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The Measurement of Respiratory Electron-transport Activity in Marine Phytoplankton[1]

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ABSTRACT

A tetrazolium-reduction method for the measurement of the respiratory electron-transport activity in plankton is described. The method is sensitive to 0.03 μg cell nitrogen and can be used to estimate total plankton respiration.

Introduction. Enzyme analysis can be used to study many biologically controlled processes in the ocean. Among these processes are photosynthesis, nitrogen fixation, oxygen utilization, sulfate and nitrate reduction, and ammonia and nitrite oxidation. Qualitative measurements of enzyme activity are useful in locating the site of a process whereas quantitative measurements are useful in estimating the rate of the process. Eppley et al. (1969) used the first approach with NADH-nitrate reductase (EC 1.6.6.1) to determine when phytoplankton assimilate nitrate. Curl and Sandberg (1961) have suggested the use of succinate dehydrogenase (EC 1.3.99.1) assays to estimate respiration rates in zooplankton. Pearre (1964) and Packard and Taylor (1968) found succinate dehydrogenase (SDH) to be a good indicator of zooplankton respiration, but Pearre (1964) found SDH to be useless as an indicator of phytoplankton respiration. Thus SDH could not be used to estimate the oxygen utilization rate in seawater because it would be sensitive to only the zooplankton fraction of the plankton community. A measurement of the activity of the respiratory electron transport system (ETS) would provide a better estimate of the potential plankton respiration rate because this enzyme system and not the component dehydrogenases controls oxygen consumption (Chance 1957, Klingenberg 1968).

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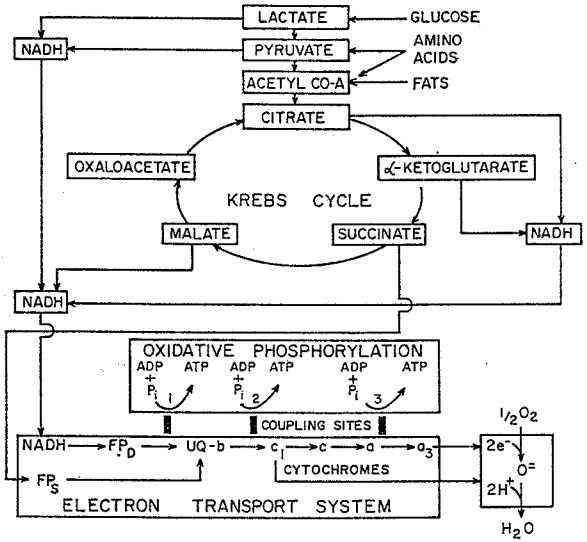

Figure 1. The respiratory electron-transport system and its relationship to the Krebs Cycle and oxidative phosphorylation. UQ-b = ubiquinone (coenzyme Q) – cytochrome-b complex, FP_D = flavoprotein (NADH reduction system), FP_S = flavoprotein (succinate dehydrogenase system), ADP = adenosine diphosphate, ATP = adenosine triphosphate.

The ETS, which is found in a cell's mitochondria and its microsomes (Fernandez-Moran et al. 1964, Strittmatter 1968), consists of a complex chain (Fig. 1) of cytochromes, flavoproteins, and metalic ions that transport electrons from catabolized foodstuffs to oxygen. To measure the activity of a complex enzyme system like the ETS, the rate-limiting step must be determined. In the ETS this step is the oxidation of the coenzyme Q-cytochrome B complex (Chance 1954, Chance et al. 1955, Green 1964) and it can be measured by its reaction with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (Kalina and Palmer 1968, Gahan and Kalina 1968). The method for measuring ETS activity described below is based on this reaction (Fig. 2).

