Radioactively labeled ALA can be readily incorporated into Chl a as well as other tetrapyrroles (14, 16). We have used both [4-\(^{14}\text{C}\)] and [3.5-\(^{3}\text{H}\)]ALA as precursors of the tetrapyrrole-based pigments, in an effort to quantify the turnover of these pigments in vivo. Three basic types of experiments were performed: (a) reverse isotope dilution, in which the radioactive precursor [4-\(^{14}\text{C}\)]ALA is incorporated into the Chl pools until steady-state labeling is reached; (b) isotope dilution, in which the pigments labeled with [4-\(^{14}\text{C}\)]ALA are chased with endogenous or exogenous ALA; and (c) dual label, in which pigments labeled with [3.5-\(^{3}\text{H}\)]ALA are chased with [4-\(^{14}\text{C}\)]ALA. We present here the results of our studies and our calculations of Chl a and chlide c turnover.

**MATERIALS AND METHODS**

**Reagents.** All solvents were spectral grade when available. Petroleum ether (b.p. 30–60 C), chloroform, absolute methanol were purchased from Mallinkrodt, Inc.; petroleum ether (b.p. 20–40 C), acetone, 1-propanol, and anhydrous diethyl ether came from J. T. Baker; petroleum ether (b.p. 63–75 C) and hexanes came from Eastman Kodak; cellulose MN 300 was supplied by Brinkmann Instruments; Aquasol, [4-\(^{14}\text{C}\)]ALA (55.1 mCi/mmol), and [3.5-\(^{3}\text{H}\)]ALA (1,194 mCi/mmol) were purchased from New England Nuclear.

**Culture Conditions.** *S. costatum* (Grev.) Cleve, Woods Hole clone SKEL, was grown on a 14:10 light-dark cycle at 15 C as previously described (10). The cultures were continuously mixed by stirring, and gently bubbled with sterile air by negative pressure. After 4 to 5 days of growth (cell density of about 2.5 \times 10^6 cells/ml) a 500-ml aliquot was inoculated into 3 liters of fresh media containing 0.5 to 0.6 \mu M [4-\(^{14}\text{C}\)]ALA or [3.5-\(^{3}\text{H}\)]ALA. The reinoculation resulted in a shift-up (12) in the permeability of the cell to ALA, allowing for enhanced uptake of ALA (see under "Results"). Following ALA uptake in isotope dilution and dual label experiments the cultures were continuously centrifuged at 12,000 g at 15 C with a flow rate of about 200 ml/min. The packed cells were washed with filtered seawater and resuspended in fresh media containing either 0.5 \mu M unlabeled ALA (isotope dilution) or 0.5 \mu M [4-\(^{14}\text{C}\)]ALA (dual label). Cells were not broken or visibly damaged by the centrifugation procedure.

**Pigment Determinations.** To determine the intracellular pigment content, cultures were subsampled frequently (~ hourly) for cell counts and pigment extraction. Cells were counted with a Spears-Levy hemocytometer, replicate counts had an error within a range of ±10%. A 10- to 25-ml aliquot of culture was filtered on a Gelman type A glass fiber filter and pigments were immediately extracted into 4 ml dry acetone, mixed with 6 ml hexane, 2 ml distilled H\(_2\)O, and 20 \mu l 50 mm NaOH. After phase separation, the absorbance of the pigments in each layer was measured at 664 and 630 nm before (A\(_0\)) and after (A\(_f\)) the addition of 1 drop of 10% HCl. Phytolated pigments retained in the hexane fraction
(nmol/sample) were determined using the following experimentally derived equations:

\[
\text{Chl a} = 26.3(\text{A}_{664} - \text{A}_{644}) \times \text{vol hexane fraction}
\]

\[
\text{pheao} = 26.3(2.18 \text{A}_{664} - \text{A}_{644}) \times \text{vol hexane fraction}
\]

