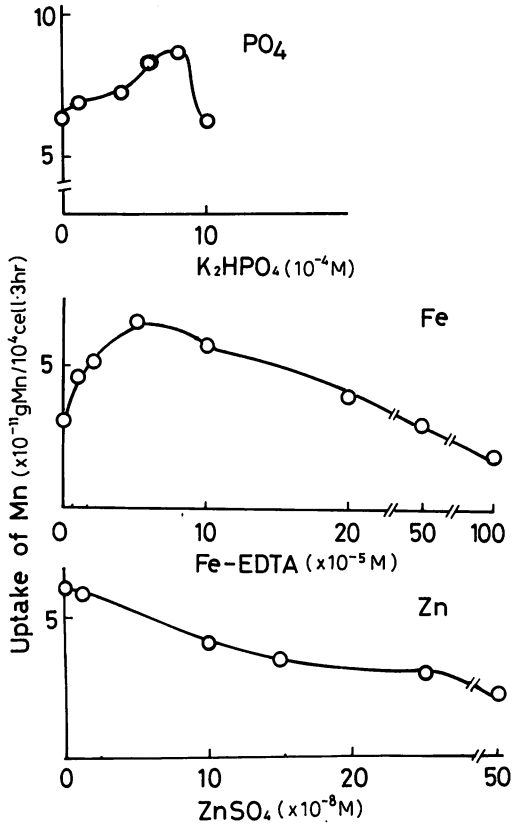

Fig. V-16. Effect of PO_4-P, Fe and Zn on Mn uptake by *Dunaliella tertiolecta*. Detail method was described previously in Fig. V-13.

were studied.

D. tertiolecta was cultured in JOHNSON'S medium and harvested in exponential phase as described before (see p. 195). On the day of the exponential growth, triplicate samples of *D. tertiolecta* were incubated in the medium containing KCN (0-50 mM), TPAC (0-10 mM) and DCMU ($0\text{-}10^{-5}$ M) at 20℃, 5,000 lux for 3 hrs (Table V-2).

Fig. V-17 shows the effect of the respiration inhibitor, KCN, on the uptake of Mn by *D. tertiolecta*. It is clear that the uptake of Mn is strongly inhibited by 40 mM KCN. This involves respiratory chain providing energy for Mn transport in the cells.

As described later, Mn absorption process may need the electronpotential of membrane (p. 217). Theoretically, cell membrane must keep -50 to -70 mV of electronpotential to take up divarent anion (CLARKSON, 1974, see Fig. 3. 1). ASANO (1974) used tetraphenylarsonium chloride (TPAC) as a metabolic inhibitor of electronpotential process in cell membrane. To prove the role of electronpotential in Mn uptake mechanisms, the TPAC was used.

As shown in Fig. V-18(A), Mn uptake was inhibited with 1 mM TPAC. On the other

Table V-2. The inhibitors used, their concentration, and metabolic effect.

Inhibitor	Effective concs. (M)	Metabolic effect
Dichlorophenyl-1,1-dimethylurea (DCMU)	$1x10^{-5}$	Inhibits the photosynthetic evolution without effect on absorption of K
Tetraphenylarsonium chloride (TPAC)	$1x10^{-2}$	Inhibits the electropotential of membrane
KCN	$5x10^{-2}$	Forms complexes with metaloenzymes and attacks the cytochrome oxidases of phosphorylation

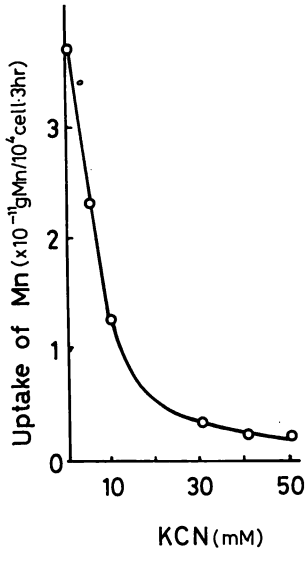

Fig. V-17. Effect of the respiration inhibitor, KCN, on Mn uptake by *Dunaliella tertiolecta*. *D. tertiolecta* was inoculated at 20℃, 3,000 lux in the modified JOHNSON'S medium which contain Mn^{54} and various amount of KCN. After 3 hrs, cell was filtrated to analyzed Mn. The uptake of Mn was inhibited at the concentration of 10-50 mM KCN.

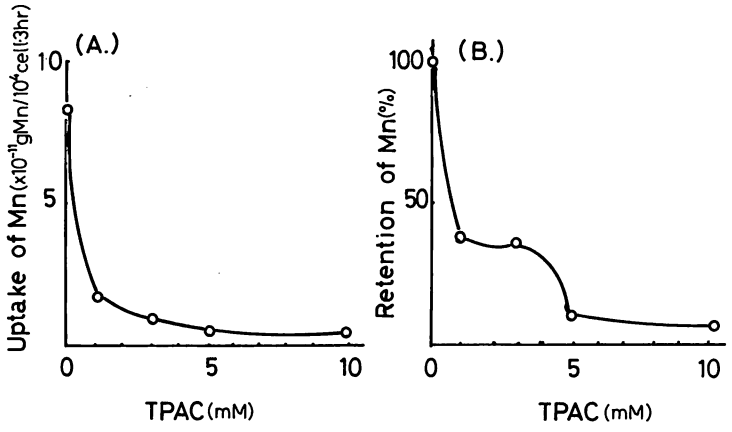

Fig. V-18. Effect of TPAC on the uptake and retention of Mn by *Dunaliella tertiolecta*. (A) : *D. tertiolecta* was incubated in JOHNSON'S medium with TPAC and Mn^{54}. Mn uptake by cell was inhibited with 1 mM TPAC. (B) : *D. tertiolecta*, which was incubated with Mn^{54}, was transfered to TPAC-added JOHNSON'S medium. The cell discharged Mn to the culture medium.

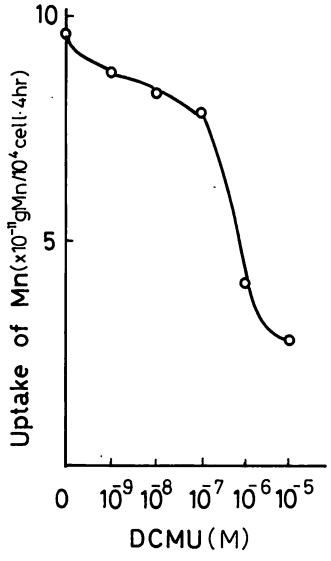

Fig. V-19. Effect of the photosynthesis inhibitor, DCMU, on Mn uptake by *Dunaliella tertiolecta* which is incubated in DCMU-added medium, at 20℃, 3,000 lux, for 4 hrs.

hand, Mn ion in cell was discharged to outside by incubating cells with TPAC (Fig. V-18(B)).

Fig. V-18 shows the effect of photosynthesis inhibitor, 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU) on the light-dependent uptake of Mn. As ORR *et al.* (1976)

suggested DCMU was an inhibitor of noncyclic photophosphorylation. Mn uptake is slightly reduced from 10^{-9} to 10^{-7} M DCMU and strongly in 10^{-5} M DCMU. This result means that the metabolic relationship between photosynthesis and Mn absorption exists. Similar inhibition was reported in Zn accumulation by *Dunaliella* (PARRY & HAYWARD, 1973).

(10) Effect of EDTA on Mn uptake

Ethylenediaminetetraacetic acid (EDTA) is most widely used as chelator in marine medium, and is not readily metabolized by microbes (JOHNSTON, 1964).

