DENSITY GRADIENT PROCEDURES FOR THE SELECTION OF SYNCHRONOUS CELLS
OF SYNECHOCoccus LIVIDUS AND Chlorella Sorokiniana, AND THE
APPLICATION OF THE ISOPYCNIC TECHNIQUE TO THE STUDY
OF THE PATTERNS OF PHOSPHORIBOSYLGLYCINAMIDE SYNTHETASE
DURING THE CELL CYCLE OF
CHLORELLA

by

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Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment for the degree of
DOCTOR OF PHILOSOPHY
in
Biochemistry and Nutrition

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June 1971
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ACKNOWLEDGEMENTS

The author expresses his gratitude to his major professor, Dr. Robert R. Schmidt, for his capable guidance and encouragement during the course of these studies.

He is grateful to all present and past members of the group, especially to Deanna Talley.

Special thanks go to the members of his graduate committee, particularly to Dr. N. R. Krieg.
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INTRODUCTION

Cultures of cells have been artificially synchronized since 1953 (1). Since that time, techniques have changed as better methods were developed, however, the two major categories of synchrony methods, induction and selection techniques, have remained the same. The various methods for obtaining synchronous cultures have been reviewed in detail by James (2) and Morse (3).

Induction as a means of synchronizing cells is achieved by different methods which involve shifts in or inhibition of the metabolism of the cells. The advantage of methods such as starvation, thymidine block, inhibition of division, temperature shocks, and intermittent illumination is that large populations of synchronized cells can be obtained. However, all these methods induce changes in the metabolism of the cell which may be temporary or may continue for many cell cycles. Therefore, it is best to avoid these methods unless selection methods can not be developed (2).

The other general method, selection synchrony, takes advantage of some physical variations in the cells (size, surface charge, sticky surface, etc.) to select uniform age group. Morse (3) examines these techniques extensively, but in these experiments only two techniques have been used. They are
differential centrifugation (separation on basis of size) and isopycnic centrifugation (separation on a density basis).

Differential centrifugation, the most widely used of all the selection techniques for microorganisms, separates cells of different age because of their different rates of sedimentation during centrifugation (4). Generally, linear gradients are used to stabilize the liquid columns. Cells selected by the method have minimum perturbations of their metabolic pools; however this rate separation technique is limited both by reproducibility and low yield of cells.

If cells could be separated on the basis of density, the problems of reproducibility and low yield could be eliminated. Lief (5) found that erythrocytes increased in density as they increased in age. By using bovine albumin solutions for gradient material Lief could centrifuge the cells to their equilibrium positions. Morse (3) lists the characteristics of ideal materials for making isopycnic gradients and various compounds that have been used.

The purpose of the present study was to develop a technique to select synchronous procaryotic and eucaryotic microorganisms without the limitations of reproducibility and cell yield; also without metabolically perturbing the cells. This technique was
developed with the eucaryote Chlorella sorokiniana and subsequently tested on the procaryote Synechococcus lividus.

The procaryote, S. lividus, was chosen for these studies because it was a thermophilic blue-green alga that divided by simple binary fission and could be cultured on inorganic medium (6). This organism separates completely after divisions and does not have a thick capsule, as compared to other blue-greens which form chains and have thick sticky capsules which makes them difficult to culture. The minimum compartmentation of blue-green algae, i.e. no nucleus, chloroplast, and mitochondria, made Synechococcus an ideal organism to compare to Chlorella. Blue-green algae have not been synchronized until recently, when Anacystis nidulans was synchronized by temperature shifts (7,8).

In Chlorella, phosphoribosylglycinamide synthetase, the second enzyme in the purine biosynthetic pathway, was assayed through the cell cycle being careful to optimize activity and stability of the enzyme. This enzyme was used to study what role substrates might play in vivo and in vitro on the stability of this enzyme, and to determine what the pattern of this biosynthetic enzyme would be in the cell cycle.
Part I

The Purification and Synchrony of the Blue-Green Alga

*Synechococcus lividus*
MATERIALS AND METHODS

Organism and Culture Conditions - The thermophilic blue-green alga *Synechococcus lividus* was cultured in medium described by Dyer and Gafford (6) which was modified by doubling the concentration of all components to allow higher concentrations of cells to be cultured. The cells were cultured with a 4% CO$_2$-air mixture in 2.54 or 5.08 cm glass culture tubes and in 0.635 cm (inside diameter) Plexiglass chambers, as described for *Chlorella* (9). A constant temperature water bath (9) was used to maintain the temperature at 45 or 50$^\circ$.