Nonphytolated pigments were similarly determined in the aqueous-acetone phase (~80% acetone):

\[
\text{chlide} a = [22.4(\text{A}_{664} - \text{A}_{644}) - 0.784 \text{A}_{680}] \times \text{vol aqueous-acetone}
\]

\[
\text{phbide} a = 22.4(2.27 \text{A}_{664} - \text{A}_{644}) \times \text{vol aqueous-acetone}
\]

\[
\text{chlide} c = 24.0(\text{A}_{664} - 0.15 \text{A}_{644}) \times \text{vol aqueous-acetone}
\]

**Chromatography.** Concentration and separation of the various Chl pigments to determine total isotopic incorporation and specific radioactivity were accomplished according to the general procedures of Jeffrey (7) with some modification. All steps were carried out at 0 to 4°C in dim light. A 150-ml aliquot of culture was filtered on a glass fiber filter and immediately extracted in dry acetone. The extract was clarified by filtration, diluted to 10 ml Aquasol in a Beckman LS 3150 scintillation counter.

The aqueous-acetone phase was reextracted with hexane until the hexane fraction showed no fluorescence under long wavelength UV light. The combined hexane fraction was washed with distilled H₂O to remove remaining acetone; the wash was combined with the aqueous-acetone fraction. The hexane was then exhaustively washed with 80% methanol to remove most carotenoids (11) followed by two distilled H₂O rinses. The hexane was then evaporated to dryness in a flash evaporator. The pigments were dissolved in ethanol, spotted on a MN 300 (1.5-mm thickness) TLC plate (5 × 20 cm), and developed in 10% fresh diethyl ether in petroleum ether (b.p. 35-60°C) to separate the remaining carotenoids, Chl a, and phaeo a. Pigments were scraped from the dried plates and counted in 10 ml Aquasol in a Beckman LS 3150 T liquid scintillation counter.

The aqueous-acetone phase was concentrated and pigments separated according to Jeffrey (7) using 2% i-propanol in petroleum ether (b.p. 20-40°C) and 28% chloroform in petroleum ether (b.p. 35-60°C) for the first and second dimensions, respectively. Dried pigments were counted as described above.

Scintillation counts were corrected for color quench with an external standard ratio using pure Chl a as a standard. In addition, [3,5-²H]ALA and [4-¹⁴C]ALA were used in conjunction with ¹⁴C and ²H quench standards (Beckman) to generate quench curves.

Initially, chromatographic analysis was performed immediately after filtration of samples. This severely limited the frequency of sampling as the method is somewhat tedious. In later experiments, samples were frozen in liquid N₂ and stored at -20°C over silica gel until analyzed (1-4 weeks). No loss of pigment was detected using this method.

**Units.** Calculation of Chl turnover times in cultures exhibiting exponential cell division requires the use of volume specific units to compensate for the non-steady-state conditions. The Chl pool size has units of µmol pigment/150 ml culture. The volume specific activity of the pool (S) has units of dpm/150 ml culture. Pigment specific activities (S) have units of µCi/µmol pigment.

**RESULTS**

**Pigment Extraction and Chromatography.** Our initial adherence to the procedures of Jeffrey (7) led to some noteworthy problems with the pigments of _S. costatum_. Extraction of pigments in 90% acetone led to high variability in the ratio of phytolated to nonphytolated pigments. Our results (unpublished) indicate that _S. costatum_ contains a highly active chlorophyllase. Dephytolization was minimized by extraction in dry acetone and removal of precipitated proteins by filtration before dilution to 90% acetone. Under these conditions, chlide a and phbide a were present in trace amounts and accounted for less than 5% of the total label incorporated.

Loss of Mg²⁺ from chlide (ide) a during concentration of pigments for chromatography was also encountered. Phaeo a was initially the major pigment on TLC plates although only traces were detected spectrophotometrically. The partitioning of phytolated pigments into hexane rather than diethyl ether gave chromatographic results in good agreement with the spectrophotometric determinations. Phaeo a frequently overlapped with Chl a after chromatography and subsequently the two pigments were treated as a single pool in all calculations.

