Use of Tritiated Substrates in the Study of Heterotrophy in Seawater

F. Azam and O. Holm-Hansen

Institute of Marine Resources, University of California, San Diego; La Jolla, California, USA

Abstract

An improved method is described for the study of heterotrophic utilization of dissolved organic substances by marine microorganisms. The method is based on the use of 3H-labelled organic substrates of very high specific activity, rather than the conventionally used 14C-labelled substrates. Direct measurement of the rate of tracer uptake at near ambient concentration can thus be made instead of extrapolation using the Michaelis-Menten equation. The method also permits comparison between the rates of tracer uptake in sub-samples exposed to different physico-chemical conditions (temperature, light, pollutants, etc.) without the necessity of determining the ambient substrate concentration. The method was applied to the determination of D-glucose uptake by nearshore and pelagic natural microbial populations, and was found to be sensitive and convenient.

Introduction

A major difficulty in measuring heterotrophic activity in aquatic environments is that the concentrations of the utilizable organic compounds are generally very low, often in the order of $10^{-9}$ to $10^{-8}$ M. Analytical procedures are rarely sensitive enough to determine such low concentrations. Also, the conventionally used 14C-labelled tracers have to be added in relatively large amounts due to their low specific activity (10 to 100 mCi/mM). An extrapolation of the observed rate to the rate at the ambient concentration is, therefore, necessary, and a direct measurement of the rate of uptake is not possible.

Parsons and Strickland (1962) and Wright and Hobbie (1966) applied the principles of enzyme catalysis as a basis for such extrapolation in the study of the uptake of organic substances by mixed populations. This approach greatly stimulated research in the field, and is the basis of the most commonly used method (Wright and Hobbie, 1966). Conceptually, a mixed population is treated as a finite number of equivalent responsive metabolic sites conforming to Michaelis-Menten-type saturation kinetics, with a half-saturation constant $K$ and a maximum rate of reaction $V$ (maximum rate of uptake in the case of a mixed population). Kinetically, the rate of uptake $v$ at the ambient concentration $S$ is given by

$$v = V S/(K + S).$$

If 14C-labelled tracer is added at a concentration $A$, then

$$v = V(S + A)/(K + S + A).$$

If a fraction $f$ of the added tracer is taken up in time $t$, then

$$v = f/t \times (S + A),$$

or

$$t/f = A/V + (K + S)/V.\quad (2)$$

In this method, increasing concentrations of 14C-labelled substrate are added to several sub-samples and the rate of tracer uptake, $f/t$, at each substrate concentration is determined. Plotting $t/f$ versus $A$, the value of $V$ and, more importantly, the “turnover time” $S/v$ are calculated. If $S$ is known, then $v$ can also be calculated.

Validity of the use of the Michaelis-Menten equation to describe the behavior of a mixed population has never been established. In fact, several authors have found that the natural oceanic populations exhibit marked departure from the behavior predicted by the equation. Thus, Vaccaro (1969) found that the “incidence of uninterpretable kinetic pattern is excessively high” and that “interpretable kinetic responses appear to be confined to occasional samples collected in eutrophic waters...”. Williams (1973) used a computer simulated model of mixed populations, and concluded that the error in estimating the turnover time from the kinetic equation under unfavorable circumstances (large range of $K$ values) could be two-fold to three-fold. A direct determination of the tracer uptake is, therefore, highly desirable.

Tritium-labelled organic compounds with high specific activity ($> 10$ Ci/mM) are available, such that the addition of sufficient radioactivity would not significantly alter the ambient substrate concentration. This paper describes a method based on the use of high specific-activity tritiated substrates. While the method should be applicable to a variety of organic substrates, D-glucose-6-3H was chosen to demonstrate the feasibility and potential of the method.
Theoretical Considerations

If the concentration of the added tracer, \( A \), is negligible compared with \( S \), then, from Eq. (2) above,
\[
v = \frac{1}{t} \times S; \quad S/v = t/f.
\]
\( t/f \) being a directly measurable quantity, the turnover time \( S/v \) can be determined without the use of the kinetic equation. If \( S \) is known, then the rate of uptake \( v \) can also be determined.

Also, if \( S \) is a constant (as in the case of sub-samples from the same sample) then
\[
S/v_1 = \frac{t_1}{f_1}; \quad S/v_2 = \frac{t_2}{f_2}, \quad \text{or} \quad \frac{f_1}{t_1} = \frac{v_1}{v_2}, \quad \text{keeping} \quad t \text{ constant.}
\]
Thus, the method also permits a direct comparison between the rates of uptake by several sub-samples exposed to different physicochemical environments (light, temperature, pollutants, etc.). This comparison between the rates of uptake can be made even though the ambient concentration and the rates of uptake are not known.

