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EXTRACELLULAR ORGANIC PRODUCTION BY A PICOPLANKTON, *STICHOCOCCUS BACILLARIS*

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Key Word Index—*Stichococcus bacillaris*; Chlorophyceae; picoplankton; glycollic acid; exudates.

Abstract—A marine isolate of the picoplankton *Stichococcus bacillaris* was cultured in an artificial seawater medium, and production of extracellular dissolved organic substances investigated by the ^{14}C-tracer technique. Extracellular ^{14}C production represented up to 10% of ^{14}C incorporation into cell material and fractionation by ion-exchange membrane electrodialysis showed that up to 98% of this material was acidic. In contrast, intracellular material was dominated by neutral components, ionic species representing no more than 34%. The predominant extracellular product was glycollic acid, with negligible amounts of other compounds.

INTRODUCTION

The production of extracellular dissolved organic matter by marine phytoplankton has been of considerable interest in recent years. Much of the earlier work has been reviewed [1], but new studies continue to add to our understanding of the processes of excretion, and the chemical nature of extracellular products. In an earlier study [2], we examined the extra-cellular products of *Hymenomonas carterae*, developing for this purpose a general approach for the analysis of algal exudates. In the present work, this methodology has been extended to laboratory cultures of *Stichococcus bacillaris* Naegeli (Class Chlorophyceae, Order Ulotrichales; see ref. [3]). This species lies at the upper end of the cell size range included under the term 'picoplankton' [4]. While *S. bacillaris* is reported to grow well in either fresh- or seawater media [5] with one authority [6] regarding it as a freshwater organism capable of tolerating high salinity media, the strain we have studied is one of several marine isolates collected recently from the Caribbean Sea. The picoplankton in general are currently attracting considerable attention, especially in terms of their role in the marine ecosystem. The current state of knowledge concerning the picoplankton has recently been reviewed [4, 7, 8], and the present work contributes to this in characterizing extracellular production by *S. bacillaris* under defined laboratory conditions.

RESULTS

The initial objective was to measure total ^{14}C uptake and excretion by *Stichococcus bacillaris* after 4 hr incubations with $NaH^{14}CO_3$ (5.2 μCi per 100 ml cuture). In this context 'excretion' encompasses both 'passive' diffusion or 'active' transport across the cell membrane, whilst 'lysis' will be used to mean leakage from dead or dying cells due to partial or complete failure of the cell membrane. Figure 1 illustrates the data for two relatively dense batch cultures differing only in the size of the inoculum; under these conditions the organism grew at a constant arithmetic rate (Fig. 1a). The representative data expressed in Fig. 1b and c represent amount of radioactivity incorporated and excreted per cell over 4 hr. This is used in preference to amount of radioactivity per unit culture volume since the latter unit does not make allowance for growth or the physiological status of the culture. The total ^{14}C uptake decreased in both cultures (Fig. 1b). In contrast, the total ^{14}C excretion appeared to reach a maximum after 7–13 days of growth before declining (Fig. 1c). The amount of excretion expressed as a percentage of uptake increased from 1.5% after two days to 10.8% after 13 days, then decreased to 3.2% after 20 days (Fig. 2). In continuous culture ($D = 0.0284$ hr^{-1}) at steady state the total rate of $^{14}CO_2$ uptake over 4 hr was 0.007 fCi/hr and the rate of excretion of acid stable products over this period was 0.001 fCi/hr; i.e. 15% of uptake. These rates are comparable to the figures obtained with organisms in the final stage of growth (*ca* 15 days) in batch culture (Fig. 2).

The distributions of ^{14}C in cell material and acid-stable extracellular material from *S. bacillaris* in batch and continuous culture fractionated by electrodialysis are shown in Table 1; these are expressed as percentages of recovered activity. Preliminary investigations of dichloromethane extracts of cell-free medium indicated that the lipid component of ^{14}C-labelled extracellular material was negligible. Consequently lipids were removed from the total cell extracts prior to electrodialysis to enable the most appropriate comparison with extracellular material. The bulk of the ^{14}C in the lipid-free cell material resided in neutral components. In contrast, the ^{14}C recovered in extracellular material was predominantly in the anionic fraction.