To study the effect of chelator on Mn uptake, *Dunaliella tertiolecta* was suspended in the modified JOHNSON'S medium containing different concentrations of EDTA from 0 to 20 x 10^{-6} M. After 0.1 ppm Mn was added, the cultures were incubated at 20℃, 3,000 lux for 3 hrs. Then the amount of the Mn taken up by cell was measured by NaI scintilation counter.

Manganese uptake was markedly stimulated by the addition of EDTA (Fig. V-20). It was evident that *D. tertiolecta* was capable of accumulating Mn from pure and chelated forms. KANNAN & JOSEPH (1975) has also reported the occurrence absorption of Mn with EDTA by germinating sorghum. Theoretically, a chelate should combine with a metal in a 1:1 molar ratio. The data shown in Fig. V-20 exhibited the same conclusion. However, it is not known whether the molecule of EDTA is transported to inside the cell with manganese ion.

If Mn uptake were stimulated by the addition of chelate substances, the organic substances which were released from algae would promote the Mn uptake process. According to HUNTSMAN & BARBER (1975) and MURPHY *et al.* (1976), algae released ion-selective chelators to the culture solution. So, used culture medium were added to Mn absorption experimental culture. As expected, Mn accumulation was stimulated by this

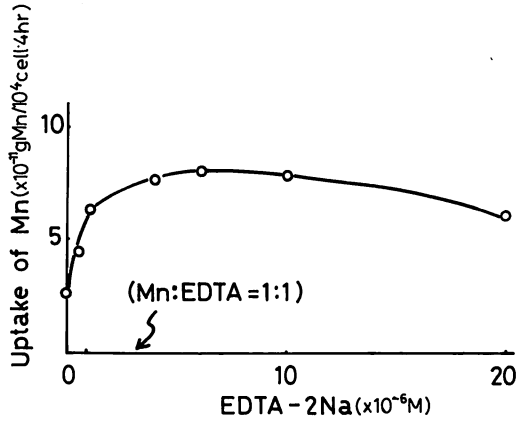

Fig. V-20. Effect of EDTA on Mn uptake by *Dunaliella tertiolecta*. After *D. tertiolecta* was suspended in modified JOHNSON'S medium containing different concentration of EDTA. The culture were incubated at 20℃, 3,000 lux for 4 hrs. Then the amount of Mn taken up by cell was measured by NaI scintilation counter.

Table V-3. Effect of used medium and EDTA on Mn uptake by *Dunaliella tertiolecta*. Culture medium which was separated from stationary phase of culture by filtration was added to JOHNSON'S medium as used medium. After *D. tertiolecta* was cultured at 20℃, 3,000 lux for 3 hrs, Mn^{54} absorbed cell was analyzed.

Medium added	Mn uptake ($\times 10^{-11}$ gMn/10^4cells. 4hrs)
J'M	4.5±1.3
J'M+0.1m*l* of used medium	6.3±0.4
J'M+0.5m*l* of used medium	7.0±1.3
J'M+10^{-5} M EDTA	11.2±2.6

J'M : JOHNSON'S medium, ±95% C. I.

used medium and it means metabolic organic substances are able to act as metal chelators (Table V-3.).

V-4. Discussion

Metabolic aspects of manganese uptake

Data obtained support the hypothesis that Mn transport in *D. tertiolecta* is energy-dependent. Mn transport is dependent on endogenous sources of energy, glucose, but is inhibited by DCMU, KCN and TPAC. These energy-dependent transport of Mn is known in yeast cell (OKOROKOV *et al.*, 1977). As with the present study on *D. tertiolecta* the previous investigations generally demonstrated the two distinct processes in accumulation of the cation. First, there is a rapid but limited, energy- and temperature-independent system binding to the cell surface. The amount of such binding system was low in comparison with values reported in other similar studies (FAILLA *et al.*, 1976). These authors have shown that cations compete for non-specific anionic sites on the cell surface.

The second process is the slower, but sustained, energy- and temperature-dependent translocation across the cell membrane. Energy-dependent Mn uptake is a highly specific process that exhibits saturation kinetics. In *D. tertiolecta*, Mn was mostly accumulated by this second process. According to GUTKNECHT (1961), physical process of adsorption of cation exchange in *Ulva lactuca* is primarily responsible for Zn uptake. Results of the present experiments indicate that Mn uptake is affected by several metabolic effects.

If it is assumed that 80 % of each cell content consists of water and that all of the intracellular Mn exists as the free cation, the uptake of Mn from the solution under 0.1 ppm Mn represents the order of 1×10^2 of concentration factor. Further, when the electrical potential of biological membrane is -40mV (BARBER, 1968), approximately 10 to 100 of the concentration factor is necessary for active transport (Nernst potential equation ; CLARKSON, 1974, p. 59).

As a conclusion, Mn is transported actively in the solution at the concentration less than 1 ppm (Fig. V-8). While the cation may be exhausted actively in the solution at the concentration larger than 5 ppm. This result means algae/phytoplankton can accumulate Mn actively in natural sea water.

The fact which Mn uptake is inhibited by KCN, DCMU and TPAC also proves the active transport in *D. tertiolecta*.

Kinetic approach

In conclusion, it has been shown that *D. tertiolecta* has a high ability to concentrate Mn from a medium. The uptake of Mn appears to be proportional to the amount of the metal in the solution to a certain degree but in the concentration more than about 0.1 ppm no further accumulation occurs.

D. tertiolecta incubated in the medium containing 0.1 ppm Mn, absorbed 7×10^{-11} gMn/10^4 cell (Fig. V-7). If the cell was regarded as an oval (10 μm x 6 μm), the volume of 10^4 cell would be:

$$4/3 \times (5 \times 10^{-4}) \times (3 \times 10^{-4})^2 \times 10^4 = 1.9 \times 10^{-6} \quad (\text{m}l/10^4 \text{ cells})$$

And the amount of Mn absorbed by this alga was calculated as follow:

$$\frac{7 \times 10^{-11}}{1.9 \times 10^{-6}} = 37 \text{ ppm Mn (wet weight)}.$$

RILEY (1971) reported that *D. tertiolecta* accumulated 3.8 ppm Mn as dry weight. Considering that the dry weight in this organism was about 10 % of the wet weight, these values were considerably different.

In biological systems ions can be considered to be acted on by two physical forces: the chemical potential gradient and the electropotential gradient. Together, they constitute the electrochemical potential gradient:

$$\bar{u} = d(RT \ln a)/dx + zF \, d\psi/dx.$$

In this equation, $\bar{u}$ is the elctron chemical potential gradient, R is the gas constant, T is the absolute temperature, a is the chemical activity, z is the algebraic valency, F is the Faraday constant. and ψ is the electrical potential.

If an ion moves "uphill", from a lower to a higher electrochmical potential during its transport through a membrane, the process requires an input of energy and this transport process is said to be active. When the tendency for an ion to move down its chemical potential gradient in the opposite direction, the equilibrium state is described as Nernst equation. The expression has the form:

$$En = \frac{RT}{ZF} \ln \left(\frac{C_{out}}{C_{in}} \right)$$

where En represents the Nernst potential and C_{in} and C_{out}, the concentrations in inside and outside the cell. The Nernst potential En=-74 mV was calculated from the Nernst equation using C_{in}=3.7 ppm, C_{out}=0.1 ppm, z=-2 and T=293 K. Although the membrane potential of *D. tertiolecta* was lacking, BARBER (1968) measured the membrane potential of -40 mV in an unicellular green alga, *Chlorella pyrenoidosa*. The membrane potential of *D. tertiolecta* must be supposed to be not far from -40 mV. The difference between -40 mV and -74 mV means the system was not in equilibrium and energy must be expended to maintain the non-equilibrium state. (i.e, it would require a metabolically driven active transport).