Materials and Methods

Radiochemical

D-Glucose-6-\(^{3}\text{H}\) (specific activity 8.57 Ci/mM, in 90% ethanol) was purchased from New England Nuclear, Boston, Mass., USA. Ethanol was removed by vacuum evaporation and 10 \( \mu \)Ci/ml solutions were made in artificial seawater (25 g NaCl + 8 g MgSO\(_4\)/l). The solutions were standardized by radioassay of 0.1 ml of 1:10 dilution. Aliquots sufficient for 1 day's work were lyophilized in order to remove any tritiated water formed due to isotope exchange. The lyophilized samples were stored below 0 °C, and were reconstituted with glass-distilled water just before use.

Radiochemicals at such high specific activities are liable to undergo significant decomposition unless protective measures are taken. For tritium-labelled compounds, an effective and convenient method is to use a "diluent" in order to harmlessly dissipate the energy of the \( \beta \)-particles (Amersham-Searle Corp., 1972). One may add "carrier", pure unlabelled compound, or a different substance. Addition of carrier, in this case, would negate the advantage of our method by lowering the specific activity. The choice of artificial seawater, prepared from analytical reagent grade salts, is ideal for use in the study of heterotrophy. The artificial seawater was irradiated with ultra-violet light (Armstrong et al., 1966) to destroy any organic impurities before making the solutions.

Procedure for \(^{3}\text{H}-\text{Glucose Uptake}\)

Sea-water samples were collected in polyvinyl chloride (PVC) Niskin bottles. Coarse detritus and larger plankton were removed by prefiltration through a 183 \( \mu \)m mesh net. To 100 ml aliquots in 125 ml BOD (biological oxygen demand) bottles, 0.5 \( \mu \)Ci (about 0.01 \( \mu \)g) D-glucose was added with an automatic pipette. Control samples were prepared by adding formalin to a concentration of 5% before the addition of \(^{3}\text{H}-\text{glucose}\). Test and control samples in duplicate were incubated at simulated \textit{in situ} temperature (± 2 °C) and light intensity for periods of 30 min to 4 h; they were then filtered through glass-fiber filters (984 H, Reeve Angle) or membrane filters (0.45 \( \mu \)m pore size, Millipore Corp.) under mild vacuum (maximum pressure differential was 190 mm Hg). The material on the filter was washed thoroughly with about 20 ml iced seawater which had been prefiltered through a glass-fiber filter. Iced seawater was used in order to minimize the loss of labelled intracellular pool material during washing. For the same reason, the test samples were not "killed" with formalin prior to filtration. The washed samples were radioassayed as described below.

Radioassay

The wet filter, along with the cells filtered onto it, was transferred into a scintillation vial containing 10 ml of the scintillator [6 g 2,5-diphenyloxazole (PPO) in 11 of 1:1 \((\nu/\nu)\) toluene-triton-X-100]. The samples were allowed to remain in the scintillator for at least 12 h before they were radioassayed, in order to ensure maximum dispersion and solubilization of the radioactive material. The samples were radioassayed in a Beckman LS 100C Liquid Scintillation System (Beckman Instruments, Inc., Fullerton, California, USA) using the \(^{3}\text{H}+{\text{H}}^\text{4}\) isoset. The efficiency of scintillation counting was determined by using a \(^{3}\text{H}\)-toluene internal standard, and was found to be 27%, for the least quenched standard in the cocktail used. Quenching due to the wet filter and the sample, in different samples, was found to be 4.1 to 6.3%. This was determined by first counting the samples in the scintillation counter and then adding a known amount of \(^{3}\text{H}\)-toluene and counting again. The percent recovery of the disintegrations/min (dpm), as the increase in the counts/min (cpm), was used to correct the counts in the samples for the quenching.

Uptake after Size Fractionation

Sea-water samples were prefiltered through 183 \( \mu \)m mesh net. One aliquot was used to determine the rate of tracer uptake, as described. Another aliquot was filtered through a membrane filter (3 \( \mu \)m pore size, Millipore Corp.) and the rate of tracer uptake by the filterable population was determined under identical conditions.

Adenosine Triphosphate (ATP) and Chlorophyll

Adenosine triphosphate (ATP) was determined using the firefly luminescence method (Holm-Hansen