**ALA Incorporation.** Normal cells of _S. costatum_ take up significant extracellular ALA (Fig. 1) even though these cells are thought to be impermeable to primary amino acids (17). Shifting-up results in a 25-fold increase in the equilibrium cellular uptake of ALA largely due to an increase in the initial rate of uptake (Fig. 1). In all cases, only 1 to 2% of the total ALA uptake was incorporated into Chl a or chlide c. Previous analysis (10) showed that after a 24-h exposure to 0.5 µM [¹⁴C]ALA, 40% of the label was recovered in soluble proteins and free amino acids. From the same analysis, the equilibrium intracellular pool size of intact ALA was found to be 8 × 10⁻¹⁵ µmol ALA/cell (1 × 10⁻³ dpm/cell).

**Pigment Pools.** The addition of 0.5 µM ALA to the culture media did not alter the rates of cell division or Chl a and chlide a accumulation from those of untreated cells.

The intracellular Chl a and chlide c pools in _S. costatum_ are characterized by a marked diel ⁶ periodicity in exponential and stationary growth (Fig. 2). The intracellular Chl a pool was smallest at the dark-to-light transition and largest at the light-to-dark transition, varying between 2.5 and 5.1 × 10⁻¹⁰ µmol/cell.

The intracellular chlide c pool paralleled Chl a but showed consistently greater variation between 3.6 and 11.5 × 10⁻¹⁰ µmol/cell. The Chl a to chlide c ratio averaged 4.07 ± 0.81 (SD) and decreased with increasing cell density. There was little evidence that the periodicity in intracellular pigment pools was the result of synchronous cell division.

The total radioactivity incorporated into Chl a and chlide c per volume culture (Sₐ and Sₐ, respectively) was roughly equal in all experiments (~4 × 10⁻³ dpm/150 ml culture); however, the pigment specific activity of chlide c (Sₐ) was invariably greater than that of Chl a (Sₐ) owing to the smaller intracellular chlide c pool size. Sₐ/Sₐ ratios remained relatively constant in experiments where the dilution of previously labeled pools was monitored, but Sₐ/Sₐ increased in experiments where the incorporation of label into pigments was examined (Fig. 3).

[¹⁴C]ALA was not directly incorporated into carotenoid or xanthophyll pigments, but the eventual labeling of all cellular intermediates (10) led to minor labeling of these pigments after 24 h.

**Reverse Isotope Dilution Experiments.** The incorporation of [4-¹⁴C]ALA into Chl a and chlide c pools in stationary cultures of _S. costatum_ is rapid and reaches equilibrium after 10 h (Fig. 4). In these experiments the Chl a and chlide c pools approximated steady-state conditions. Equilibration of the chlide (ide) pools lagged behind equilibrium whole cell uptake by about 8 h (compare Figs. 1 and 4).

**Isotope Dilution Experiments.** Cells which had been exposed to [¹⁴C]ALA for 48 h and had attained a cell density of 1 × 10⁸ cells/ml were harvested and resuspended in fresh media at a density of 3 × 10⁵ cells/ml. Cellular label decreased from 6.0 to 1.1 × 10⁻¹⁴ µmoles ALA/cell upon resuspension of cells into media containing

---

⁶ Dieλ: involving a 24-h period that includes a day and the adjoining night.
no ALA. A smaller decrease (8.3 to 6.1 × 10^{-12} \text{ mol ALA/cell}) was noted in cells resuspended in media containing 0.5 \text{ mol unlabeled ALA. Exponential cell division and pigment pool expansion began immediately after resuspension and were accompanied by a rapid loss of label from the Chl a and chlide c pools. No loss of ^{14}\text{C} into the cold media was detected, but a gradual decrease in the cellular ^{14}\text{C} content was seen throughout the 48 h following resuspension (Fig. 5).