Methods. Prepare all solutions with deionized distilled water.

i. Potassium dihydrogen phosphate (0.1 *M*). Dissolve 13.6 g of KH_2PO_4 in water and make up to 1 L.

ii. Disodium hydrogen phosphate (0.1 *M*). Dissolve 14.20 g of Na_2HPO_4 in water and make up to 1 L.

iii. Phosphate buffer (0.1 *M*). Mix 106 ml of 0.1 *M* KH_2PO_4 solution with 894 ml of 0.1 *M* Na_2HPO_4 solution and adjust the *p*H to 7.7.

iv. Stock homogenizing buffer. Dissolve 9 mg $MgSO_4$ and 1.5 g of polyvinyl pyrrolidone (PVP) in 1 L of 0.1 *M* phosphate buffer. Keep frozen.

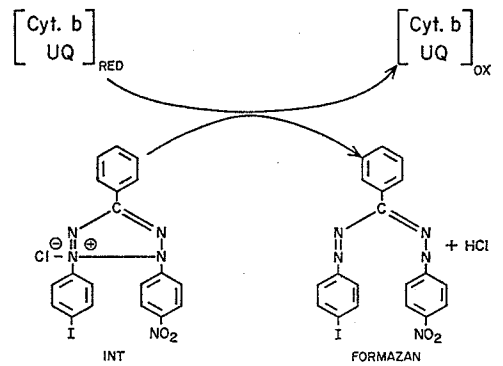

Figure 2. The oxidation reduction reaction between INT and the ubiquinone-cytochrome-b complex.

v. Homogenizing buffer. Dissolve 5 mg of glutathione in 33 ml of the stock homogenizing buffer. Prepare daily and store at 0–4°C.

vi. Stock substrate solution. Dissolve 3.6 g of sodium succinate in 100 ml of 0.1 *M* phosphate buffer. Keep frozen.

vii. Substrate solution. Dissolve 19 mg of reduced nicotinamide adenine dinucleotide (NADH) and 6 mg of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in 30 ml of stock substrate solution. Prepare daily and store at 0–4°C. Use 3 ml per assay.

viii. INT solution (0.2%). Dissolve 100 mg of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride in 50 ml of water and adjust the *p*H to 7.5 with Na_2CO_3 and HCl. Filter off any undissolved tetrazolium, then freeze the solution until needed. Use 1 ml per assay.

ix. Ferric chloride (0.15 *M*). Dissolve 12 g of $FeCl_3$ in water and make up to 500 ml.

x. Solvent solution. Mix 400 ml of tetrachloroethylene with 600 ml of acetone.

INT is reduced by bacterial action in solution unless the solutions are kept frozen. Solutions of glutathione, NADH, and NADPH can not be stored longer than a day without loss of activity. All biochemical reagents were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Procedure. Seawater in an amount of 4–8 L or an algal culture of 100–200 ml is filtered through 47-mm glass-fiber filters (Gelman, Type A). The filters are ground in 3 ml of solution v [0.1 *M* phosphate buffer, (*p*H 7.7), 75 μM $MgSO_4$, 0.5 m*M* glutathione and 1.5 mg/ml polyvinylpyrolidone] at 0°–4°C. One ml of the crude homogenate is incubated for 20 min, at *in situ* temperature or at a predetermined optimum temperature, with 0.5 m*M*

NADH, 0.15 m*M* NADPH, 80 m*M* sodium succinate, 60 m*M* phosphate buffer (*p*H 7.7), and 0.04% (w/w) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). Three ml of solution vii and one ml of solution viii are used; the final volume is 5 ml. The reaction is stopped by adding 1 ml of a 37%-formaldehyde solution, 1 ml of 0.15 *M* $FeCl_3$, and 4 ml of a 1.5:1 solution (v/v) of acetone and tetrachloroethylene and by chilling the reaction tubes in an ice bath. Controls were run in the same way, using solution iii instead of solution vii. The formazan (reduced INT) solution is cleared by centrifugation, drawn off by syringe, diluted to 8 ml with solution x, and determined colorimetrically at 490 mμ. The assay is standardized by the method of Packard and Healy (1968). The ETS activity (A) is expressed in μl O_2 L^{-1} hr^{-1} and may be calculated from the expression: A = corrected absorbance $\times 3 \times H \times F/V$, where 3 converts the activity to hour units, H is the homogenate volume, V is the volume of water filtered, and F is a factor that is determined during standardization.