During electrodialysis, the neutral fraction of the cell material was sufficiently desalted to allow concentration for 2D-TLC with detection by autoradiography. The autoradiogram showed that the bulk of the ^{14}C had been incorporated into three unidentified carbohydrates all showing chromatographic behaviour similar to that for the hexose, glucose, used as a reference. The remaining

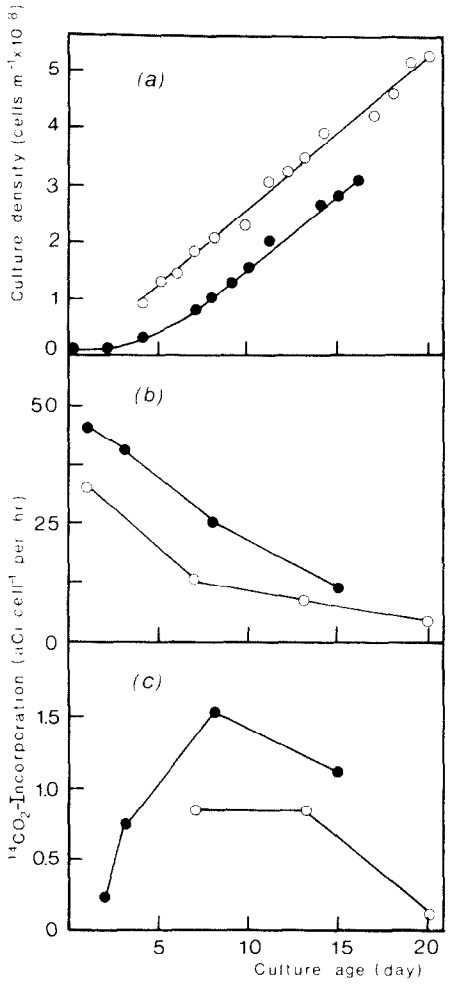

Fig. 1. *Stichococcus bacillaris* in batch culture: (a) cell density of growing cultures used to obtain the data in b and c. Samples were taken at the times indicated from these cultures and incubated for 4 hr with $Na_2{}^{14}CO_3$. Uptake of ^{14}C and its appearance in the exudates was linear over this time. Incorporation into (b) cell material; and (c) acid-stable dissolved extracellular material was determined. Data from two separate experiments are given.

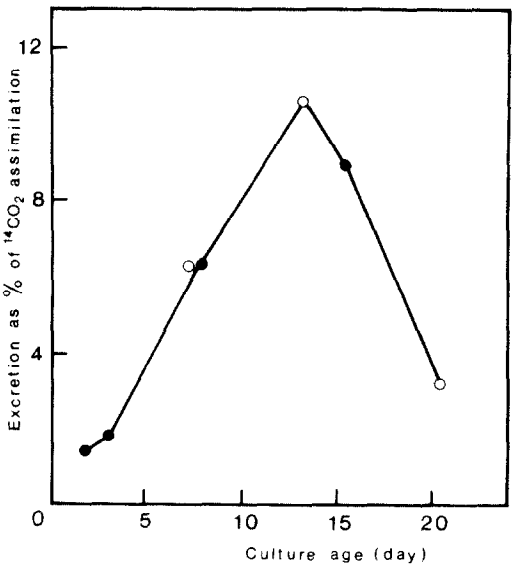

Fig. 2. *Stichococcus bacillaris* in batch culture: ^{14}C incorporation into acid-stable dissolved extracellular material expressed as a percentage of ^{14}C incorporation into cell material. The data for 4 hr incubations from two separate experiments are shown.

activity was distributed between *ca* 20 minor zones, including several amino acids. The most prominent of the latter was an amino acid identified from R_f values as valine. The neutral fraction of the extracellular material was also desalted during electrodialysis, enabling analysis by 2D-TLC. The autoradiogram exhibited nine zones, of which five eluted in the region corresponding to carbohydrates. One of these spots was reactive with a *p*-anisidine/periodate spray specific for sugar alcohols [9] but these compounds were present in insufficient amounts to enable further investigation of this fraction.