Complete dilution of the labeled pigment pools was not observed. The initial rapid loss of label was followed by a period characterized by little or no loss and an S' of about 30% of the maximum attained before resuspension. Beyond this period (15 h after resuspension), the pigment pools showed a trend of increased labeling.

**Dual Label Experiments.** The dilution of Chl a and chlide c pools labeled with [3,5-^{3}\text{H}]ALA and the coincident incorporation of [4-^{14}\text{C}]ALA into these pools are shown in Figures 6 and 7.
**Fig. 5.** Loss of $^3$H label from cells of *S. costatum* in dual label experiments. Cells were initially exposed to 0.5 μM [H]ALA for 24 h. Heavy broken line represents harvesting and resuspension of cells into media containing 0.5 μM unlabeled ALA (▲) cellular ALA incorporation; (●): total label uptake per volume culture.

**Fig. 6.** Labeling pattern of Chl a pool in exponentially dividing cells of *S. costatum* in dual label experiments. Cells were exposed to 0.5 μM [H]ALA for 48 h. Following centrifugation, the cells were washed and resuspended in media containing 0.5 μM [C]ALA (time = 0 h). The Chl a pool is expressed as equivalent ALA (8 × Chl a). (□): Chl a pool size; (●): $^3$H label in Chl a pool; (▲): $^4$C label in Chl a pool.

**Fig. 7.** Labeling pattern of chlide c pool in exponentially dividing cells of *S. costatum* in dual label experiments. Conditions are identical to Figure 6. Chlide c pool is expressed as equivalent ALA (8 × chlide c). (□): chlide c pool size; (●): $^3$H label in chlide c pool; (▲): $^4$C label in chlide c pool.
experiments. Tritium counts were corrected for losses of label which occur during pigment synthesis (78% loss in Chl \(a\), 84% in chlide \(c\)). The \(^3\)H decrease in both pools closely parallels that observed for \(^{14}\)C in the isotope dilution experiments; however, the initial incorporation of \(^{14}\)C was very slow and differed in kinetics from label incorporation in reverse isotope dilution experiments (compare Figs. 4 and 6). Rapid \(^3\)H incorporation did not begin until 20 h after resuspension and was accompanied by slight increase in \(^3\)H labeling. The pigment pools expanded exponentially during light periods but remained constant in the dark.

**Calculation of Pigment Turnover.** The change in the size of a pigment pool with respect to time can be described by a conversation of mass equation:

\[
\frac{dQ}{dt} = R_{in} - R_{out} \tag{1}
\]

where \(Q\) is the pool size and \(R_{in}\) and \(R_{out}\) represent the gain and loss of ALA from the pigment pool. The chl(id) pools are expressed as "equivalent ALA" pool size (8 \(\times\) pigment pool size). The turnover time (\(T\)) of the pools is calculated from the equation:

\[
T = 1/K = Q/R_{in} \text{ (or } R_{out}) \tag{2}
\]

where \(K\) is analogous to a first order rate constant.

In the reverse isotope dilution experiments the pigment pools were in steady-state (\(R_{in} = R_{out}\)) and the pigment specific radioactivity will increase as long as \(S_{ALA} > S_s\) (or \(c\)). Under these conditions the turnover time can be calculated from the rate constant of an equation which describes the changes in \(S\) with respect to time (t):

\[
S = S_{max}(1 - e^{-kt}) \tag{3}
\]

where \(S_{max}\) is the asymptotic value of \(S\) for a given reverse isotope dilution experiment. These calculations yield turnover times of 8 h for Chl \(a\) and 7 h for chlide \(c\).

Calculation of turnover is more difficult when the pigment pool size is increasing as a result of cell division (\(R_{in} > R_{out}\)). In this case the difference between \(R_{in}\) and \(R_{out}\) represents net accumulation of pigment supporting cell division and as such does not contribute to turnover. The calculation of \(T\) thus requires the use of \(R_{in}\) in equation 2. To relate a change in the amount of label in a pigment pool to \(R_{in}\) or \(R_{out}\), the \(S^*\) of each flux must be known.