The chlorophyll was determined by the method of Lorenzen (1967) and the particulate nitrogen by the method of Barsdate and Dugdale (1965).

Standardization. INT is reduced by coulometric titration, and a standard curve is constructed by plotting formazan absorbance versus millicoulombs of current (Packard and Healy 1968). The slope (S) of the line is calculated and reported in units of millicoulombs per absorbance unit. The factor F is then calculated from the slope: F = S/17.2, where 17.2 is the electrochemical equivalent in millicoulombs of 1 μl O_2. F = 3.9 was used in this study.

Assay Development. Analysis of the ETS in plankton requires (i) the disintegration of both the cell walls and the mitrochrondrial membranes of planktonic organisms and (ii) the preservation of ETS activity during the process. Homogenization in distilled water readily accomplishes the disintegration but it does not preserve that activity of the ETS enzymes (Chance et al. 1968, Laties 1954). Preservation of enzyme activity frequently requires the use of a buffered solution of glutathione or another reduced sulfur compound (Chance et al. 1968, Eppley et al. 1969). Various buffer preparations were tested (Table I), but ETS activity was best preserved by a combination of glutathione, polyvinylpyrrolidone (PVP), and $MgSO_4$ in a phosphate buffer. Dithiothreotol and cysteine did preserve activity, but they reduced the INT non-enzymatically and thus could not be used. The dependence of the ETS activity upon the concentration of glutathione and PVP is shown in Fig. 3.

Table I. The enhancement of ETS activity in *Cyclotella nana* (0.64 mg cell N/L) by the use of different buffer mixtures during the preparation of homogenates. The concentrations were: phosphate, 01 *M*; glutathione, 1 m*M*; polyvinyl pyrrolidone (PVP), 3 mg/ml; and $MgSO_4$, 75 μM. Maximum ETS activity (100%) was 0.56 ml O_2 hr^{-1} mg cell N^{-1}.

Buffer contents	ETS activity (% of maximum)	Blank
Water	38	4
Phosphate	29	4
Phosphate, glutathione, $MgSO_4$	40	15
Phosphate, PVP, $MgSO_4$	66	8
Phosphate, glutathione, PVP	92	13
Phosphate, glutathione, PVP, $MgSO_4$	100	13

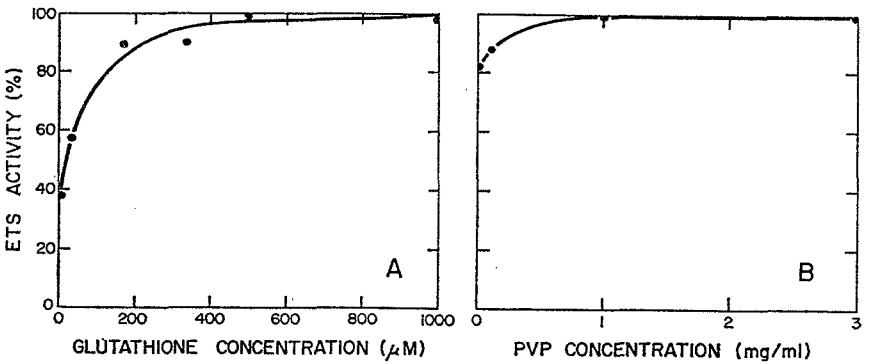

Figure 3. The effect of the glutathione (A) and polyvinylpyrrolidone, PVP (B) concentrations in homogenates (19 μg cell N/ml) of *Cyclotella nana* upon the ETS activity. Maximum ETS activity (100%) was: (A) 0.57 ml O_2 hr^{-1} mg cell nitrogen^{-1}; (B) 0.25 ml O_2 hr^{-1} mg cell N^{-1}.

