

## Radioactive contamination of the marine environment: Uptake and distribution of $^3\text{H}$ in *Dunaliella bioculata*

S. Strack\*, S. Bonotto & R. Kirchmann

Department of Radiobiology, C.E.N.-S.C.K., 2400 Mol, Belgium

**ABSTRACT:** The marine flagellate *Dunaliella bioculata*, which is easily cultivated under laboratory conditions, is a suitable organism for assessing the importance of the radioactive contamination by  $^3\text{H}$  bound to organic molecules. We have studied the uptake of the following tritiated precursors: thymidine-methyl- $^3\text{H}$ , adenine-2- $^3\text{H}$ , uridine-5- $^3\text{H}$ , l-leucine-4- $^3\text{H}$ , glycine-2- $^3\text{H}$ , l-arginine-3-4- $^3\text{H}$ , l-aspartic acid-2-3- $^3\text{H}$ , l-phenylalanine-2,3- $^3\text{H}$ , D-glucose-2- $^3\text{H}$  and D-glucose-6- $^3\text{H}$ . Under the experimental conditions (2000 lux; incubation time 30 min), all tritiated molecules are taken up by *D. bioculata*. Their intracellular concentration may reach that of the external medium. However, leucine and adenine accumulate in the algae: their respective concentrations are 10 and 100 times higher than in the culture medium. The molecular distribution of  $^3\text{H}$  has been studied by various biochemical techniques and by sieve chromatography on sepharose 4B. It has been found that more l-leucine-4- $^3\text{H}$  is incorporated into acid and acetone soluble substances than into proteins. Adenine-2- $^3\text{H}$  is mainly incorporated into macromolecules of biological significance (RNA, DNA). CsCl gradient centrifugation has shown that the total DNA of *Dunaliella* is constituted by a major ( $\rho = 1.707 \text{ g/cm}^3$ ) and by a minor ( $\rho = 1.693 \text{ g/cm}^3$ ) component.

### INTRODUCTION

Tritium from nuclear facilities is released into the environment mainly as tritiated water (HTO). Most  $^3\text{H}$  is finally dispersed into the oceans, but a significant amount may enter living organisms; it is incorporated into organic molecules by isotopic exchange with hydrogen or by photosynthetic processes in aquatic and terrestrial plants.

Tritium bound in organic compounds behaves differently from that present in HTO (Strack, 1978). The fate of organically bound  $^3\text{H}$  will largely depend on the chemical form of the "carrier" molecule. Organic tritium may present a risk to man, because it could be transported along the food chains. Moreover, particular tritiated compounds may be preferentially incorporated into radiosensitive cell constituents of living organisms and cause genetic damage. Organic compounds are used not only by animal but also by plant cells. It is well known that planktonic algae can utilize dissolved organic compounds (Fogg, 1972; Neilson & Lewin, 1974; Darley et al., 1979). Freshwater algae may accumulate organic tritium present in nuclear effluents (Kirchmann et al., 1977a).

Since the amount of tritiated water released into the aquatic systems is rapidly increasing and because the ocean may become a disposal area for large amounts of tritium originating from inland as well as coastal reprocessing plants (Bonka, 1979),

\*Fellow of the Commission of the European Communities

investigations on the fate of  $^3\text{H}$  in marine organisms are urgently needed. This work is an attempt to gain more insight into the radioecological behaviour of organically bound tritium. Data obtained under controlled laboratory conditions may be used in connection with those from field experiments for developing mathematical models on the biological behaviour of tritium in the marine environment.

## MATERIAL AND METHODS

### Culture of algae

The planktonic marine alga *Dunaliella bioculata* (Fig. 1), a wall-less flagellate, was cultivated in Miquel's sea-water medium (Izard & Testa, 1968) in 250-ml Erlenmeyers at 20 °C under continuous light (2000 lux from 40 W Phytotubes, ACEC). Cell density and