In experiments in which the loss of label from a pigment pool was monitored in cells previously exposed to \(^{14}\)C- or \(^{3}\)H-ALA (isotope dilution and dual label experiments), \(R_{in}\) is calculated using the measured \(S^*\) of the pigment pool. Results from isotope dilution experiments, using data from the first 5 h after resuspension, give \(T = 9\) h for Chl \(a\) and 20 h for chlide \(c\). Similar calculations from dual label experiments (first 5 h after resuspension) yield turnover times of 3 h and 8 h for Chl \(a\) and chlide \(c\), respectively.

Pigment turnover was also detected during the dark cycle. In this 10-h period, cell division continued at the rates comparable to those in the light cycle while pigment accumulation ceased. Using \(^3\)H dilution data from the dual label experiments, turnover times of 6 h for Chl \(a\) and 26 h for chlide \(c\) are calculated.

Calculation of \(R_{in}\) from the rate of \(^{14}\)C incorporation in dual label experiments is accomplished using the following equation:

\[
\frac{dS^*}{dt} = R_{in}(S_{ALA} - S_s) \tag{4}
\]

where \(S_{ALA}\) has a value of 3.28 \(\times\) 10\(^3\) dpm/150 ml. Calculations of \(R_{out}\) determined from data obtained during the exponential increase in \(S^*\) during the later hours of dual label experiments and equations 1 and 4 give turnover times of 5 h and 11 h for Chl \(a\) and chlide \(c\), respectively.

As previously noted, complete washout of label in dilution experiments was not observed for either Chl \(a\) or chlide \(c\). Calculation of turnover time in the period following rapid loss of label gives times on the order of 100 h for both pigments.

**DISCUSSION**

The rapid uptake of exogenous ALA, enhanced by the shift-up phenomenon, reaches a maximal whole cell uptake of 2.1 \(\times\) 10\(^{-11}\) \(\mu\)mol ALA/cell after 2-h incubation with ALA. At equilibrium, intact labeled intracellular ALA accounts for only 8.0 \(\times\) 10\(^{-14}\) \(\mu\)mol/cell. Our previously reported results (10) agree with those of Trotler and Brown (16) that exogenously applied ALA is not committed to porphyrin synthesis but may appear to a large degree in amino acid and protein fractions. The reversible transamination and reduction between \(\alpha\)-ketoglutarate and ALA via dioxovaleric acid may be one pathway linking ALA and amino acids.

The condensation of two ALAs to form porphobilinogen and subsequent reactions of Chl synthesis are thought to be largely restricted to the chloroplast (3, 6). The incorporation of exogenous ALA into Chl thus requires the transport and partitioning of exogenous ALA between the cytoplasm and chloroplast. Within the chloroplast, the labeled ALA is diluted with endogenously produced ALA. The amount of dilution is estimated to be \(\times\) 10\(^4\) from the maximum specific radioactivity (\(S\)) of the Chl pool. ALA is not known to accumulate in untreated cells and our results (unpublished) indicate that the cellular ALA content (largely restricted to the chloroplast) does not exceed 10\(^{-13}\) \(\mu\)mol/cell. The high degree of dilution of label in the ALA pool thus implies slow transport of exogenous cytoplasmic ALA into the chloroplast and may explain why most of the incorporated label appears in fractions other than Chl.

Equilibration of the ALA pool with respect to exogenous and endogenously ALA is required for calculation of turnover time in experiments in which the incorporation of label into Chl pools is monitored (reverse isotope dilution and dual label). Chl synthesis, to support cell division (no turnover), would require about 2 \(\times\) 10\(^{-10}\) \(\mu\)mol ALA/cell.h and implies that the chloroplastic ALA pool turns over 1,000 times/h. Thus, equilibration of the chloroplastic ALA pool should occur rapidly after reaching maximal whole cell uptake of ALA. Also, the forcing of Chl turnover by the exogenous ALA appears unlikely.