Isolation of a Pure Culture of *Synechococcus lividus* - The culture, when received from Argonne National Laboratories as a liquid innoculum, was contaminated by two types of rod-shaped thermophilic bacteria. However, I was able to develop two techniques, selective centrifugation and isopycnic banding, that were employed to enrich the culture in algae prior to their purification.

In the first procedure, a culture was centrifuged at room temperature 4 times to selectively pellet the algae in a 100 ml Goetz tube. The tubes were centrifuged in a number 240 rotor at 1,000 rpm for 5 min in an International centrifuge (Model UV) using medium to resuspend the pellet. After the fourth centrifugation, the pellet was mixed with 0.5 ml of medium and streaked on agar plates.
In the isopycnic banding technique, the cells were washed once with medium at $2^\circ$ and the pellet was resuspended in 40% w/w solution of Ficoll (Pharmacia Fine Chemicals) and deionized water. This cell suspension was then layered on the bottom of a linear gradient (40% to 15%, 30 ml) of dialyzed Ficoll which was prepared by the method of Lakshaman and Lieberman (40). After centrifugation in a Model L2-65B, Beckman ultracentrifuge, with a SW-27 rotor at 25,000 rpm and $2^\circ$, the tube was punctured at the bottom and the first fractions containing algae were collected and washed with cold medium. The cells obtained by both of these techniques were then streaked on agar plates containing culture medium plus 1.5% agar. These plates were maintained in an incubator at 45$^\circ$ with an atmosphere of 4% CO$_2$ and a light intensity of 500 foot-candles. After a week colonies were isolated and streaked on agar slants and maintained in an atmosphere of 4% CO$_2$-air, until checked for purity.

Procedures for Synchronizing Synechococcus lividus - Three procedures were used in an attempt to synchronize S. lividus: intermittent illumination (11), differential centrifugation (4), and isopycnic centrifugation (12).

The light-dark regime which provided the best synchrony was a 3-hour light period followed by a dark period of 4.25 to 4.5 hours.
The procedure for selective centrifugation involved the use of linear Ficoll gradients (7.5 to 5%, w/v, 40 ml), which were used only to stabilize the liquid columns. Prior to layering on the gradient, the cells were harvested, washed in cold medium (2°), and suspended in a small volume of cold culture medium. A 2 ml aliquot was gently layered on each gradient. The tubes were centrifuged in a HB-4 rotor in a Sorvall centrifuge at 3,000 rpm for 10 min. Two ml of cells were taken from the top of the bank in each gradient and washed twice with cold medium and cultured.

The isopycnic technique was similar to the one described for isolating the pure culture of the organism except that linear gradients ranging from 30 to 20% w/v were used. Approximately 1 ml fractions were collected and uniformity of cell size was examined using the Coulter Counter, Model B.

General Methods - Because of problems encountered using crude Ficoll, growth inhibition and clumping of cells on gradients, a procedure was developed to remove low molecular weight material from these commercial preparations. Dialysis was found to be the best method of removing small molecules from this compound. One to 2 liters of a 15% aqueous solution of Ficoll was dialyzed against 20 liters of distilled water for 4 days at 2°. The water was changed every 12 hours the first day, then every 24 hours for the next 3 days. The water outside the dialysis sac was circulated by a magnetic
stirrer. After dialysis, the Ficoll solution was passed through a Millipore filter (0.45 μm) to remove lint and bacteria and flash evaporated at 40° to a concentration of approximately 45%. The exact concentration of this solution was determined at room temperature with a 50 ml volumetric flask used as a pycnometer.

Appropriate dilutions could be made from this purified Ficoll, which was used as a stock solution, and then autoclaved at 121° for 5 min for the preparation of sterile gradients.

To prepare agar plates and slants, 1.5% agar in culture medium was autoclaved, cooled at 45°, and the CaCl₂ and MgSO₄ solutions were added. This mixture was then placed in a 5.08 cm culture tube through which was bubbled 4% CO₂-air at 45° for 30 min. The slants and agar plates were then poured and allowed to cool at room temperature.