The optimal substrate conditions of the enzyme assay are shown in Fig. 4. Maximum activity was obtained with a combination of 0.08 *M* sodium succinate, 0.5 m*M* NADH, and 0.15 m*M* NADPH. Sodium succinate and

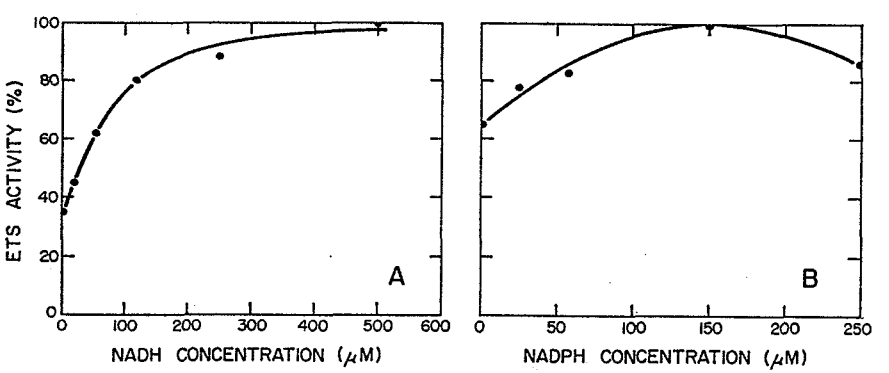

Figure 4. The effect of the NADH (A) and NADPH (B) concentrations upon the ETS activity in extracts of *Cyclotella nana* (23 μg cell N/ml homogenate). Maximum ETS activity (100%) was: (A) 0.60 ml O_2 hr^{-1} mg cell N^{-1}; (B) 0.29 ml O_2 hr^{-1} mg cell N^{-1}.

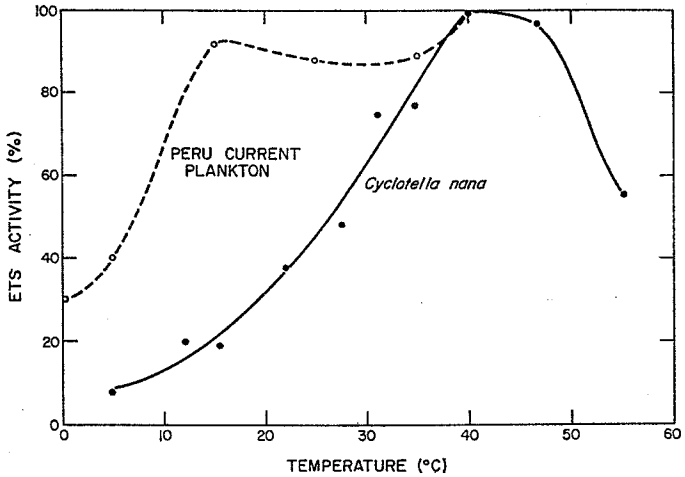

Figure 5. The temperature dependence of the ETS activity in *Cyclotella nana* and in Peru Current plankton. The plankton was dominated by diatoms; a silico flagellate, *Dichtiocha fibula*, and an unidentified tintinnid. The five most numerous diatoms were: *Chaetoceros compressus*, *Asterionella japonica*, *Coscinodiscus* sp., *Chaetoceros affinis*, and *Chaetoceros debilis*. Maximum ETS activity (100%) was: 1.1 ml O_2 hr^{-1} mg cell N^{-1} for *C. nana* and 0.5 μl O_2 L^{-1} hr^{-1} for Peru Current plankton.

NADH are known electron donors for the mitochondrial ETS (Klingenberg 1968), and NADPH is the electron donor for the microsomal ETS (Stritmatter 1968). ADP was not required because the oxidative phosphorylation system is uncoupled from the ETS by INT (Clark et al. 1965, Packard 1969).