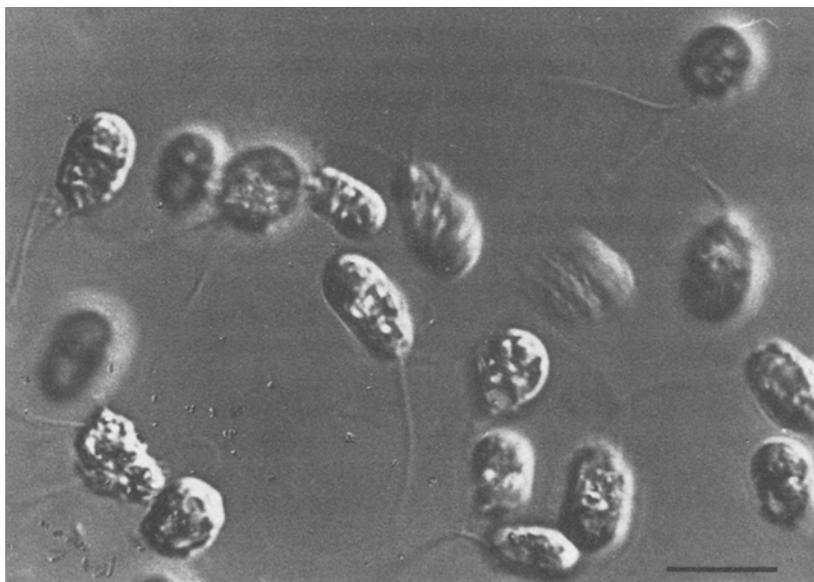


Fig. 1. *Dunaliella bioculata* cells showing the two flagella (Nomarski interference optics). Scale = 10  $\mu\text{m}$

volume ( $1.2 \times 10^{-10}$  ml/cell) were determined with a Coulter Counter (Model ZF). The algal cultures were routinely checked for bacterial contamination by plating on Zobell's solid medium for marine bacteria.

### Incorporation of tritiated molecules

Tritiated molecules, obtained from CIS Association, were added to 8-day-old cultures with about  $2 \times 10^6$  cells/ml. In initial experiments, the uptake was stopped by adding 1% formalin. The suspension was then rapidly centrifuged and the pellet washed with ice-cold culture medium. TCA-soluble and insoluble radioactive sub-

stances, collected on glass-fibre filters, were measured in a liquid-scintillation spectrometer using internal standards for quenching correction (Bonotto & Kirchmann, 1971). The kinetics of  $^3\text{H}$ -adenine uptake was studied by centrifuging the cells into a layer of dibutylphthalate (Eilam & Stein, 1974) after addition of an excess of the unlabeled precursor. The intracellular concentration of radioactivity was calculated on the basis of the weight of packed cells, pelleted by centrifugation ( $230 \mu\text{g}/10^6$  cells).

### Molecular sieving chromatography

A crude cell lysate of *Dunaliella bioculata* was subjected to molecular sieving on a Sepharose 4B column ( $40 \times 2.5$ ) and eluted with  $0.1 \times \text{SSC}$  ( $0.015 \text{ M NaCl}$ ,  $0.0015 \text{ M Na citrate}$ ). Such sieving was successfully used for the purification of DNA extracted using a modified Marmur technique (Marmur, 1961). The cell lysate, obtained with 2% SDS, was extracted with ethanol and treated with proteinase K and RNAase before being sieved. The DNA was eluted with  $2 \text{ M NaCl}$  at room temperature. Absorbance was monitored with an Isco Model UA2 apparatus connected to a Kipp & Zonen BD9 recorder and to an Isco fraction collector model 568.

### Determination of buoyant density of DNA

Buoyant density of DNA was determined by CsCl density gradient centrifugation (33,000 rpm, 65 h,  $20^\circ\text{C}$ ). The slope of the gradients was determined graphically by using *Clostridium perfringens* DNA ( $\rho = 1.691 \text{ g/cm}^3$ ) and phage 2C DNA ( $\rho = 1.742 \text{ g/cm}^3$ ) as density markers.

Abbreviations: RNAase = ribonuclease, SDS = sodiumdodecylsulfate; SSC =  $0.15 \text{ M NaCl} + 0.015 \text{ M Na citrate}$ ; TCA = trichloroacetic acid.

## RESULTS

### Comparison of uptake and incorporation of various tritiated organic compounds

Various tritiated organic compounds were supplied singly to 8-day-old cultures of *Dunaliella bioculata* (about  $2 \times 10^6$  cells/ml) for 30 min. Their final concentration in the culture medium ranged between 0.8 and  $2.2 \times 10^{-7} \text{ M}$  except that of glucose-1- $^3\text{H}$  ( $1.1 \times 10^{-6} \text{ M}$ ). The results obtained are reported in Table 1, which gives the concentrations of  $^3\text{H}$  in the medium and in the algae as well as the concentration ratios. The concentration ratios for total activity (fourth column) show that: (a) the intracellular concentration of most of the tritiated compounds tested approaches that of the external medium, being slightly higher for uridine-5- $^3\text{H}$  and lower for arginine-3.4- $^3\text{H}$ , phenylalanine-2.3- $^3\text{H}$  and glucose-1- $^3\text{H}$ ; (b) the respective intracellular concentrations of leucine-4- $^3\text{H}$  and of adenine-2- $^3\text{H}$  are about 10 and 100 times higher than in the external medium. A comparison of the figures of column 2 (total activity) with those of column 3 (soluble activity) reveals that, after 30 min of incubation, significant amounts of the radioactivity are present in the soluble "pool". The figures also show that all the used substances become incorporated into the TCA-insoluble fraction of the cells.