Cell number per ml was determined by use of a hemocytometer. Turbidity was measured with a Spectronic 20 (Bausch and Lomb) colorimeter at 550 nm in a 1.3 cm colorimeter tube. Percent cross-walls in synchronous cultures was determined microscopically by taking a ratio of the number of cells with cross-walls to the total number of cells (a total of 200 cells were counted per measurement). In this organism there was only one cross-wall per cell immediately before division.
RESULTS AND DISCUSSION

Isolation of a Pure Culture of Synechococcus lividus - S. lividus was contaminated by two thermophilic bacteria which existed in a symbiotic fashion with the alga. When the mixed culture was streaked on agar plates, colonies of algae only developed around bacterial colonies, suggesting that the bacteria were supplying some compound(s) necessary for growth of the algae. Culture medium that stimulated growth of algae on agar plates was obtained by culturing a mixed culture for a few days, removing the organisms by centrifugation, and passing this pre-cultured medium through a 0.45 μm Millipore filter. If this medium was autoclaved it failed to stimulate growth of the algae. Many heat labile compounds (vitamins, etc.) were tested on the growth of the algae without any observed stimulation of growth. The active compound was subsequently found to be CO₂ or HCO₃⁻ which was driven off the pre-cultured medium by the high temperature of autoclaving. A high concentration of CO₂ (4% CO₂-air) allowed subsequent isolation of pure colonies of the algae. Isolated colonies of bacteria would not grow in the inorganic medium in the absence of the algae. In the native environment (hot springs) the bacteria would presumably maintain a high CO₂ or HCO₃⁻ level and the algae would supply the bacteria with various organic compounds necessary for their existence.
**Culture Conditions** - If the alga was cultured at 50°C in a 5.08 cm tube with an atmosphere of 4% CO₂-air, it grew exponentially (3.8 hour doubling time) until a turbidity of 0.90 was reached, then the culture grew in a linear fashion (0.166 A₅₅₀ units/hour) to a turbidity of at least 3.5 (Fig. 1, 2). When cultured in a thin Plexiglass chamber in which the effective light intensity was higher, the organism grew exponentially (3.7 hour doubling time) to a turbidity of 3.5. The linear growth phase could be eliminated by lowering the temperature to 45°C. At this lower temperature, the cells cultured in a 5.08 cm tube grew at an exponential rate (4 hour doubling time) until the turbidity of 0.8 was reached, then the algae grew at a new lower exponential rate (5.75 hour doubling time). By culturing the organism in a thin Plexiglass chamber, the faster doubling time (3.8 hour doubling time) was maintained until a turbidity of 5.3 was reached. When the turbidity of the culture increased, the growth rate decreased and the culture shifted to a deeper blue-green color. This color shift may be due to an increase in phycocyanin or a decrease in the chlorophyll pigments.

**Synchronization Procedures** - The synchronization of the blue-green alga was approached with three different methods; however, only one of these, differential centrifugation, gave acceptable synchrony. The other two methods, intermittent illumination and isopycnic centrifugation, showed a partial synchrony which was not considered to be acceptable for cell cycle experiments.
Fig. 1. *Synechococcus lividus* was cultured at 50°C in a 5.08 cm glass culture tube, aerating with 4% CO₂-air. The light intensity was 1,100 footcandles. Samples of the culture were harvested every 15 min, measuring the turbidity at 550 nm with a Spectronic-20 colorimeter (Bausch and Lomb) in 1.3 cm colorimeter tubes. The arrow indicates the break in the growth curve at an absorbance of 0.90.
Fig. 1
Fig. 2. *Synechococcus lividus* was cultured and its growth rate was determined as in Fig. 1. The starting absorbance was 0.30.
Fig. 2
Three hours of light followed by a dark period of 4.5 hour resulted in the best results with intermittent illuminations (Fig. 3). Exposure of the cells to 6 cycles of alternating light and dark had no apparent effect on improving this synchrony.

When fractions from isopycnic gradients were examined microscopically or by the use of the Coulter Counter, only slight differences in size were seen. The algae, harvested from the top of the buoyant band and cultured, showed a poor degree of synchrony, i.e., cross-wall never exceeded 40%.