Gel filtration on Biogel P-2 (exclusion M_r 1800) was employed as a means of comparing the M_r ranges of the lipid-free cell material, and of the anionic extracellular material (Fig. 3). In both cases the recovered radioactivity was eluted predominantly in a single sharp peak.

Table 1. *Stichococcus bacillaris* in batch and continuous culture: amount of ^{14}C in electrodialysis fractions of cell metabolites and extracellular material after 4 hr incubation expressed as a percentage of recovered radioactivity

	Culture age (days)	Cell density (Cell/ml)	Electrodialysis distributions					
			Extracellular material			Cell material		
			Anions	Neutrals	Cations	Anions	Neutrals	Cations
Batch cultures	7	—	91.9	7.0	1.1	20.0	74.9	5.1
	15	—	87.5	6.8	5.7	25.2	66.6	8.2
Continuous culture	—	6×10^8	98.0	1.7	0.3	17.0	80.5	2.5

—Not determined.

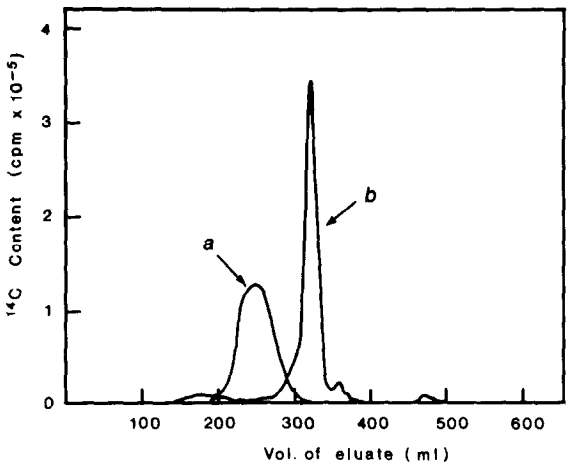

Fig. 3. *Stichococcus bacillaris* in continuous culture: gel filtration profiles of (a) ^{14}C-labelled cell extract after removal of lipid, and (b) ^{14}C-labelled extracellular material, following 4 hr incubation with $NaH^{14}CO_3$.

However, the elution profiles were sufficiently different to suggest that the molecular compositions of the samples were quite different. Following gel filtration and concomitant desalting the anionic extracellular fraction was concentrated for 2D-TLC. All the radioactivity resided in a single zone whose R_f values in the two dimensions were characteristic of glycollic acid. Though proline, tyrosine and alanine have similar chromatographic properties the extracellular product did not show any reaction to ninhydrin. For further confirmation, the sample was chromatographed in one dimension using a *n*-butanol–acetic acid–water system which clearly separates the above four compounds [10, 11]. The sample was spotted both separately and conjointly with unlabelled glycollic acid, with amino acids standards applied separately. The chromatogram was then eluted, and radioactivity located by autoradiography prior to the plate being sprayed with ninhydrin and an aniline/glucose reagent for the detection of carboxylic acids [12]. The four standards were well separated, with R_f values consistent with the literature values. The anionic extracellular material, which was reactive with aniline/glucose reagent, ran well ahead of the amino acids, and its R_f value corresponded to that of glycollic acid. Moreover, the radioactive zone for this material exactly coincided with standard glycollic acid on co-chromatography; this coincidence was also evident following two-dimensional chromatography. Finally, oxygen uptake was evident on addition of glycollate oxidase to the putative glycollic acid in an oxygen monitor. The identity of the anionic material was therefore confirmed as glycollic acid.

The very low level of radioactivity recovered in the cationic fraction of the extracellular material precluded further investigation of its chemical nature.

DISCUSSION

Excretion versus lysis

Various sources of error in the measurement of extracellular production have been discussed previously [2]. In the present work perceived sources of experimental artefacts have been minimised and any remaining would be quantitatively similar in all experiments. The assumption which is most crucial to the following discussion is that following acidification and bubbling any radioactivity found in cell-free medium after exposure of cells to $NaH^{14}CO_3$ is derived from algal cells. We believe that this assumption is justified in view of careful experimental design and inclusion of appropriate controls [see ref. 2].