It has been observed that the kinetics of Mn transport across membranes are similar to those of enzymic catalysis (Michaelis-Menten kinetics), and this showed that reversible binding carrier mediates the transport process. In general, there are two distinct

mechanisms of absorption for a given ion ; mechanism 1, operating even at low concentrations, that is, with high affinity for the ions ; and mechanism 2, which becomes evident only at concentrations higher than those giving essentially the maximal rate of absorption via mechanism 1 (EPSTEIN, 1973). But the typical saturation curve was obtained. It is possible that this is not a physiologically high concentration for this micronutrient element. BOWEN (1969) reported the same phenomena in sugarcane leaf tissue.

Factors affecting Mn uptake

In general, biological accumulation process was affected by physiological and external conditions in circumstances. As described above, Mn accumulation was influenced by physiological characteristics. For example, in exponential phase or during a period of vigorous growth cells actively took up Mn.

Cell density is also an important factor ; for high cell density makes competitive effects on Mn uptake and low cell density also makes Mn uptake. The increase of pH value in the medium accompanys with the active uptake of Mn and this tendency becomes conspicuous at pH 9, which is not appropriate for algal growth. Mn accumulation pump may be active at high pH range independent of cell growth, but the mechanism is obscure. The results obtained are almost similar to that of Zn uptake by *D. tertiolecta* (PARRY & HAYWARD, 1973).

Ⅵ. Distribution of Mn accumulated by cells of of *Dunaliella tertiolecta* BUTCHER

Ⅵ-1. Introduction

Manganese acts as an activator in glycolysis and TCA cycle. Manganese is also a prominent component of chloroplasts and participates in the reaction leading to the evolution of oxygen. Therefore, Mn is expected to be present in mitochondria and chloroplasts in which these metabolisms presented.

However, there is no report on the localization of Mn in the cell of plants. In this chapter, I present the distribution and state of Mn in *D. tertiolecta.*

Ⅵ-2. Materials and methods

D. tertiolecta was incubated for 5 days at 20℃ in modified JOHNSON'S medium of NORO (1978) contained 0.1 ppm of Mn with Mn^{54}. The cells were harvested by centrifugation and resuspended in 5 m*l* of the culture medium without Mn. After thorough stirring, the cells were centrifuged and the supernatant was decanted. This washing process was repeated five times. The radioactivity of cells after washing was measured and compared with that before washing to calculate the rate of manganese elution.

Ⅵ-3. Result and discussion

When *D. tertiolecta* accumulate Mn from the culture medium, its cell surface are regarded to plays an important role. When the Mn was loosely adsorbed on the cell surface, it would be easy to eliminate by washing with medium. Mn which was strongly bound to the cell membrane was difficult to remove by the washing as described above, but it was eluted with

Table Ⅵ- 1. Elution of Mn from *Dunaliella tertiolecta* to the various solutions after repeated washings. After culturing *D. tertiolecta* in JOHNSON'S medium with Mn^{54} at 20℃, 3,000lux for 5 days, the cells were harvested by centrifugation and were washed with various kinds of washing solution to elucidate the residual Mn in the cells.

Washing solution	Activities in cells after washing (cpm)	Residual activities after washing (%)
Control (Befor washing)	6,290	100.0
JOHNSON'S medium (pH 8.0)	1,881	27.2
JOHNSON'S medium (pH 4.0)	1,434	20.7
Distilled water	618	8.9
0.1 N HCl	617	8.9
10 mM EDTA	143	2.1

Table Ⅵ- 2. Mn distribution in *Dunaliella tertiolecta* after accumulation from the medium. Mn content in tonoplast was estimated from the Mn which was eluted from cell by washing with JOHNSON'S medium (pH 8.0). Bounded Mn in protoplasm was estimated from the Mn which was not eluted from cell by washing 10 mM EDTA solution. Free ion or low molecule compound Mn was calculated from the difference:

Free ionic Mn = 100(%) - Mn in tonoplast - bounded Mn in protoplasm

Compartment		Mn content (%)
Tonoplast		72.8
Protoplasm	free ion or low molecule compound	25.1
	bounded	2.1

EDTA solution. Mn which had penetrated beyond the cell membrane would be difficult to elute even with EDTA solution.

The manganese taken up by cells was eluted by washing with JOHNSON'S medium (pH 8.0) and about 72.8 % of the Mn was removed. The results showed that about 72.8 % of the Mn taken up by cells might be adsorbed loosely to tonoplast. By washing with 10 mM EDTA solution which has a strong tendency to combine with Mn, about 97.9 % of the Mn taken up was eluted out. The difference between these two fractions (25.1 %=97.9−72.8 %) seemed relatively firmly adsorbed on the cell membrane. The residual Mn, 2.1 %, which could not be eluted even by EDTA solution, might be accumulated incide the cell and it was not possible to eliminate. (TableⅥ-1.).

From these results it can be concluded that 72.8 % of accumulated Mn was bounded to surface membrane, 25.1 % as free ion or low molecule compound in protoplasm and 2.1 % firmly to the organella of protoplasm (TableⅥ-2.).

In yeast cells, about 75 % of the Mn accumulated is found in protoplasts and nearly 25 %

in the cell wall (OKOROKOV *et al.*, 1977). But the cell of *D. tertiolecta* is different from yeast, because *D. tertiolecta* lacks cell wall.

According to von KAMEKE & WEGMANN (1978), the following two distinct manganese-containing subchloroplast particles could be isolated from *Dunaliella*: (1) one is green fraction and has mol. weight 480,000, containing chlorophylls -a and -b and relatively weakly bound manganese in the molecular ratio Mn : chl. -a : chl. -b : protein =2 : 6 : 4 : 1 and (2) another is a yellow fraction and has mol. wt. 600,000, containing neoxanthin and manganese in the molecular ratio Mn : neoxanthin : protein =1 : 10 : 1. The yellow fraction contains tightly bound Mn and showed superoxide dismutase activity.

Ⅶ. Manganese sensitivity of ATPase in *Dunaliella tertiolecta* BUTCHER

Ⅶ-1. Introduction

In 1957, SKOU isolated an enzyme system from crab nerves. It was ATPase which hydrolyzes ATP to ADP and inorganic phosphate. This ATPase required Mg, Na and K to being fully active, and its specification was assumed to be a system mediating the reciprocal movements of K and Na across the nerve cell membrane. Since then, such "Na-K transport ATPases" have been found in many tissues of animals and bacteria. Studies on this subject have been followed by SKOU(1965), RACKER(1976), ROTHSTEIN *et al.* (1976)WILBRANDT(1975) and WILSON *et al.* (1976) and show that the Na pump that moves Na from inside to outside constitute membrane proteins being powered directly by hydrolysis of ATP. It comes in several versions and the most thoroughly studied of which is a Na-K exchange pump that moves three Na out coupled in an obligate way to the uptake of two K and the hydrolysis of one ATP. The responsible protein when isolated referred to as a Na, K-stimulated ATPase, for it can hydrolyze ATP only in the presence on Na and K.

Na, K-ATPase in plant has been also studied with peanut seedlings (BROWN & ALTSCHUL, 1964), oat roots(FISHER & HODGE, 1969), barley(HALL, 1971), fungus (ZONNENVELD, 1976) and diatom (SULLIVAN & VOLCANI, 1974 ; 1975). These evidences are well summarized by HODGES(1976).