The optimum temperature of an enzyme-catalyzed reaction is not a constant. It varies with the biological source of the enzyme and with the characteristics of the enzyme assay, i.e., ionic strength, *p*H, and incubation time. Hammen and Lum (1966) used 25°C, Ryan and King (1966) used 35°C, and Raymont et al. (1967) used 18°C in their studies of respiratory enzyme activity in marine organisms. For the ETS assay described here, the optimum temperature for ETS activity in Peru Current plankton extended from 15° to 40°C (Fig. 5). In *Cyclotella nana* the optimum occurs at 40°C. The relative complexity of the temperature dependence of ETS activity in plankton probably reflects the different characteristics of the isoenzymes involved.

The dependence of INT reduction on incubation time was tested. The rate of reduction was constant for 20 min; then it decreased (Fig. 6). This precluded the use of longer incubation times to gain sensitivity. The 20-min incubation was chosen because the reaction rate is constant over this time period, with a measurable quantity of INT produced. INT reduction during the 20-min incubation varies directly with the amount of enzyme preparation (Fig. 7).

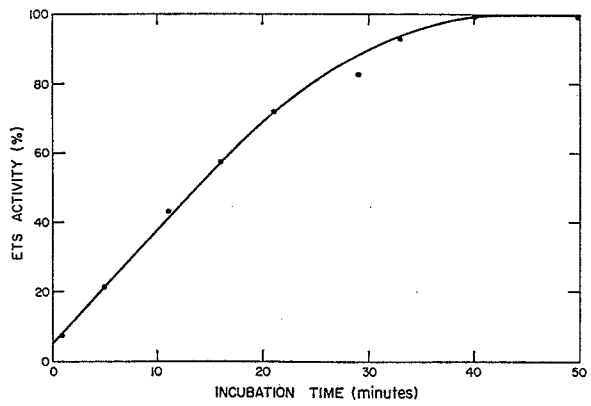

Figure 6. The dependence of the ETS activity on incubation time in extracts of *Cyclotella nana* (7.6 μg cell N/ml homogenate). Maximum ETS activity (100%) was 0.24 ml O_2 mg cell N^{-1} per incubation time.

The precision of the ETS assay was determined from replicate samples of a homogenate prepared from the diatom *Skeletonema costatum* ($1.8.10^8$ cells/L). The mean value and standard deviation of six samples were 198.8 ± 0.5 lO_2 L^{-1} hr^{-1}. At the 95%-confidence level, ETS measurements vary $\pm 1\%$ of the mean value. This error will be larger when filtering is considered.

The estimation of metabolic activity or the oxygen utilization rate by the measurement of the ETS activity requires that INT reduction be negligible in nonliving particulate matter. In dead cells from oxygenated waters (killed by heating or freezing), the ETS activity was found to be negligible. However, dead cells and other particulate matter from sulfide-rich waters are contam-

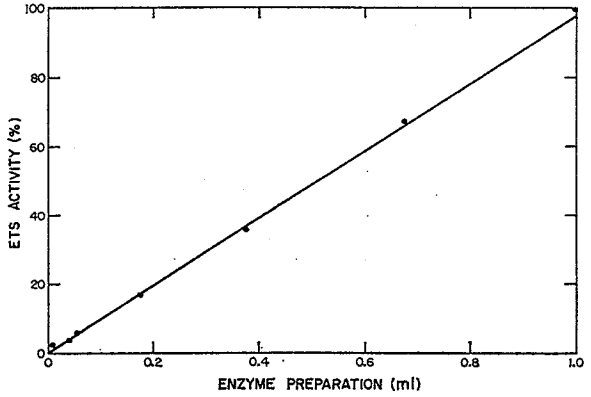

Figure 7. The dependence of the ETS activity on enzyme concentration in extracts of *Cyclotella nana* (2.4 μg cell N/ml homogenate). Maximum ETS activity (100%) was 2.6 μl O_2 hr^{-1}.

inated with $S^=$, which causes considerable INT reduction. Unless the sulfide is oxidized before analysis, particulate matter from anoxic waters cannot be assayed for ETS activity.