Selective centrifugation yielded the best synchrony of all methods evaluated. When taken from the top of the gradients, cells showed a short division period and a high cross-wall index of 85% (Fig. 4). During the second division, the cross-wall index dropped to 65%. Cells from fractions taken deeper in the gradients showed earlier division times. The low yield of cells achieved by this technique limits the types of biochemical studies that can be performed. The zonal rotor undoubtedly could be used to scale up the differential centrifugation procedure for biochemical studies requiring large amounts of cell material.
Fig. 3. *Synechococcus lividus* was cultured at 45° and subjected to intermittent illumination. The culture was cycled through two periods of 4.5 hours dark and 3 hours light, harvesting samples every 15 min during the light periods. Cell number, $\bullet$, was determined with a hemocytometer, while $\%$ cross-wall index, $\Delta$, was determined by microscopic examination. The dark and light areas at the top of the figure represent the duration of dark and light periods.
Fig. 3
Fig. 4. A synchronous culture of *Synechococcus lividus* was selected from random cells by differential centrifugation. About 800 ml of the culture was cultured as in Fig. 1 at 45°. The culture was harvested when its turbidity reached 0.38, and was washed once with 0.1 M CaCl₂, suspending the pellet in 10 ml of 0.1 M CaCl₂. Two ml of this cell suspension was layered on 4 linear gradients of Ficoll (40 ml, 7.5-5% w/v) which were centrifuged in a HB-4 rotor at 3,000 rpm in a Sorvall (Model RC-2B) centrifuge for 10 min. Two ml samples were harvested from the band of algae, and resuspended in a 1-inch tube with 150 ml of medium. Cell number, *, and % cross-wall index, Δ, were determined as in Fig. 3.
Fig. 4
Part II

An Isopycnic Technique for the Selection of Synchronous Cells, and the Application of This Technique to Study the Stability and Activity of Phosphoribosylglycinamide Synthetase during the Cell Cycle of *Chlorella sorokiniana.*
MATERIALS AND METHODS

Organism and Culture Conditions - The thermophilic strain 7-11-05 of the unicellular alga *Chlorella pyrenoidosa* Chick, recently renamed *Chlorella sorokiniana* Shihira and Krauss (13), after its isolator, was used in these experiments. With the exception of the light intensity, which was reduced to 550 f.c.\(^1\) to give a division number of four, the cells were cultured by the method of Hare and Schmidt (9).

Methods of Synchrony - Two techniques were used to obtain synchronous populations of cells for these experiments: modifications of the intermittent illumination method of Baker and Schmidt (11) and an isopycnic selection technique using Ficoll density gradients (12,14).

In the first method, cells were given at least four successive periods of 10 hours of light and 8 hours of dark. These cells were then placed in continuous light for cell cycle experiments, and they were diluted every hour with medium preequilibrated for temperature and CO\(_2\), to keep the turbidity constant (11,15).

The isopycnic technique involved the use of linear gradients of Ficoll (12) or a modification of this procedure which used a single concentration of Ficoll (14). A modification of the manufacturing process made the purification of Ficoll unnecessary (16). Linear density gradients of Ficoll (32 to 27%, w/v, 26 ml) in deionized water were used to select the daughter cells. Asynchronous cells were layered on the top of the gradients, centrifuged

\(^1\)The abbreviation used is: f.c., foot-candle.
for one hour at 25,000 rpm in a SW-27 rotor in a Beckman Model L2-65B ultracentrifuge. From the top of the buoyant band, 5 ml of cells were removed, washed with cold medium, and cultured. A modification of this technique which does not require pre-formed gradients was developed. Cells were harvested, resuspended in 18.5% w/w Ficoll, and then 35.5% w/w Ficoll was added to give a final concentration of 26.8% w/w. This cell suspension was transferred to 2.54 x 8.89 cm cellulose nitrate tubes, 34 g per tube. Five ml of water was layered on the top of the suspension and partially mixed with a glass rod to form a partial gradient. This procedure prevented a "turnover effect" from occurring. Anderson (17) observed this effect and said that it was due to the layer made denser by the particles it now contains and "turns over". The layer of material will move as a body to the bottom of the tube. The tubes were centrifuged for 2 hours as before. The buoyant cells at the top of the Ficoll were removed, washed, and cultured.