Table 1. *Dunaliella bioculata*. Uptake and incorporation of various  $^3\text{H}$  labeled organic compounds

Compound	Activity medium ( $\mu\text{Ci/ml}$ )	Intracellular activity		Concentration ratios	
		total ( $\mu\text{Ci/g}$ fresh weight)	TCA-soluble	total	TCA-soluble
Thymidine-methyl- $^3\text{H}$	5.64	4.75	3.84	0.84	0.68
Adenine-2- $^3\text{H}$	1.30	159.57	138.79	122.75	106.76
Uridine-5- $^3\text{H}$	3.50	7.15	3.07	2.04	0.88
L-leucine-4- $^3\text{H}$	2.84	32.50	18.56	11.44	6.53
Glycine-2- $^3\text{H}$	3.60	4.25	3.79	1.18	1.05
L-arginine-3,4- $^3\text{H}$	1.60	1.10	0.65	0.68	0.41
L-aspartic acid-2,3- $^3\text{H}$	2.04	2.20	2.14	1.08	1.05
L-phenylalanine-2,3- $^3\text{H}$	3.68	1.89	1.62	0.51	0.44
D-glucose-1- $^3\text{H}$	4.14	1.71	1.44	0.41	0.35
D-glucose-6- $^3\text{H}$	0.83	0.82	0.77	0.99	0.93

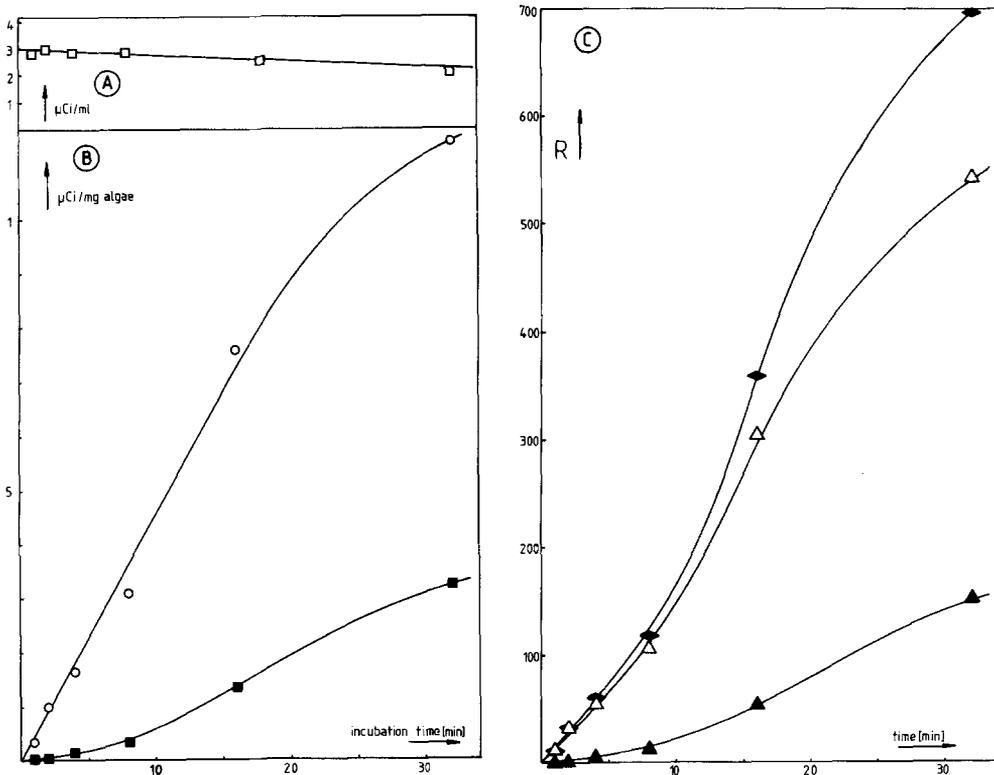


Fig. 2. *Dunaliella bioculata*. Time course of  $^3\text{H}$ -adenine uptake. (A) decrease of  $^3\text{H}$ -adenine concentration in the external medium; (B) kinetics of  $^3\text{H}$ -adenine uptake in the TCA-soluble (○) and in the TCA-insoluble (■) fraction; (C) concentration ratio (intracellular/external  $^3\text{H}$  concentration) for TCA-soluble (Δ), TCA-insoluble (▲) and total (◆) activity in the cells. The initial  $^3\text{H}$ -adenine concentration in the medium was  $10^{-7}\text{M}$ . The algae (end of exponential growth phase) were incubated in the light (2000 lux) at  $22^\circ\text{C}$