The histochemical demonstration of ATPase was first described by POAUX (1967). HALL (1971) and HALL & DAVIE (1975) have also developed a method for the ultrastructural localization of ATPase in maize (*Zea mays*) root tips and leaves of *Suaeda maritima.*

It is well established that ATPases are involved in ion transport in animal tissues. However the role of ATPases in ion transport in plants remains controversial, good correlative evidence for the involvement of membrane bound Na, K-stimulated ATPases is now emerging (LAI & THOMPSON, 1972).

The reasons to distinguish plant cells from animal tissues in relation to ion transport are :

i) Ouabine can not inhibit ion uptake and ATPase activity simultanously in plant cells, but this inhibitor is effective in animal cells.

ii) The membrane of animal cell shows functional differentiation (e. g. erythrocyte), while there are many types of ATPase in plant cell.

The concept of energy-dependent ion transport in algae evolved from the work of

HOAGLAND *et al.* (1926), in which a light-enhanced uptake of ion in *Nitella* was observed. Since that time many studies using algae have been directed to the physiological processes and molecular mechanisms in relation to ion transport. Although the precise nature of the metabolism coupling with ion transport is unkown, generally anion transport is thought to be connected directly with electron transfer reactions or to be dependent on some reduced product; or ATP is involved in the system, and the major energy source is a membrane-bound ATPase.

A few ATPase activities in a algal membrane have been described and recently only several evidences for their possible role in ion transport in algae have been shown (FALKOWSKI & STONE, 1975; BUTZ & JOCKSON, 1977).

As described above, the role of ATPase in the mechanisms of Na, K pump has been well established. However, the role of ATPase in the uptake of other ions has been poorly reported.

The purpose in this chapter is to clarify the characteristics and localization of Mn-stimulated adenosine triphosphatases that may be a part of, or functionally associated with, Mn transport system in *D. tertiolecta*.

There is strong histochemical evidence of plasma membrane-associated ATPase in vascular tissues of plants, but plasma membrane ATPase of cell is needed to determine biochemically if the enzyme has properties in common with those of the cation ATPase believed to function in cells as an ion pump.

Ⅶ-2. Materials and methods

A. Cell growth and harvesting

Axenic clonal cultures of *D. tertiolecta* were grown in 2 *l* Erlenmeyer flasks containing 1 *l* of modified JOHNSON'S medium at 20℃ for 2 weeks. The cells in the late exponential growth phase (2-5 x 10^6 cells/m*l*), were harvested by centrifugation at 20℃, 6,000 rpm, and were washed once by resuspension in washing solution (Table Ⅶ-1).

B. Cell desruption

The pellets fraction of harvested cell are disturbed by Potter's homogenizer at 0℃. The pellets are resuspended in a 5 m*l* of 20mM Tris buffer (pH 8.5) and dialyzed in a same Tris buffer at 4℃ for 6 hrs. Microscopy confirms breakage of cell approximately 95 %.

C. ATPase analysis

The activity of the enzyme is assayed by incubation in a medium determined to give a

Table Ⅶ-1. Composition of washing solution for the preparation of harvested cell of *D. tertiolecta*. After washing with this solution, ATPase activity was analyzed.

NaCl	0.24g
Tris	0.24g
H_2O	100 m*l*
pH	8.5

maximum response of enzyme activity. The assay is initiated by the addition of 0.1 m*l* of enzyme solution containing 10-50 μg homogenate protein, to 0.9 m*l* of prewarmed assay mixture in 30 m*l* Pyrex tubes. After incubation with shaking at 30℃ for 30 min the reaction is stopped by adding 0.25 m*l* of 20% trichloroacetic acid and 0.1 M sodium acetate. The contents of protein per assay and incubation times are adjusted so as not to exceed 10% hydrolysis of substrate, yet obtain final absorbance readings in the range from 0.4 to 0.7 above the blank. After thorough mixing, the tubes are placed in an icewater bath. Then the residue is removed by centrifuge. The supernatant is collected and assayed for inorganic phosphate.

D. Phosphate determination

The phosphate released during ATP hydrolysis is determined by FISKE-SUBBAROW method (1925). Three m*l* of Solution-I was added to the 5m*l* of centrifuged or filtered sample (Table Ⅶ-2). Then 2m*l* of solution-Ⅱ was added. After the mixture was placed in the dark for 15 minutes, its optical density was measured at 720mμ.

Table Ⅶ-2. Reagnt solutions for phosphate determination (FISKE & SUBBAROW, 1925).

Solution I :	$(NH_4)_6Mo_7O_{24}.4H_2O$	4.4g
	1.6 N H_2SO_4	1,000 m*l*
Solution II :	$SnCl_2.2H_2O$	0.6g
	Hydrazine sulfate	2.0g
	0.6 N H_2SO_4	950 m*l*

E. Determination of protein

The procedure of HARTREE (1972) was used to determine the protein. Samples of protein were dissolved in 0.9 m*l* of solution-A by heating in a water bath at 50℃ for 10 min (Table Ⅶ-3). After cooling to room temperature (20-25℃), 0.1 m*l* of solution-B was added. After 10 min., 3 m*l* of solution-C was added and stirred vigorously and heated in water bath at 50℃ for 10 min. After cooling, the optical density of the mixed solution was measured at 650mμ. Bovine serum albumin was used for standard curve measurements.

Table Ⅶ-3. Reagent solutions for protein determination (HARTREE, 1972).

Solution A :	Potassium sodium tartrate	2g
	Na_2CO_3	100g
	1 N NaOH	500m*l*
	H_2O	1,000m*l*
Solution B :	Potassium sodium tartrate	2g
	$CuSO_4.5H_2O$	1g
	H_2O	90m*l*
	1 N NaOH	10m*l*
Solution C :	Folin-Ciocalteu reagent	1m*l*
	H_2O	15m*l*

F. Cytochemical determination of Mn-ATPase activity

The cells, in the form of centrifuged pellet, were fixed in 0.2 M s-collidine buffer (pH 7.6) with 4% glutaraldehyde for 30 min at 0℃. They were then washed thoroughly to remove excess fixative, and were incubated in the staining medium. The medium used in this study was modified from the solution used by HALL (1971) and HALL & DAVIE(1975) in maize (*Zea mays*) root tips and leaves of *Suaeda maritima* (Table Ⅶ-4). The cells were post fixed in 0.2 M s-collidine buffer (pH 7.6) with 1% O_sO_4 and embedded. Ultrathin sections were cut as previously described (p. 189).

Table Ⅶ-4. Staining medium of Mn-ATPase in the cell of *Dunaliella tertiolecta*. The cell was stained for 1 hr at 23℃ to detect the activity of ATPase using an electron microscope.

2mM	ATP
2mM	$MnCl_2$
3.6mM	$Pb(NO_3)_2$
48mM	s-collidine buffer (pH 7.6)

Ⅶ－3. Results

(1) ATPase activity in whole homogenate of *Dunaliella tertiolecta*

To discriminate the type of ATPase in the cell, whole homogenates of *D. tertiolecta* were incubated with ATP and various combinations of Na, K, Mg and Mn.

The result shown in Table Ⅶ-5 reveals at least 4 types of ATPase activities were recognized; (Na, K)-, (Na, K, Mg)-, Mg, and Mn-stimulated ATPase. But, the activities of Mg-, and Mn-ATPase took only one figure down from (Na, K)-, and (Na, K, Mg)-ATPase.

Table Ⅶ-5. Effect of various combination of Na, K, Mg and Mn on the ATPase activity of the whole homogenate of *Dunaliella tertiolecta*. The concentrations of various additives were ; 4 mM ATP, 4mM $MgCl_2$, 100mM KCl, 2 mM $MnCl_2$ and 100 mM Tris-HCl (pH 8.0). Reaction time was 60 min at 35℃.