The kinetics of the incorporation of labeled ALA into Chl pools is very different in reverse isotope dilution and dual label experiments (compare Figs. 4 and 6). In reverse isotope dilution experiments, labeled ALA is rapidly incorporated into Chl pools of cells which are previously untreated with ALA. In dual label experiments, label incorporation is initially very slow; rapid incorporation does not begin until some 24 h after resuspension of cells. The difference may be due to changes in the partitioning of exogenous ALA between the cytoplasm and chloroplast as a result of the previous exposure of cells in dual label experiments to exogenous ALA.

In experiments in which the loss of label from the pigment pools is monitored (isotope dilution and dual label), complete loss of label is not observed. Rather, about 30% of the maximal labeling is retained after the initial rapid loss and trends of increasing label are noted in longer incubations. This is indicative of two processes which are not mutually exclusive. First, direct recycling of Chl breakdown products to intermediates of Chl synthesis may occur. In addition, indirect recycling of label into endogenously produced ALA may occur. Conversely, retention of label in dilution experiments may indicate the presence of two distinct pigment fractions containing both Chl \(a\) and chlide \(c\), of which one turns over rapidly (10 h) and the other turns over more slowly (100 h). Shlyk (13) has presented evidence of multiple pigment fractions, each with characteristic turnover rates.

The kinetics of label incorporation or dilution is very similar for Chl \(a\) and chlide \(c\). The higher specific activity of chlide \(c\) (\(S_s\)) indicates that the synthetic pathway between ALA and chlide \(c\) is
shorter and/or contains fewer accumulating intermediates than that of Chl α. It is unlikely that chlide c is a precursor of Chl α and there is no evidence for significant interconversion of the pigments. Variation in the ratios of Sr/Ss in incorporation experiments, and not in dilution experiments, indicates that regulation of Chl α/chlide c occurs during synthesis and not degradation. Independent regulation of Chl α and chlide c concentration is required to account for changes in the a/c ratio on a diel scale and as a function of the ambient light intensity. In addition, the turnover rates of the two pigments are not related to their respective intracellular pool sizes. The metabolic relationship of Chl a and chlide c does not appear to be analogous to that of Chl a and b (13, 14).

The results of all three types of experiments (reverse isotope dilution, isotope dilution, and dual label) are summarized in Table I. These data strongly suggest that Chl a and chlide c are highly dynamic in vivo. As ALA is rapidly incorporated into the pigment pools when the pools are in steady-state, turnover must occur under stationary conditions (i.e. in stationary phase). That 14C can exchange with 3H in Chl a and chlide c when the pigment pools are increasing suggests that growth is accompanied by turnover. The turnover times presented here vary with experimental conditions, and should not be considered as hard numbers. Our calculations greatly oversimplify the complexity of Chl metabolism, and turnover is likely to be dependent on growth rate, light intensity, and nutrient supply, and these parameters vary according to the type of experiment performed. The existence of Chl turnover on a scale of hours is unquestionable. The ability to adapt to changes in ambient light intensity in less than a generation time are of great ecological significance to phytoplankton which are potentially exposed to considerable variation in light intensity in a period of hours.

Turnover times on the order of days have been reported in higher plants (9, 13, 14). Grumbach et al. (4) have demonstrated a turnover time of about 1 h for Chl a in the green algae *Chlorella pyrenoidosa*. Our results are in general agreement with those of Grumbach et al. and with our potential turnover times estimated from the in vitro activity of ALA-dehydrase in *S. costatum* (10). It is possible that planktonic algae maintain the ability to adapt to rapid changes in light intensity, a characteristic which is neither observed nor required in higher plants.

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**Table I. Summary of Chl Turnover Calculations**

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1. 10h dark period.
2. First 5h after resuspension.
3. During exponential growth.

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