## Time course of adenine uptake

On account of the observed accumulation of  $^3\text{H}$ -adenine, its uptake has been studied as a function of the incubation time. For this experiment, cultures of *Dunaliella bioculata* in a late exponential growth phase were utilized. We observed: (a) a decrease of adenine concentration in the external medium (Fig. 2A); (b) a rapid linear increase of free adenine (TCA-soluble) concentration in the cells during the first 15 min followed by reduced uptake, suggesting that the intracellular pool approaches the saturation level (Fig. 2B); (c) a slow increase of the incorporated adenine (TCA-insoluble), showing a lag period in the first minute and a beginning of saturation after 15 min (Fig. 2B, lower curve); (d) a sigmoid course of the concentration ratios (Fig. 2C), which in this particular experiment reached a very high value (700 for total activity) after 32 min of incubation.

## Adenine uptake as a function of the concentration in the external medium

*Dunaliella bioculata* cultures were incubated in media containing increasing concentrations of adenine (from  $10^{-7}$  to  $10^{-4}\text{M}$ ). The incubation time was fixed to 5 min to ensure that initial uptake velocities were measured. The hyperbolic shape of the fitted curve (Fig. 3) suggests an uptake process obeying Michaelis-Menten kinetics. From the data of Figure 3, we calculated the Michaelis-Menten constant ( $K_m = 2.87 \pm 0.72 \mu\text{M}$ ), which gives the substrate concentration for half maximal initial uptake rate. Moreover, the maximal uptake rate ( $V_{\max} = 3.83 \pm 0.23 \text{ pM} \times \text{mg}^{-1} \text{ dry matter} \times \text{min}^{-1}$ ) was calculated directly by non-linear regression analysis of the observed data.

Figure 4 shows a Hofstee plot of the data. Theoretically (Neame & Richards, 1972) a linear regression in this plot would signify a carrier-mediated transport mechanism.

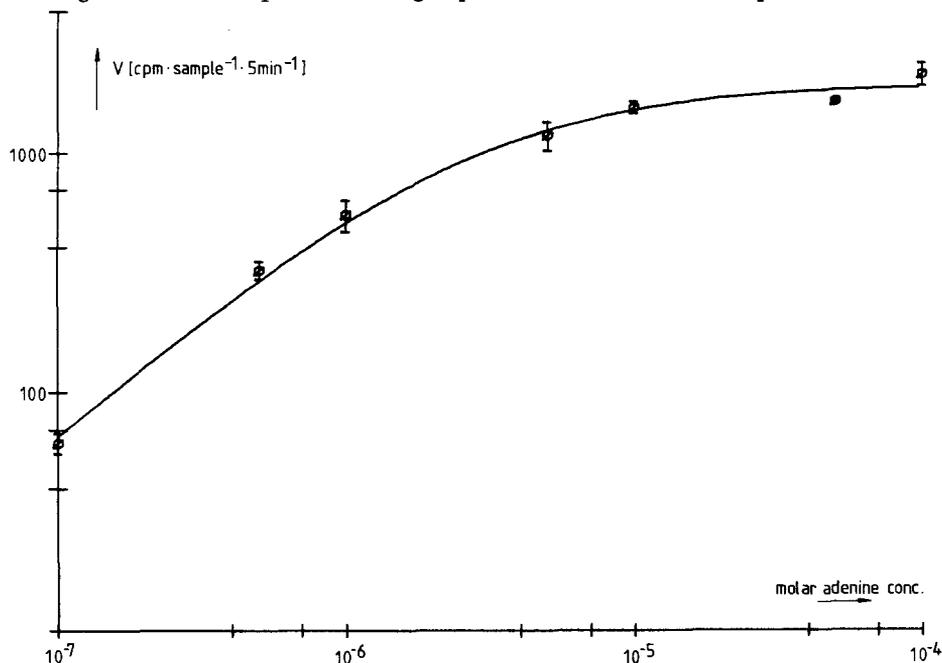


Fig. 3. *Dunaliella bioculata*.  $^3\text{H}$ -adenine uptake as a function of the concentration in the external medium (graph obtained by computer analysis). Incubation time: 5 min