Clearly, significant cell lysis would result in dissolved organic matter having a chemical composition very similar to that of the intracellular material. In the continuous culture experiment this is clearly not the case: the intracellular material here consisted largely of neutral (mainly carbohydrate) material, while up to 98% of the extracellular material consisted of a single compound having chromatographic properties characteristic of glycollic acid. Such highly specific release could not be explained in terms of cell lysis, but rather points to some selective excretion mechanism. Moreover sorbitol and proline were not present to a significant extent in the extracellular material; these solutes are osmoregulants in *Stichococcus* sp. [13]. The conclusion that the radiolabelled extracellular material observed in the continuous culture of *S. bacillaris* was the product of excretion by that organism rests on this firm foundation. This conclusion also holds for the batch culture experiments where similar differences in electrodialysis distributions of intra- and extracellular material (Table 1) were observed.

Changes in labelling patterns with culture age

Typical data for *S. bacillaris* in batch culture are shown in Fig. 1. The decrease in ^{14}C uptake per cell with culture age (Fig. 1b) is similar to the pattern shown in parallel studies with another phytoplankton *Hymenomonas carterae* [2]. The explanation for the fall in rate is not nutrient ($^{14}CO_3^{2-}$) depletion but light limitation as cells become 'shaded' in the more dense cultures; the total assimilation in the incubations increases with cell numbers at the lowest culture densities but then reaches a plateau.

A peak of extracellular production was observed, expressed both as a percentage of carbon uptake (Fig. 1c) and as amount per cell. This pattern reflects changes in the amount of excretion, rather than in the susceptibility of the cells to lysis during incubation with $^{14}CO_3^{2-}$ or during culture filtration, since cell lysis would be expected to increase in older cultures as the proportion of non-viable cells rises. The observed changes may be attributed to excretion by a number of mechanisms since different compounds may be excreted in varying amounts at different stages during the growth of the culture. For example, accumulation of extracellular carbohydrates in cultures of several microalgae occurs preferentially in the stationary phase [14, 15] and changes in the proportions of extracellular organic acids occur during the growth of cyanobacteria [16]. Alternatively, the change in excretion levels may represent a functional response to unfavourable growth conditions. During the first few days of growth, increasing excretion may reflect elimination from the cells of some intermediate which is accumulating because of 'feed-back' inhibition of a metabolic pathway. At later growth stages, lower fixation of carbon into the initial photosynthetic products might reverse this constraint, and the observed decrease in excretion levels of

the compound would follow. A third possibility is that an increasing tendency towards excretion is off-set by an increasing 'back-potential' as the product accumulates in the medium; this would not be a factor *in situ* in oceanic waters. Detailed chemical characterization of both the intra- and extracellular products at different stages in the development of the culture would be necessary to distinguish between these alternatives.

Comparison with other microalgae

The published data on measurements of excretion by microalgae has been reviewed recently [17]. For cultured algae the excretions reported ranged between 0.3 and 38.0%, expressed as a percentage of fixed carbon; for natural populations excretion was as high as 75%. In recent years it has become generally accepted that for exponentially growing microalgae excretion normally represents 0–5% of carbon fixation, rising to higher values under adverse conditions [18, 19]. The values observed in the present study are consistent with this picture. Results for the diatom *Phaeodactylum tricornutum* (Gottingen strain 1090-1b) under the same conditions give 0.3–0.9% excretion in one- to fourteen-day-old cultures (unpublished data). In contrast, results for the marine coccolithophorid *Hymenomonas carterae*, again cultured under conditions identical to those we employed for *S. bacillaris*, gave up to 64% excretion [2]. Thus excretion levels observed under a given set of circumstances are highly species-dependent, as suggested by comparative studies [see e.g. ref. 20]. Such differences may be related to the precise nature of transport mechanisms but they may also reflect differing susceptibility of different organisms to metabolic pertubation during radiolabelling experiments or, depending on the care taken, to mechanical damage in manipulative procedures; varying degress of cell breakage during filtration of 13 algal strains has been indicated [21].