Additives	ATPase activities (μgPi/0.1m*l* homogenate)
Na, K	88.4
Na, K, Mg	94.3
Mn	7.1
Mg	4.6

(2) Mn-ATPase activity as a function of incubation time

In genetal, enzymatic activity constantly increases at the early period of incubation, then its rate reduces consecutively. For comparing enzymatic characteristics, the data obtained

in this constant activity is useful.

Mn-ATPase from *D. tertiolecta* exhibits constant activity at least for the first 50 mins incubation in this experiment. So that, incubation time was set to 30 mins for further studies (Fig. Ⅶ-1.).

(3) Effect of temperature on the activity of Mn-ATPase

The Mn-ATPase activity in whole homogenate of *D. tertiolecta* was observed at different

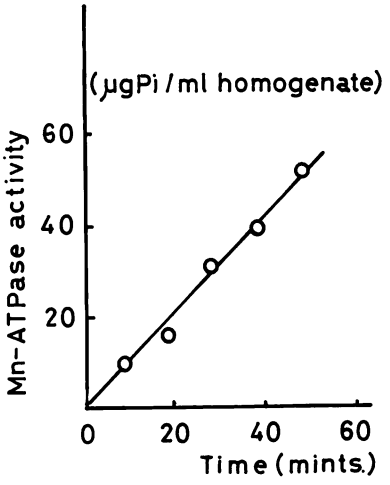

Fig. Ⅶ-1. Effect of incubation time on Mn-ATPase activity of *Dunaliella tertiolecta*. Whole homogenate was incubated for 60 mints and activity of Mn-ATPase was analyzed in every ten mints.

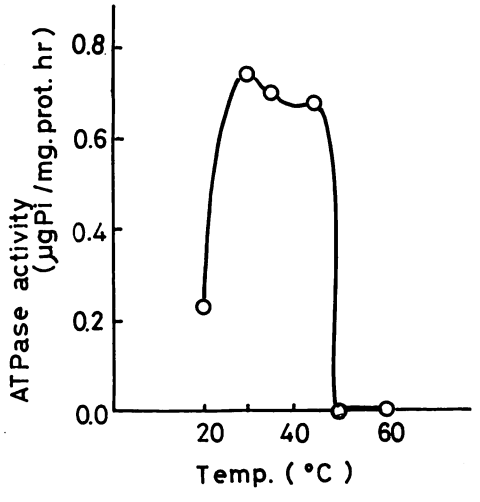

Fig. Ⅶ-2. Effect of temperature on Mn-ATPase activity of the whole homogenate of *D. tertiolecta*. The reaction mixture contained 4 mM ATP, 100 mM Tris-HCl (pH8.0) and 2 mM $MnCl_2$. Reaction time was 30 min.

temperatures from 20℃ to 60℃ (Fig. Ⅶ-2).

This enzyme activity was strongly temperature dependent. The activity attained to a maximum level at 25℃ and completely vanished at 50℃. As described before, *D. tertiolecta* grows and takes up Mn with a maximum at about 20-25℃.

Hitherto, enzymatic activity had been measured at the temperature of 37℃, but recently the temperature of 25℃ is introducing internationally. So, the Mn-ATPase was analysed at the 25℃ of incubation temperature.

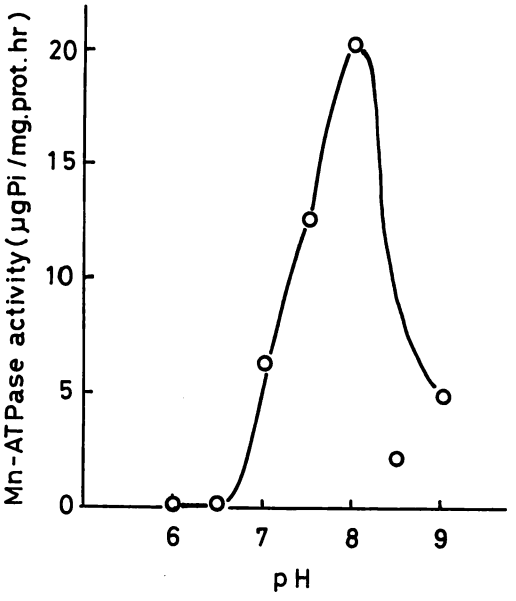

Fig. Ⅶ-3. Effect of pH on the Mn-ATPase activity by the whole homogenate of *D. tertiolecta*. The reaction mixture contained 4 mM ATP, 100 mM Tris-HCl (Proportion varied with desired pH). Reaction time was 30 min at 25℃.

(4) Effect of pH on the activity of Mn-ATPase

The activity of Mn-ATPase in whole homogenate of *D. tertiolecta* was observed at pH value from 6.0 to 9.0 (Fig. Ⅶ-3.). This enzyme was activated at pH value between 7.0 and 9.0, but the activity completely vanished at the pH of 6.5. Maximum pH for the activity was observed at pH 8.0. The maximum growth of *D. tertiolecta* occurred at the same pH. For further experiments, the Mn-ATPase activity reaction system was incubated at pH 8.0.

(5) Effect of Mn concentration on Mn-ATPase activity

The activity of Mn-ATPase in whole homogenate of *D. tertiolecta* was measured as a function of the external Mn concentrations (Fig. Ⅶ-4).

With increasing Mn concentration, the Mn-ATPase activity rose and reached a limiting value at a concentration of 1 mM Mn (=55 ppm Mn). Then, the activity slowly decreased and vanished at 8 mM Mn (=440 ppm Mn).

(6) Effect of divalent cations on the ATPase activity

TableⅦ-6 illustrates the effect of divalent cations on the ATPase in whole homogenate

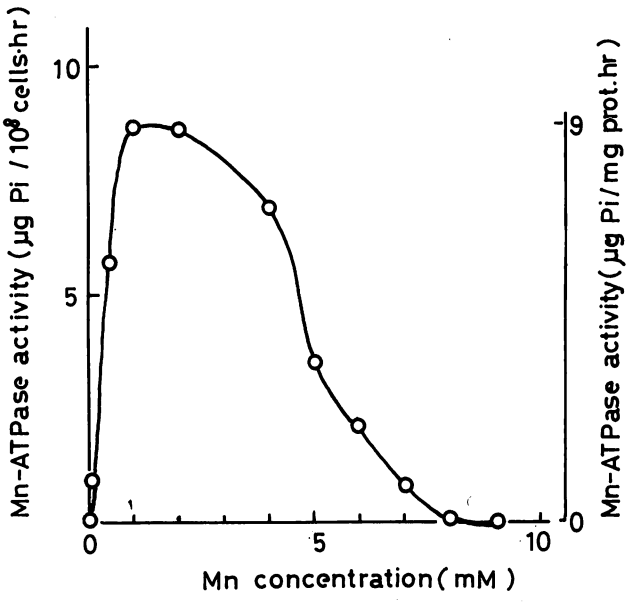

Fig. Ⅶ-4. Effect of various concentration of Mn on the activity of Mn-ATPase of *D. tertiolecta*. The assay medium contained 100 mM Tris-HCl (pH 8.0), 4 mM ATP. Reaction time was 30 min at 25℃.

of *D. tertiolecta*. The concentration of the cations was 0.1 ppm each.

ATPase of *D. tertiolecta* is sensitive to divalent cations. Each of Ca, Mn and Mg stimulated the ATPase of *D. tertiolecta*, while Zn, Cu and Fe could not activate the ATPase.