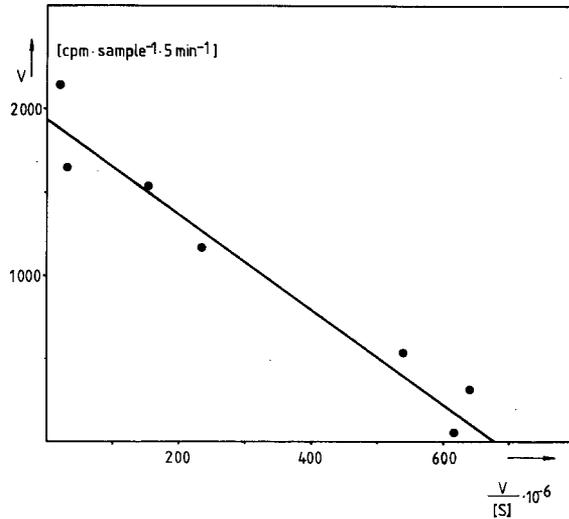


Fig. 4. Hofstee plot of <sup>3</sup>H-adenine uptake as a function of its concentration in the medium

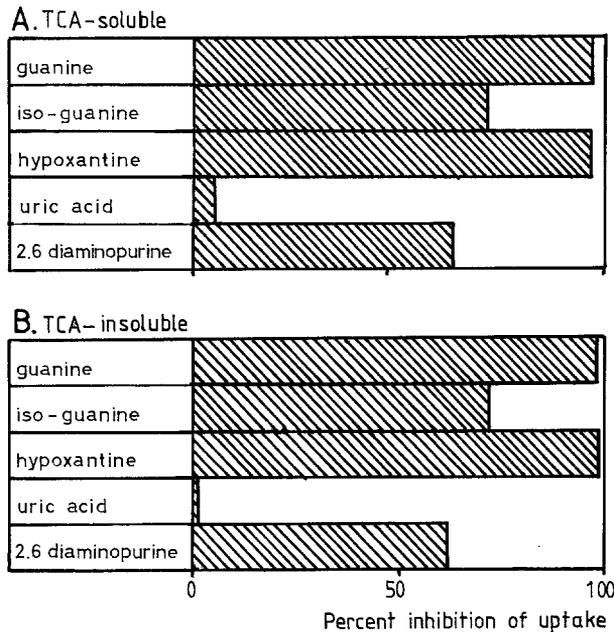


Fig. 5. *Dunaliella bioculata*. Effects of various adenine analogues on the uptake (A) and incorporation (B) of <sup>3</sup>H-adenine. Average data of three independent experiments

### Competitive experiments with adenine analogues

In order to demonstrate substrate specificity for uptake in *Dunaliella bioculata*, which would support the existence of a carrier-mediated transport mechanism, several purine derivatives (guanine, iso-guanine, hypoxanthine, uric acid and 2.6

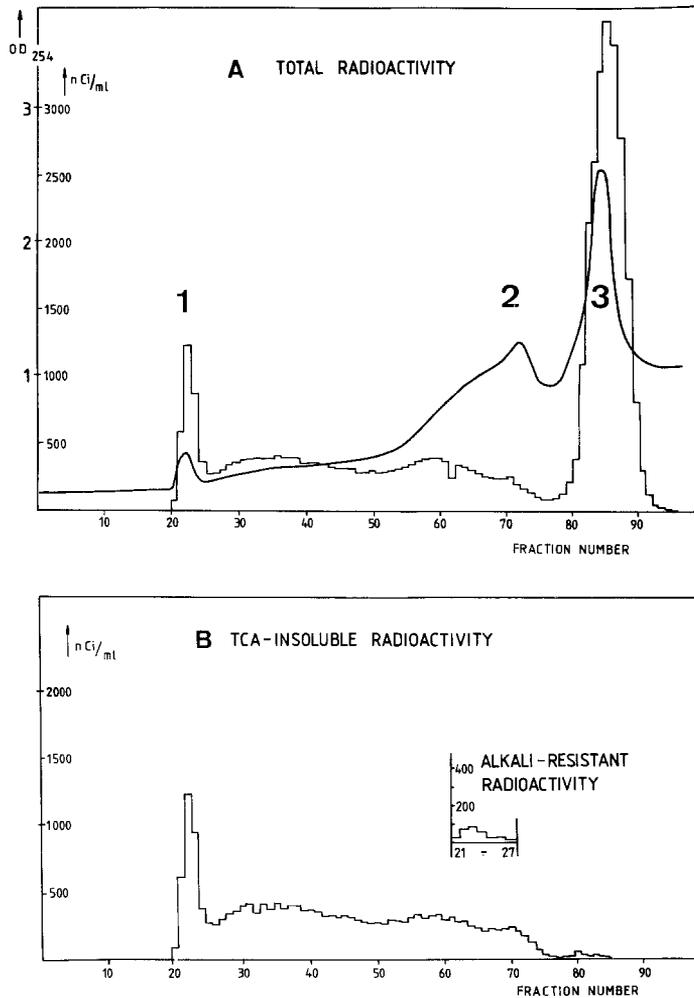
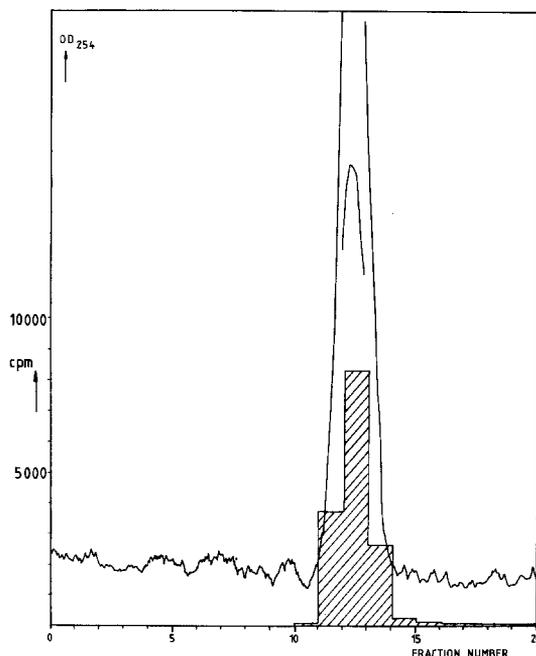