To increase the yield of cells from a culture, a combination of intermittent illumination and isopycnic techniques were used. After subjecting cells to intermittent illumination, they could be harvested in the dark, or allowed to grow in continuous light for one or two cycles and harvested after the mother cells had divided. Culturing the cells in continuous light
before harvesting should minimize the effects of the dark period. This procedure greatly increased the yield of daughter cells when the culture was subsequently placed on isopycnic gradients.

Preparation of Cell Material for Analysis - Cells were harvested by centrifugation at 10,000 X g in a Sorvall RC-2B centrifuge for 5 min and washed twice in 0.01 M Tris-HCl buffer (pH 8.1) at 2-3°. For studies on the stability of phosphoribosylglycinamide synthetase, 1 ml p.c.v. pellets were resuspended in a final volume of 15 ml of buffer (0.1 M Tris-maleate, pH 8.6) or buffer plus substrates. For smaller volumes the same ratio of p.c.v. to buffer volume was maintained.

Cells for the above studies were broken with a sonic oscillator, a French press, and by freeze-thawing. In the sonication procedure, a 1 ml p.c.v. of cells was suspended in 14 ml of the appropriate solutions and sonicated in a Raytheon 10 kHz sonic oscillator. When the cells were ruptured with the French press, pressures were maintained between 18,000 and 20,000 psi. For the freeze-thaw studies, 1 ml samples of cell suspension were placed in small polycarbonate tubes and frozen, or the cells were pelleted in the tubes then frozen at a temperature of -20° for 48 hours.

2The abbreviations used are: p.c.v., packed-cell-volume; psi, pounds per square inch.
Phosphoribosylglycinamide Synthetase - This assay measured the production of PRG$^3$ from PRA$^3$, ATP-MgCl$_2$, and glycine. The incorporation of radioactive glycine into the product was used as a measure of enzyme activity (15,18). The PRA for this assay was synthesized by a chemical reaction between ribose-5-P and NH$_4$OH (18).

The standard reaction mixture contained, in umoles, MgCl$_2$ 21.9; (1$^{14}$C)-glycine, 6.25; ATP, 21.9 (adjusted to pH 8.3 with 19.4 umoles of Tris-maleate, pH 8.6, and 47 umoles NH$_4$OH); dithiothreitol, 0.0688; Tris-maleate (pH 8.6), 50; ribose-5-P, 6.88 (adjusted to pH 8.3 with 1.1 umoles NH$_4$OH); NH$_4$OH, 6.88; and crude Chlorella enzyme preparations to a final volume of 250 ul. The PRA-forming reaction was started by the addition of 220 umoles of NH$_4$OH to ribose-5-P which had been adjusted to pH 8.3, bringing the reaction mixture to 2 ml. The mixture was incubated at 38.5$^\circ$ for 50 min. The PRA was made fresh for each set of incubations. The enzymatic reaction was started by adding 50 ul of PRA to 100 ul of substrate solution, then mixing in 100 ul of the crude enzyme. This incubation was placed in a 38.5$^\circ$ water bath, measuring the reaction rate by removing 50 ul samples every 5 min. The reaction in 50 ul samples are terminated with 100 ul of 16% trichloroacetic acid.

$^3$The abbreviations used are: PRG, phosphoribosylglycinamide; PRA, phosphoribosylamine.
In studies in which substrates were incubated with the enzyme preparation to test for enzyme stabilization effects, or when substrates were used to stabilize the enzyme during cell breakage, the final concentration of all substrates (except glycine) in the incubation mixture was increased by 50% over that of the standard incubation mixture except in cell cycle studies where it was increased by 25%. Cells were passed through the French press twice with substrate concentrations at the levels used for stability and cell cycle experiments showing identical activities.

After the reactions were terminated, they were centrifuged at 4,000 X g for 5 min, and 100 ul of each supernatant placed on a 0.5 X 6.0 cm cation exchange column (Ag50W-X4), 100 to 200 mesh, NH₄⁺ form). The PRG was eluted with 2.5 ml of 0.05 M ammonium formate buffer (pH 3.3) and collected in counting vials. Fifteen ml of a triton-toluene scintillation counting solution (19) were added and radioactivity was determined in either a Nuclear-Chicago (Model 6804) liquid scintillation counter or a Beckman (Model LS-133).

One unit of enzyme is defined as the number of umoles of product formed per min of incubation.