Discussion. The ETS assay reported here is a modification of an earlier version (Packard 1969). Homogenization has been improved by using the glutathione-PVP buffer mixture rather than water and by terminating the reaction with formalin and $FeCl_3$ instead of with HCl. The homogenization change improves the sensitivity, and the termination change facilitates storage. Although the assay has the advantage of providing a quick and simple index of metabolic activity, it is complicated by the sensitivity of the enzyme system to environmental changes. This necessitates careful handling of the homogenates. They must be used quickly and can not be subjected to heating or freezing without a loss of activity. However, these precautions do not cause serious inconvenience.

The assay is very sensitive and has been used to detect ETS activity in the plankton at 5000 m in the eastern tropical Pacific Ocean (Packard et al. 1971). Menzel (1967) has reported the occurrence of 5.10 μg/L of particulate organic carbon at this depth in the same region.

The rates of nitrate reduction and sulfate reduction in anoxic waters as well as the oxygen utilization rate in oxygenated waters may be estimated by an ETS assay. In anaerobic organisms, the nitrate, sulfate, or carbon dioxide replaces oxygen as the terminal oxidizing agent. The use of these oxidizers requires different terminal enzymes of the ETS. For oxygen the enzyme is cytochrome oxidase, for nitrate and nitrite it is nitrate reductase (EC 1.9.6.1) and nitrite reductase, respectively, and for sulfate it is adenosine-5-phosphosulfate reductase (Smith 1968). In spite of the differences in the terminal enzymes, a measurement of the preceding ETS activity should indicate the potential rate of each of these processes.

Respiratory processes are not the only ones amenable to study by enzyme techniques. Phytoplankton nitrogen assimilation (whether it be by nitrogen fixation or by nitrate, nitrite, or ammonia uptake) may be estimated from the activity of glutamate dehydrogenase (EC 1.4.1.2). Zooplankton ammonia excretion may be estimated by glutaminase activity (EC 3.5.1.2), photosynthetic carbon fixation by diphosphoribulose carboxylase activity (EC 4.1.1.39), and photosynthetic electron transport by ferredoxin-NADP oxidoreductase activity.

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On the Circulation of the Intermediate Water in the Southwestern Atlantic Ocean[1]

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ABSTRACT

In early studies of the Antarctic Intermediate Water in the South Atlantic it was assumed that the flow was mostly thermohaline and meridional. However, there is evidence that the wind-driven subtropical anticyclonic circulation in the South Atlantic Ocean extends to and includes the Intermediate Water, as in other oceans.

Introduction. Wüst (1935) has described the formation and flow of the Antarctic Intermediate Water, the North Atlantic Deep Water, and the Bottom Water in the Atlantic Ocean by means of the "core layer"; he defined the movement of these bodies of water as a thermohaline circulation with a strong meridional sense of movement, assuming that the wind-driven subtropical circulation extends only to a shallower depth. Since then, other investigators have suggested that, as in the other oceans, the anticyclonic gyre extends to greater depths, embracing the Intermediate Water. The purpose of the present work is to examine, with more data than Wüst had and by means of isopycnal analysis and relative geostrophy, the flow of the Intermediate Water in the southwestern Atlantic.

Method. The method chosen has been called isopycnal or isentropic analysis. Depth, salinity, potential temperature, and oxygen have been examined along surfaces (or strata) defined by a density parameter. The method was first used by Parr (1938) and subsequently by Montgomery (1938), Clowes (1950), Riley (1951), Taft (1963), Reid (1956), Lynn and Reid (1968), and Reid and Lynn (in press). The descriptions and limitations have been discussed by the above-mentioned authors, especially by Montgomery, Lynn and Reid, and Reid and Lynn.

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