Fig. 6. *Dunaliella bioculata*. Molecular sieve chromatography on sepharose 4B (column:  $2.5 \times 40$  cm) of a crude lysate of cells, prelabeled 2 hours with  $^3\text{H}$ -adenine ( $10^{-7}\text{M}$ ). Elution with  $0.1 \times \text{SSC}$ . (A) Total radioactivity; (B) TCA-insoluble and alkali-resistant (insert) radioactivity

diaminopurine) were utilized in combination with  $^3\text{H}$ -adenine. The concentration of the analogues was about 1000 times higher ( $1.25 \times 10^{-4}\text{M}$ ) than that of  $^3\text{H}$ -adenine ( $10^{-7}\text{M}$ ). The results (Fig. 5A, B) show that: (a) guanine and hypoxanthine almost completely inhibit  $^3\text{H}$ -adenine uptake; (b) uric acid shows almost no effect on the uptake of  $^3\text{H}$ -adenine; (c) iso-guanine and 2,6 diaminopurine cause 70 % and 62 % inhibition of  $^3\text{H}$ -adenine uptake, respectively; (d) the incorporation of  $^3\text{H}$ -adenine seems directly dependent on the uptake mechanism; in fact, both uptake and incorporation are equally affected by the analogues. These results support the existence of a specific transport mechanism with an affinity for adenine and its structural analogues guanine and hypoxanthine.

Table 2. *Dunaliella bioculata*. Distribution of  $^3\text{H}$  into three different fractions, after 30 min incubation with tritiated leucine

Compound	pCi/ml	%
TCA-soluble fraction	4359	67
Acetone-soluble fraction	1899	29
TCA-insoluble fraction	260	4
Total	6518	100

Fig. 7. *Dunaliella bioculata*. Molecular sieve chromatography of DNA, labeled 2 hours with  $^3\text{H}$ -adenine. Elution with 2 M NaCl

### Molecular distribution of $^3\text{H}$ -adenine

On account of the large amounts of  $^3\text{H}$ -adenine taken up by *Dunaliella bioculata*, it was of interest to know in which cell constituents this tritiated molecule becomes incorporated. Molecular sieving of a  $^3\text{H}$ -adenine labeled cell lysate revealed a considerable TCA-soluble pool (Fig. 6A, peak 3). However, an appreciable amount of radioactivity was also present in fractions where nucleic acids and proteins are eluted (Fig. 6A, peaks 1 and 2). The presence of alkali-resistant radioactivity in peak 1 (Fig. 6B) suggested that  $^3\text{H}$  was bound to DNA. To confirm the hypothesis that  $^3\text{H}$ -adenine may serve as a precursor for the biosynthesis of the genetic material of *Dunaliella*, the DNA was purified from proteins and RNA by enzymatic treatments (proteinase K, RNAase) and by molecular sieving (Fig. 7). Figure 7 shows that the DNA has become heavily labeled. CsCl density gradient centrifugation of the DNA in the presence of density markers

demonstrated the presence of a main component and of a satellite band with respective buoyant densities of 1.707 and 1.693 g/cm<sup>3</sup> (Fig. 8, top). The asymmetry of the corresponding radioactivity peak (Fig. 8, bottom) may be taken as an indication that both DNA are labeled.