Purification of (1-¹⁴C)-glycine - Commercial preparations of radioactive glycine (New England Nuclear) were contaminated with a labeled compound which eluted with PRG from the cation exchange columns, giving high backgrounds. However, this compound could be removed by placing 1 ml of a glycine solution (0.258 mg/2.5 ml),
acidified with 100 ul of 16% trichloroacetic acid, on a 0.5 X 6.0 cm column (50W-X4, 100 to 200 mesh, NH4+ form) and eluting with 4 ml of ammonium formate buffer (pH 3.3). The glycine was then removed with 0.05 M NH4OH. The fractions which contained the radioactive glycine were combined and concentrated by flash evaporation.

General Procedures - Cell number per ml of culture was determined either by a hemocytometer or by a Coulter Counter, Model B. Turbidity was measured with a Spectronic 20 Colorimeter (Bausch and Lomb) at 550 nm in a 1.3 cm colorimeter tube.

DNA was measured by a modification of the diphenylamine procedure (20). The modification consisted of extracting cells for 12 hours with 1 N NaOH at room temperature, neutralized with cold HCl (2°), and precipitated by the addition of trichloroacetic acid to a final concentration of 10% wt/vol. After washing twice with cold 10% trichloroacetic acid, the precipitate was extracted with 5% trichloroacetic acid for 20 min at 90°, cooled to 2°, and centrifuged. The DNA in the supernatant was measured by the Burton method (21). Samples were taken from the 12-hour base extraction and measured for protein by the procedure of Lowry et al. (22) using bovine serum albumin as a standard.
RESULTS AND DISCUSSION

Isopycnic Techniques for Selecting Synchronous Cells - Differential centrifugation of many types of cells in density gradients has been used to select small uniform cells from asynchronous populations for cell cycle studies (4, 23, 24, 25). These gradients were designed to stabilize the liquid column and were not sufficiently dense to behave as isopycnic gradients. In rate separation procedures the volume and concentration of the cell suspension and the time of centrifugation greatly influence the separation. Because of these problems, difficulty is often encountered in obtaining homogenous cell populations and sufficient concentration of material for cell cycle experiments.

An isopycnic technique was reported to select young erythrocytes from mature erythrocytes on the basis of their differences in density (5). This suggested that other cell types might differ in density during the cell cycle sufficiently to allow the use of isopycnic gradients which would eliminate most of the problems encountered with differential centrifugation.

Initial attempts with *Chlorella* using isopycnic gradients of aqueous sucrose failed to float any cells from an asynchronous population. The cells, showing an apparent high density, appeared to be plasmolyzed resulting from dehydration by the high osmolarity of the sucrose.

- 23 -
When Ficoll was used to replace the sucrose, these problems were overcome. The high molecular weight of Ficoll gives it a much lower osmotic strength relative to a given density of sucrose.

Asynchronous cells, when layered on either the top or bottom of identical gradients of Ficoll, were buoyant in the same density region (Fig. 5, A and B). This indicated that the cells were at true equilibrium position. Additional periods of centrifugation could not change this position. Cells could be removed from the top and bottom of a buoyant zone, washed, mixed, and placed on another gradient. When centrifuged again, the cells distributed into two distinct bands corresponding to their positions on the original gradients (Fig. 5, C). This indicated that the Ficoll procedure did not significantly alter the density of Chlorella cells.

Microscopic examination of cells in fractions, taken progressively down a gradient, revealed that cells increased in size down the buoyant band. Thus it would appear that Chlorella cells increased in density as they developed in their cell cycle.

A gradient designed to float daughter cells and sediment other age cells, could be loaded up to approximately 2 g of cells per gradient (Fig. 5, D). Daughter cells taken and cultured from such gradients showed a high degree of synchrony, a periodic increase in total cellular DNA, and an exponential increase in total cellular protein during a synchronous cell cycle (Fig. 6).
Fig. 5. Centrifugation of asynchronous cells of *Chlorella* to equilibrium on isopycnic gradients of aqueous Ficoll.