The sample used here was the whole homogenate of *D. tertiolecta*. So, several types of ATPase were expected in whole homogenate. And my result could not conclude that Mn-ATPase was not stimulated by another divalent cation, Ca or Mg. The purification of

Table Ⅶ-6. Effect of divalent cations on the ATPase of *Dunaliella tertiolecta*. Reaction mixture contained 100 mM Tris-HCl (pH 8.0) and 4 mM ATP.

Ion	ATPase activity	
	μgPi/10^8cells. hr	μgPi/mg. prot. hr
Mg^{2+}	4.49	4.63
Mn^{2+}	8.61	8.87
Ca^{2+}	11.79	12.15
Co^{2+}	2.13	2.19
Zn^{2+}	0.00	0.00
Cu^{2+}	0.00	0.00
Fe^{2+}	0.00	0.00
Mo^{2+}	0.18	0.19

Mn-ATPase is necessary to verify the function of Mn-ATPase.

(7) Effect of metabolic inhibitor on the activity of Mn-ATPase

In animal cell there are many evidence for active K-influx and Na-efflux. This active transport of Na, K-ATPase are sensitive to metabolic inhibitors, ouabain and oligomycin.

To know the contributions of these metabolic inhibitors on Mn-and (Na, K, Mg)-ATPase activities of *D. tertiolecta*, ouabain and oligomycin were added on ATPase reaction mixrure (Table Ⅶ-7).

Na, K, Mg-ATPase in whole homogenate was insensitive to ouabain (10^{-3} to 10^{-4} M) ; however inhibition of this enzyme activity by oligomycin (10^{-4} to 10^{-5} M) was 90%. On the other hand, the activity of Mn-ATPase was inhibited at the rate from 15 to 25% by 10^{-4} M ouabine, but 100% inhibition of Mn-ATPase activity was experienced by 10^{-4} M oligomycin. This is an interesting difference between Na, K, Mg-ATPase and Mn-ATPase of *D. tertiolecta*.

Table Ⅶ-7. Effect of metabolic inhibitors on ATPase activity of *Dunaliella tertiolecta*. Whole homogenate of *D. tertiolecta* was incubated with Na, K, Mg, or Mn. Reaction mixture contained several concentrations of inhibitors, ouabain and oligomycin. The assay medium also contained 100 mM Tris-HCl (pH 8.0), 4 mM ATP. Reaction time was 30 min at 25℃.

ATPase activity +metabolic inhitit.	concentration of inhibitors				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}M
Na, K, Mg-ATPase					
+Ouabain	100	−	122	122	100
+Oligomycin	100	119	12	11	0
Mn-ATPase					
+Ouabine	100	−	107	75	85
+Oligomycin	100	100	115	0	0 %

(8) Localization of Mn-ATPase in the cell of *D. tertiolecta*

Electron microscopic enzyme cytochemistry is concerned with the localization of specific enzymes at their normal sites within intact cells. The majority of these methods relys on the formation of an insoluble, electron-dense reaction product at the site of enzyme activity, which may then be located by direct observation of ultra thin sections. Using this approach, normal cellular structure is preserved, and thus enzyme activity may be shown to be related to a specific site within an identifiable cell.

To preserve fine structure, it is necessary to fix appropriately the tissue. Modern fixatives, ex. glutaraldehyde or OsO_4 form molecular link in a macromolecular network. Fixation, thus, stabilizes cellular structure and fixes diffusible components in their original position. This process involves the formation of covalent bonds with a variety of functional groups on proteins, lipids, nucleic acids and other molecules. From the morphologist's point of view, the more fixatives penetrate into the cell the more clear image is observed using electron microscope. However, the enzyme cytochemist is confronted with the

additional requirement for preserving the activity of ATPase. These two objectives, fixation of cell and preservation of enzyme activities, are often incompatible, since the formation of covalent bonds with the enzyme during fixation will usually reduce its activity. Thus, it is often found that the better the structural preservation, the lower the enzyme activity, and vice versa. It is not always possible therefore to adopt the normal fixation procedures used in morphological work for enzyme cytochemistry. The method of fixation is thus of paramount importance, and the effect of the fixative on the enzyme under investigation must be known as precisely as possible.

Formaldehyde, as a popular fixative, would be expected to have less effect on enzyme activity than the more reactive glutaraldehyde. However, the results shown in Table Ⅶ-8 indicate that Mn-ATP activity of *D. tertiolecta* was totally destroyed by 1.0 % glutaraldehyde and formaldehyde, but enough activity remained after the use of 0.5 % glutaraldehyde

Table Ⅶ-8. Effects of fixatives on the activity of ATPase from *Dunaliella tertiolecta*. Whole homogenate of *D. tertiolecta* was incubated with glutaraldehyde and formaldehyde for 30 min at 25℃. The assay medium also contained 100 mM Tris-HCl (pH 8.0) and 4 mM ATP.

Fixatives and their concentrations	Mn-ATPase activity
Control	100.0%
0.1% Glutaraldehyde	100.9
0.5% Glutaraldehyde	27.5
1.0% Glutaraldehyde	0.0
0.1% Formaldehyde	114.1
0.5% Formaldehyde	10.3
1.0% Formaldehyde	0.0

to allow the localization of this enzyme. The values show that more than 70 % of the original activity was lost after 0.5 % glutaraldehyde fixation, whilst 90 % of the enzyme was destroyed by 0.5 % formaldehyde fixation.

Mn-ATPase have been localized by lead precipitation techniques in 0.5 % glutaraldehyde fixation for 30 mins at 0-4℃. Electron-dense deposits of Mn-ATPase in *D. tertiolecta* are associated with plasma membrane (Fig. Ⅶ-5). To preserve enzyme activity, fixative procedure makes the morphological images on photograph worse than that of Fig. Ⅲ-1-4.

By methods of electron microscopic cytochemistry, it is generally believed that Na, K-ATPase is localized on plasma membranes (ERNST, 1975) or vacuole membrane (HALL, 1971). However, BELITSER *et al.* (1981) found ATPase activity on plasmodesmata, lysed protein bodies and nuclei but lately detected on the plasmalemma (BELITSER *et al.*, 1981).

Ⅶ-4. Discussion

The energy source for ion transport in plants was unknown. Respiratory-inhibitor studies

Fig. Ⅶ-5. Localization of ATPase activity in *D. tertiolecta*. Electron-dense deposits are associated with plasma membrane. Ultra thin section of cell was stained with the staining medium of Table Ⅶ-4.

implicate oxidative phosphorylation in the transport process; however, the coupling mechanism is controversial. On the basis of arsenate and oligomycin inhibition of transport in plant roots, it can be seen that ATP serves as the actual energy source for transport (FISHER *et al.*, 1970).

The most convincing evidence for transport ATPases in plant tissue comes from the laboratory of HODGES. FISHER and HODGES (1969) isolated ATPases from the particulate fraction of homogenates from oat roots, *Avena sativa*. The enzymes were activated by Mg and further activated by monovalent salts, KCl being most effective. However, there was no synergistic effect from the simultaneous presence of KCl and NaCl, nor was there inhibition by ouabain. So far, these findings are not different from that in animals.

In green algae, anion transport is more closely linked to the photosynthetic electron transport process whereas cation transport is coupled to ATP formation and utilization (MACROBBIE, 1965 ; RAVEN, 1968 ; RAVEN *et al.*, 1969).