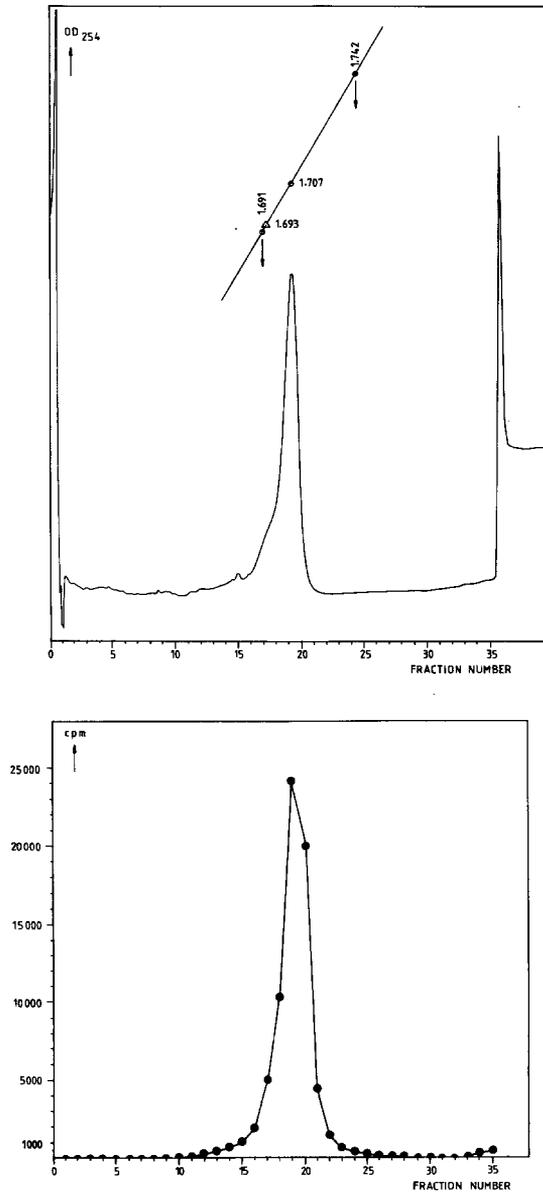


Fig. 8. CsCl density gradient centrifugation of DNA extracted from *Dunaliella bioculata* and purified by enzymatic treatment (proteinase K, RNAase) and by molecular sieving. Absorbance at 254 nm of main ( $\rho = 1.707$  g/cm<sup>3</sup>) and of satellite component ( $\rho = 1.693$  g/cm<sup>3</sup>); the arrows indicate the position of density markers

Molecular distribution of  $^3\text{H}$ -leucine

Preliminary experiments have shown that after an incubation period of 30 min,  $^3\text{H}$ -leucine is preferentially incorporated into acetone-soluble substances of *Dunaliella bioculata* rather than into proteins (Table 2). The nature of the labeled substances has not yet been investigated.

## DISCUSSION

The results reported in this paper clearly show that *Dunaliella bioculata*, like other planktonic algae (Fogg, 1972; Neilson & Lewin, 1974; Darley et al., 1979), is able to utilize various dissolved tritiated compounds. This heterotrophic potential may be of ecological significance. *Dunaliella* cells may use exogenous organic substances for satisfying part of their carbon and nitrogen requirements. However, our results show that in *Dunaliella* the uptake system has a low affinity for the tested organic substrates, except for  $^3\text{H}$ -leucine and  $^3\text{H}$ -adenine. Since *Dunaliella* species synthesize and release glycerol (Beardall et al., 1976), it is possible that particular dissolved organic substances are used for this biosynthetic pathway. Experiments with  $^3\text{H}$ -leucine seem to support this idea as most of the incorporated radioactivity is present in acidic or acetonic fractions (see Table 2), where labeled glycerol would be dissolved. Only 4 % of  $^3\text{H}$ -leucine was incorporated into acid insoluble substances, probably proteins.

Table 1 shows that *Dunaliella bioculata* is able to concentrate  $^3\text{H}$ -adenine even when its concentration in the external medium is rather low ( $10^{-7}\text{M}$ ). Experiments on  $^3\text{H}$ -adenine uptake as a function of time and of its concentration in the external medium suggest that this organic molecule penetrates the cells by a carrier-mediated transport mechanism. Results not reported in this paper have shown that a similar amount of  $^3\text{H}$ -adenine was taken up by *Dunaliella* under light or under dark conditions.