Gradient A, 1 ml of cell suspension (1.71 \times 10^9 cells) in culture medium was layered upon a 34-ml linear density gradient of Ficoll (25-45\%) in deionized water. An additional 1 ml of 45\% Ficoll in water was layered with a syringe on the bottom of the cellulose nitrate centrifuge tube (2.54 by 8.89 cm) to make gradient A equivalent to gradient B; gradient B, linear density gradient was identical to A with the same cell number in 1 ml of 45\% Ficoll in water layered with a syringe on the bottom of centrifuge tube; gradient C, linear density gradient was identical to A with the cell suspension layered on top, also as in A. The suspension was composed of cells removed with a syringe from the top and bottom of the zone of buoyant cells in a gradient similar to A; gradient D, 5 ml of cell suspension (8.55 by 10^9 cells) in culture medium was layered on a 30-ml linear density gradient of aqueous Ficoll (27-36\%). The linear gradients were prepared and maintained at 2^\circ C, as were the cells from their time of harvest. Centrifugation was performed in a model L2-65B, Beckman-Spinco ultracentrifuge, with a SW-27 rotor at 25,000 rpm. Cells layered on top of the gradients came to equilibrium between 30 and 60 min, while those layered on the bottom required approximately 120 min. The dotted lines on the centrifuge tubes were used to indicate 30- and 34-ml levels during gradient preparations.
Fig. 6. Changes during synchronous growth of daughter cells of *Chlorella* selected from an asynchronous culture by an isopycnic density-gradient procedure. (Ο) Periodic increases in cell number; (⊙) total cellular DNA; and (△) approximately an exponential increase in total protein per ml of culture. One cell cycle is defined as the time at which 50% of the cells have divided. The initial concentrations of cells, DNA, and protein per ml of culture were $140 \times 10^6$, 9.30 ug, and 355 ug, respectively; the light intensity was 550 footcandles. The culture apparatus of Hare and Schmidt (9) was used along with their continuous dilution procedure, which was modified by reducing the dilution rate to 1.35% per hr; DNA was measured by a modified diphenylamine procedure (20) while cellular protein was estimated by the procedure of Lowry et al (22); linear density gradients of Ficoll (27-32%, 26 ml) in deionized water were used. The top 5 ml of buoyant cells from these gradients were those used.
Fig. 6
The time consuming process of preparing linear gradients was eliminated by developing a procedure in which cells were suspended in a single concentration of 26.8% w/w Ficoll. All buoyant cells are removed, making this modification more reproducible.

The isopycnic procedure has eliminated most of the problems encountered with differential centrifugation, and has allowed selection of daughter cells from cultures growing in continuous light. By maintaining cells at 2°C, from the time of harvest until they are transferred to pre-equilibrated culture medium, their pools of metabolizable intermediates are not as likely to be perturbed as in the intermittent illumination synchronization procedure (11) where the cells are in the dark at 38.5°C for 6-8 hours.