ATPases stimulated by Na-K and Mn were confirmed their presence in whole homogenate of *D. tertiolecta*. The Mn-ATPase activity was one-tenth lower than that of Na-K-ATPase. The cytochemical confirmation showed the distribution of this Mn-ATPase on plasma membrane. On the other hand, many types of ATPases from *D. tertiolecta* which were stimulated by many ions, Na-K, Co, Ca, Mg and Mn, were not biochemically purified enzyme. So, it is not clear whether Mn-ATPase and Na-K-, Ca-, and Mg-ATPase are the same enzyme or not. However, it is concluded that Mn-stimulated ATPase was present on the plasma membrane of the cell.

Possible correlation could be tested between Mn-stimulated ATPase and rates of ion flux for plasmalemma. A highly siginificant correlation was observed ($P<0.025$; $r=0.896$) between plasmalemma enzyme activity and influx over the range of 0 to 5 ppm Mn (Fig. Ⅶ-6 .).

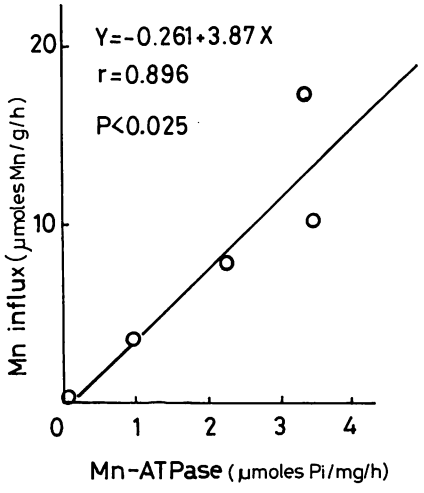

Fig. Ⅶ-6. Correlation between Mn-ATPase and Mn influx of *Dunaliella tertiolecta*, using the data of Figs. Ⅴ-7 and Ⅶ-4.

Ⅷ. General discussion : Manganese uptake mechanism by *Dunaliella tertiolecta*

Reviews dealing with aspects of algal nutrition have been published at intervals (KRAUSS, 1958 ; PROVASOLI, 1958 ; LEWIN, 1962 ; GERLOFF, 1963 ; NICHLLAS, 1965 ; HUNTER & PROVASOLI, 1964 ; HEALEY, 1973 ; EPSTEIN, 1973). The recent well distributed review by O'KELLEY (1974) dealt specifically with the essentiality and metabolic roles for mineral nutrient. Pollution studies of heavy metals with algae were also reviewed by DAVIES (1976) and RAI *et al.* (1981).

Date regarding heavy metal concentrations of algae were actively accumulated during the past decade, but the absorption mechanisms of heavy metal has not been extensively studied until recently.

Reports on the uptake of heavy metals by algae are relatively few, but the following elements have studied: Zn by *Ulva* (GUTKNECHT, 1961); Fe, Mn and Zn by *Porphyra* (TSUKIDATE, 1974); Fe, Zn, Mn, Cu, Pb and Cd by *Porphyra* and *Enteromorpha* (MAEDA & FUJIYAMA, 1977).

However, the uptake mechanism was not mentioned by any author except GUTKNECHT (1961) and VEROY *et al.* (1980), by whom Zn uptake by *Ulva* and Pb and Cd uptake by *Eucheuma* were explained as not the active transport but the ion exchange mechanism.

Fifteen species of marine phytoplankton were analysed for their endogenous trace metals by RILEY & ROTH (1971). From their data, concentration factors of the trace metals in *D.*

Table Ⅷ-1. Concentration of heavy metals in *Dunaliella tertiolecta* and their concentration factors. The data was calculated from RILEY & ROTH (1971).

heavy metal	concentration in *D. tertiolecta.*			concentration in medium (ppm)	concentration factor
	dry weight (ppm) a)	wet weight (ppm) b)	wet weight (μM) c)	d)	(e
Mn	3.8	0.12	2.2	5.0	0.02
Zn	285	9.12	139.4	0.15	60.8
Cu	57	1.82	28.7	0.034	53.5
Ag	7.8	0.25	2.3	0.008	31.3
Pb	8.1	0.26	4.1	0.031	8.4
Sn	17.5	0.56	4.7	0.010	56.0
Ni	4.3	0.14	2.4	0.008	17.5
V	2.9	0.09	1.8	0.020	4.5
Cr	3.6	0.12	2.3	0.012	10.0

b=a×0.032
c=b/molecular weight
e=b/d

tertiolecta were calculated (Table Ⅶ-1). In these results, the concentration factor of Mn was unreasonably low, probably by the reason why the concentration of the trace metal in the medium would be too high to be absorbed normally. However, each metal was absorbed in different concentration factors. This means that trace metal uptake by *D. tertiolecta* is not only physicochemical adsorption but also physiololgical active absorption.

The results obtained in this study showed that Mn uptake by *D. tertiolecta* was affected by several environmental factors, e.g. temperature, light intensity, pH etc. So, active absorption was expected in Mn uptake mechanisms in this alga.

To clarify the active transport, many criteria have been proposed (LEHNINGER, 1970). For example;

(a) uphill accumulation against gradient of solute,
(b) inhibition by metabolic inhibitor
(c) competitive uptake of solute
(d) uptake rate holds Michaelis-Menten equation
(e) high temperature coefficiency (Q_{10}).

The manganese uptake mechanism, which was studied here, was sufficient for these criteria (p. 202—213). Furthermore, the most clear evidence of active transport that *D. tertiolecta* accumulates more manganese is explainable by the Nernst equation (p. 218). These are the reasons for concluding that the Mn uptake mechanism is an active transport process.

On the other hand, metabolic inhibitors make the cellular Mn discharge (p. 214) and dead cells also loose the Mn easily (p. 202). Therefore, chemical form of Mn in *D. tertiolecta* was expected to be ionic or of low molecular weight compounds. ITOH (1970) studied the Mn function in chloroplast, and according to him, Mn is the reducer in photosynthesis (Ⅱ). Hence, Mn of different valences would be present in chloroplast and protoplasm. VON KAMAKE & WEGMANN (1978) isolated Mn-protein compounds from *D. tertiolecta*. However more detail studies on chemical form of Mn in cell are needed.

In the early uptake process of ionic Mn from the medium, *D. tertiolecta* adsorbs it on the surface of cell membrane. Similar adsorption of Zn was reported in yeast cell by FAILLA *et al.* (1976) and PARRY & HAYWARD (1973) proposed the presence of proteinaceous compounds as binding sites in the cell for the Zn uptake by *D. tertiolecta*. This kind of compounds would act as binding site in early process of adsorption of heavy metal. It has been postulated that metabolism-independent binding of divalent cations is an essential step in their energy-dependent accumulation. However, the relationship between metabolism-independent Mn binding and metabolism-dependent Mn accumulation is thus not clear from existing data (REYNAFARJE & LAHNINGER, 1969). It is also unknown whether the binding site is able to distinguish Mn from Fe or Zn. Nevertheless, PATON & BUDD (1972) thought that Mn appeared to be transported into the cytoplasm via the same site on the Zn carrier though Zn uptake by mycelium of a fungus was inhibited in the presence of Mn.