Competition experiments with adenine analogues revealed that *Dunaliella* possesses a specific transport mechanism which has a high affinity for adenine and its structural analogues guanine and hypoxanthine.

Analysis of the molecular distribution of  $^3\text{H}$ -adenine in *Dunaliella* shows that it may be a good precursor for the synthesis of macromolecules of important biological significance (RNA, DNA). Molecular sieving and CsCl gradient centrifugation demonstrate that  $^3\text{H}$  is incorporated into the genetic material of the cells. The total DNA extracted from the algae is comprised of a major ( $\rho = 1.707 \text{ g/cm}^3$ ) and a minor ( $\rho = 1.693 \text{ g/cm}^3$ ) component. The major component probably originates from the nucleus, whereas the minor could be of plastidial origin. It is known, in fact, that *Dunaliella* cells contain only one chloroplast. Satellite DNAs have also been found in other planktonic microalgae (see Charles, 1977).

Apart from theoretical and ecological considerations, the results reported in this paper are of interest for a better understanding of the radioecological behaviour of organically bound tritium in the marine environment. The utilization of an experimental food chain (Kirchmann et al., 1977b) to study the transfer of organic tritium may allow evaluation of the possible risk to man.

*Acknowledgements.* We thank Mr. A. Bossus and Mr. G. Nuyts for assistance, Mrs. I. Nolzen for preparing the graphs and Mrs. J. Romeyer-Luyten for typing the text. We are grateful to Dr. G. Gerber for computer analysis, to Dr. A. Lüttke for fruitful discussions and to Professor S. Puiseux-

Dao who kindly provided *Dunaliella bioculata*. The financial support of the Commission of the European Communities to one for us (S.S.) is acknowledged.

## LITERATURE CITED

- Beardall, T., Mukerji, D., Glover, H. E. & Morris, I., 1976. The path of carbon in photosynthesis by marine phytoplankton. – J. Phycol. 12, 409–417.
- Bonka, H., 1979. Production and emission of tritium from nuclear facilities and the resulting problems. In: Behaviour of tritium in the environment. IAEA, Vienna, 105–122 (IAEA-SM-232).
- Bonotto, S. & Kirchmann, R., 1971. Effets des radiations gamma sur la synthèse des acides nucléiques et des protéines chez *Acetabularia mediterranea*. – Bull. Soc. r. Bot. Belg. 104, 125–135.
- Charles, D., 1977. Isolation and characterization of DNA from unicellular algae. – Plant Sci. Lett. 8, 35–44.
- Darley, M. W., Ohlmann, C. T. & Wimpee, B. B., 1979. Utilization of dissolved organic carbon by natural populations of epibenthic salt marsh diatoms. – J. Phycol. 15, 1–5.
- Eilam, Y. & Stein, W. O., 1974. Kinetic studies of transport across red blood cell membranes. In: Methods in membrane biology. Ed. by E. D. Korn. Plenum Press, New York 2, 283–354.
- Fogg, G. E., 1972. Organic substances plants. In: Marine ecology. Ed. by O. Kinne. Wiley-Interscience, London 1 (3), 1551–1563.
- Izard, C. & Testa, P., 1968. Recherches sur les effets de la fumée de cigarette sur la motilité de *Dunaliella bioculata*. – Anns S. G. I. T. A. 6, 121–156.
- Kirchmann, R., Piron, C., Meurice, M., Fagniard, E. & Sironval, C., 1977a. Le tritium des effluents liquides d'installations nucléaires. – Anns Ass. Belge Radiopr. 2, 163–182.
- Kirchmann, R., Bonotto, S., Bossus, A., Nuyts, G., Declerck, R. & Cantillon, G., 1977b. Utilisation d'une chaîne trophique expérimentale pour l'étude du transfert du  $^{60}\text{Co}$ . – Revue int. océanogr. méd. 48, 117–124.
- Marmur, J., 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. – J. molec. Biol. 3, 208–218.
- Naeme, K. O. & Richards, T. G., 1972. Elementary kinetics of membrane carrier transport. Blackwell, Oxford, 144 pp.
- Neilson, A. H. & Lewin, R. A., 1974. The uptake and utilization of organic carbon by algae: an assay in comparative biochemistry. – Phycologia 13, 227–264.
- Strack, S., 1978. Biokinetische Untersuchungen über organisch gebundenes Tritium in Algen-Kulturen. Reimer, Berlin, 97 pp.