During our investigations of *S. bacillaris* it became apparent that several features strongly contrasted it with *Hymenomonas carterae*. In continuous culture, at least 98% of the extracellular material produced by *S. bacillaris* consisted of small molecules ($M_r < 1800$), mainly glycollic acid. In contrast, *H. carterae* excreted polysaccharides of $M_r > 1800$, which comprised 20% or more of the extracellular material, together with a variety of small molecules including carbohydrates and amino acids. The electrodialysis distributions reflect this difference, the extracellular material of *H. carterae* being dominated by neutral components, that of *S. bacillaris* by anionic (acidic) components. Such considerable differences in the extracellular products of these two organisms suggest that quite different release processes operate in the two cases. Glycollic acid is a commonly encountered extracellular product of marine microalgae [1], and has been identified as a component of sea water [22, 23]. Factors which have been shown to affect glycollate release include carbon dioxide concentration, culture age and pH [24, 25]. These latter [25] concluded that glycollate release in the freshwater diatom *Navicula pelliculosa* is an artifact of growth on high concentrations of carbon dioxide. Glycollate release by *S. bacillaris* cannot be attributed to this cause as available carbon dioxide was not significantly enhanced over environmental levels. The extent of glycollate release by *S. bacillaris in situ* is uncertain since in laboratory cultures it may in whole or part originate from enhanced photorespiration due either to the effect of oxygen [26] or a photoinhibition of photosynthesis [27]; these effects have been seen with (prokaryotic) cyanobacteria [28]. However, in the present work the (relatively dense) laboratory cultures were not aerated during the incubation with $^{14}CO_3^{2-}$ and their growth was light-limited and not nutrient-limited; the irradiance levels used were not sufficiently high to give photoinhibition with other phytoplankton. For example, *H. carterae* did not similarly release glycollate as a quantitatively significant product when grown under identical conditions [2]. Discounting glycollate, *S. bacillaris* exhibits a very small exudate release. Presumably, for a particular alga the nature of the component(s) excreted arises from inbalance in a metabolic pathway(s) and the consequent disturbance of interactions of this with other pathways within the cell; clearly these events underlie biochemical mechanisms of environmental adaptation.

Our studies of *S. bacillaris* and *H. carterae* emphasize the importance of detailed chemical characterization of extracellular products as an aid to understanding excretion processes. We have found that a combination of ion exchange electrodialysis and gel filtration can be used to differentiate general patterns of extracellular production, and through comparison to the compositions of intracellular metabolic pools can be used in determining the relative contributions of cell lysis and excretion to this process.

EXPERIMENTAL

An axenic culture of *Stichococcus bacillaris* (strain 10/01) was provided by Professor P. J. Syrett (University College of Swansea) originally isolated by Professor R. A. Lewin from Lameshur Bay, St. John, Virgin Islands. This strain is now held at the Scottish Marine Biological Association, Oban. Experimental batch cultures were grown at 20° in 1 l flat-sided glass bottles containing 900 ml modified 0–1 medium [2], gassed with compressed air (30 ml/min) by open 2 mm dia. tube and illuminated from both sides by 2 × 80 W cool-white fluorescent tubes providing 100 $\mu E/m^2/sec$ at each vessel surface. It was also grown in continuous culture (dilution rate 0.0284 hr^{-1}) at 20° in a 1 l vessel containing 600 ml modified 0–1, gassed with compressed air (300 ml/min) through a 1.5 cm dia. AG 25X0 sinter and illuminated by a circular 32 W fluorescent tube providing 50 $\mu E/m^2/sec$ at its focus. Full details of culture methods, together with detailed protocols for radiolabelling experiments, extraction and preparation of samples, ion-exchange membrane electrodialysis, gel filtration and TLC and autoradiography, have been given in [2]. It should be noted that in the incubations with $NaH^{14}CO_3$ estimates of $^{14}CO_2$ assimilation and excretion were made at hourly intervals and were linear with time over the 4 hr on which quoted rates are based.

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