Various ways of plotting data on the binding of small molecules to macromolecules have been reviewed by EDSALL & WYMAN (1958). The relative affinities and number of binding sites can be extrapolated (REYNAFARJE & LEHNINGER, 1969) ; it is similar to the Eadie plot

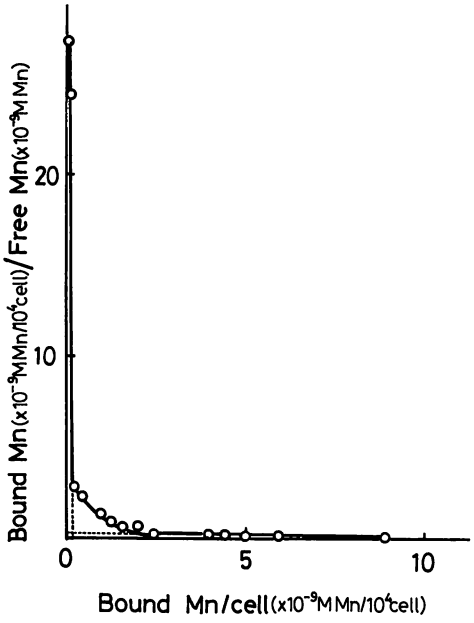

Fig. Ⅷ-1. Scatchard plots of Mn binding. The data of Fig. Ⅴ-7 were plotted in this figure.

for enzyme kindtics (EADIE, 1967). Fig. Ⅷ-1 shows a Scatchard plot of the data of Fig. Ⅴ-7. The ratio of the amount of Mn bound to the concentration of free Mn was plotted against the amount of Mn bound. This plot shows that *D. tertiolecta* have at least two types of Mn-binding sites that differ greatly in their affinity for Mn. From the derivation of SCATCHARD *et al.* (1957), if such a plot is linear, all of the binding sites for Mn function independently and are equal in affinity. The intercept on the ordinate is nk, where n is the number of binding sites per particle and K is the formation or stability constant of the Mn-binding site complex ; the intercept on the abscissa is n, the number of binding sites.

If the Scatchard plot is biphasic, two classes of binding sites different in affinity is indicated to be present. In the latter case, the extrapolated intercepts of the two linear segments of the plot can be used to arrive at the number and affinity of each type of binding site. Fig. Ⅷ-1 shows that the Scatchard plot of the Mn binding data is biphasic. The two linear segments of the plot differ markedly in gradient, suggesting the occurrence of two types of Mn-binding sites differing in number and affinity for Mn.

The Mn ion on binding site may be actively transported to the interior of the cell. From the biochemical knowledge, this active transport needs proper energy. And this energy is derived from the hydrolysis of ATP.

From this study the presence of ATPase that is stimulated by Mn was confirmed (Mn-ATPase). Mn-ATPase distinguishes Mn from other heavy metal and hydrolyzes the ATP to derive energy for Mn uptake. But, in this experiment this enzyme could not

separated and purified. So, it is doubtful whether Mn-ATPase is different from Na, K-ATPase. In the whole homogenate of *D. tertiolecta*, the ATPase that was activated by other heavy metals, e. g. Fe, Zn and Ca, was detected. But, it is not clear that Mn-ATPase can be stimulated by other kind of heavy metals.

Using these results, Mn uptake mechanism of *D. tertiolecta* is illustrated (Fig. Ⅷ- 2). On the cell membrane, there is a channel or a pump which is activated by the energy of Mn-ATPase. This pump takes up Mn from outside to inside. Most of the manganese taken up are stored in the form of ion or low molecular compounds near the cell membrane. But some of Mn are carried to organella, such as chloroplast. When metabolic inhibitors prevent supply of energy to the channel or supply of the electrical membrane potential, most of the low molecular Mn in the cell are released to outside. Without energy, this channel can not prevent the Mn to release. However small amount of Mn which is bound to organella remains inside of the cell.

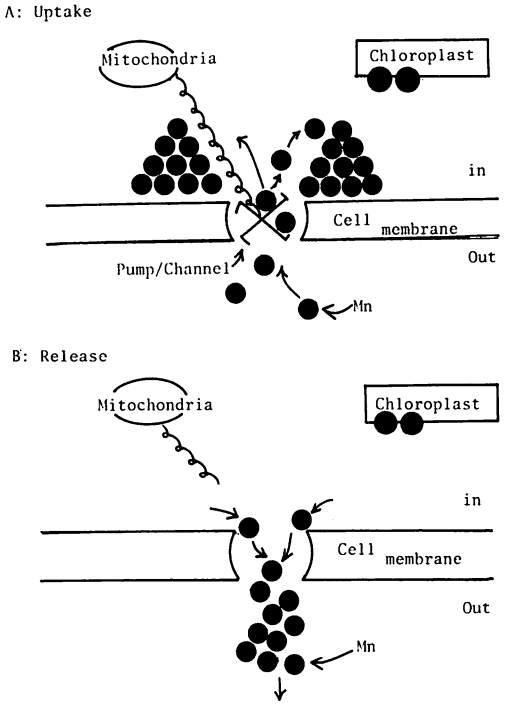

Fig. Ⅷ-2. Proposed scheme for the transport of Mn by *D. tertiolecta*. (A) : On the cell membrane, there is a channel or a pump which is activated by the energy of Mn-ATPase. This pump takes up Mn from outside to inside. Most of the manganese taken up are stored in the form of ion or low molecular compounds near the cell membrane. But some of Mn are carried to organella, such as chloroplast.
(B) : When metabolic inhibitors prevent supply of energy to the channel or supply of the electrical membrane potential, most of the low molecular Mn in the cell are released to outside. Without energy, this channel can not prevent the Mn to release. However small amount of Mn which is bound to organella remmains inside of the cell.

ROTHSTEIN *et al.* (1958) and JENNINGS *et al.* (1958) studied active transport of Mg and Mn in yeast cells. ROTHSTEIN *et al.* (1958) concluded that Mg and Mn were actively transported into the cell by mechanism involving a phosphate and protein constituent. JENNINGS *et al.* (1958) also found out that the synthesis of a carrier for the transport of Mn and Mg involves a phosphorylation step closely coupled with reaction in the absorption of phosphate and they offered a simplified scheme for the transport of Mn and P into the yeast cells. The Mn uptake mechanism shown in Fig. Ⅷ- 2 agrees with those proposed by the above authors in a great extent.

The uptake of other heavy metals may show almost similar mechanisms, but this study was restricted to Mn. Of course, different elements show different patterns in physiological functions. But Ni, Co and Zn can be taken up into a nonexchangeable pool by yeast cells, by a system that also transports Mg and Mn (FUHRMANN & ROTHSTEIN, 1968).

Experimental organism for this study was an unicellalar green alga, *D. tertiolecta*. And heavy metal accumulation in other algae would follow same or similar mechanism that was found in *D. tertiolecta*.

Acknowledgements

I wish to express my deepest gratitude to Professor Tomitaro MASAKI of the Laboratory of Marine Botany, Faculty of Fisheries, Hokkaido University for his encouragement and guidance throughout this study. My sincere thanks are also devoted to Professor Isamu TSUJINO and Associate Professor Hiroshi YABU of the same university for critical reading of the manuscript.

I am indebted to Dean and Professor Koji NOZAWA, Faculty of Fisheries, Kagoshima University, Dr. Yuzuru SAITO and Dr. Hirotoshi YAMAMOTO and all the members of the Laboratory of Marine Botany, Faculty of Fisheries, Hokkaido University, for their fruitful discussions and criticisms.

I thank the following people. Dr. Y. NISHIHAMA of Shikabe Mariculture Center, Hokkaido, who kindly offered *Dunaliella tertiolecta* to me; Dr. J. TSUKIDATE of the Nansei Regional Fisheries Research Laboratory, Hiroshima, who helped in R. I. techniques; Prof. S. TSUNOGAI, Associate Professors T. HARADA and K. ARAI, and Dr. K. KONNO of Hokkaido University who assisted me in the form of advice or use of equipment.

Special thanks are also given to my wife, Reiko, who always showed great fortitude and love this work.

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