Stabilization of Phosphoribosylglycinamide Synthetase - Molloy (15) showed phosphoribosylglycinamide synthetase activity to decay rapidly at 38.5°C after Chlorella cells were frozen (once) at -20°C and then thawed. However, when substrates of the enzyme were added to frozen-thawed whole cells or to sonicated cells for assay of enzyme activity, a linear accumulation of product was observed with incubation time, suggesting that the substrates stabilize the activity of this enzyme. Molloy (15) did not take precautions to stabilize the enzyme after breakage of the permeability barriers of the cells by freeze-thawing or by sonic oscillation; the pattern of enzyme activity which he determined during the cell cycle of Chlorella needed to be re-evaluated.
Since Herrmann (26) and Vassef (27) reported that freezing whole cells once at -20\(^\circ\) allowed subsequent measurement of total cellular aspartate transcarbamylase activity without breakage of the cells, cells were frozen at -20\(^\circ\) for 48 hours, as a pellet or suspended in buffer, and then thawed and then assayed for phosphoribosylglycinamide synthetase activity. Both of these treatments showed similar initial levels of activity and similar rates of decay at 38.5\(^\circ\) (Fig. 7). However, when cells were frozen suspended in buffer containing ATP-MgCl\(_2\) or all of the substrates, they showed lower initial activities upon thawing than cells which were frozen as pellets and then resuspended in these same agents; but, upon incubation at 38.5\(^\circ\), the activity of the enzyme in the former preparation increased to that of the latter and the activities subsequently decayed at essentially the same rate (Fig. 7). The crude enzyme in the presence of all of the substrates remained stable. Apparently freezing in the presence of the substrates delays the complete destruction of permeability barriers to small molecules until the cells are incubated at 38.5\(^\circ\) for approximately 20 min. Thus, to use the freeze-thaw procedure most effectively, the cells should be frozen as a pellet, thawed, and immediately resuspended in buffer containing the substrates prior to assay.
Fig. 7. The stability of phosphoribosylglycinamidine synthetase was examined in the presence of various substrates after different methods of freeze-thawing. A culture of Chlorella, synchronized by intermittent illumination, was inoculated into a thin (0.635 cm, inside diameter) Plexiglass chamber at a turbidity of 1.8. Medium was added to the chamber every hour to maintain a constant turbidity. The cells were harvested at the fourth hour and washed with buffer (0.1 M Tris-maleate, pH 8.6). One ml p.c.v. were resuspended in a final volume of 15 ml of buffer or buffer plus substrates. For smaller volumes the same ratio of p.c.v. to buffer volume was maintained. One ml samples of the cell suspension in buffer were placed in 1.5 ml polypropylene tubes and centrifuged at 4,000 X g for 5 min. The pellets were frozen at -20° for 48 hours, allowed to thaw at room temperature, then suspended in 0.93 ml of buffer (0.1 M Tris-maleate, pH 8.6), ○; buffer plus ATP-MgCl₂ (21.9 umoles/50 ul, □; buffer plus ATP-MgCl₂ (21.9 umoles/50 ul), PRA (ribose-5-P, NH₄OH) (6.88 umoles/50 ul), and glycine (3.125 umoles/50 ul) △. Other samples were first suspended in buffer, △; buffer plus ATP-MgCl₂, □; and buffer plus all the substrates, ○; and then freeze-thawed. All samples were incubated at 38.5° harvesting 100 ul samples every 30 min and assaying for phosphoribosylglycinamidine synthetase activity.
Fig. 7
The freeze-thaw procedure still did not allow the measurement of total phosphoribosylglycinamide synthetase activity as revealed by a 40% increase in activity after sonication of freeze-thawed cells in buffer containing all of the substrates. Also, when the procedure for freezing and thawing identical samples was not exactly reproduced, approximately a 20% variation in activity between samples was observed.

A comparison was then made of enzyme activity from fresh cells sonicated in the presence of buffer, buffer containing PRA and glycine, ATP-MgCl₂, or all of the substrates (Fig. 8). The initial activities in buffer alone or buffer plus PRA and glycine were similar and the activities were stable. In contrast, the initial activities in buffer containing ATP-MgCl₂ or all of the substrates were similar to each other but lower than the former treatments, and surprisingly they exhibited a linear decay in activity. The presence of PRA and glycine, along with the ATP-MgCl₂, reduced slightly the ATP-dependent linear loss of activity. Cells which were frozen-thawed and then sonicated gave essentially the same results as sonicated, unfrozen cells.

This ATP-MgCl₂ dependent loss in enzyme activity is only associated with sonication as a method of cell breakage. Several possibilities exist to explain this phenomenon: (a) the sonication procedure may be solubilizing a membrane-bound enzyme which either
Fig. 8. Stability of phosphoribosylglycinamide synthetase was examined in cells broken in the sonic oscillator in the presence of various substrates. *Chlorella* was cultured and harvested as in Fig. 7. The cells were suspended in either buffer, ○; buffer plus PRA and glycine, ●; buffer plus ATP-MgCl₂, □; or buffer plus all the substrates, Δ; and broken by sonic oscillation. One ml samples were incubated at 38.5°C and assayed for enzyme activity as before.
Fig. 8
attacks the phosphoribosylglycinamide synthetase directly or
(b) an enzyme is released which converts ATP to an inhibitor of
phosphoribosylglycinamide synthetase or requires ATP to convert
some endogenous compound to an inhibitor of the enzyme activity
Hershki and Tompkins (28) have evidence which suggests that ATP
is playing an active role in the degradation of tyrosine aminotransferase in rat liver. Regardless of the mechanism for the
loss in synthetase activity in Chlorella, sonication cannot be used
as a method of breakage because ATP-MgCl₂, a substrate of the enzyme,
results in the linear loss of activity during enzyme assay.

Microscopic comparison of Chlorella cells broken by sonica-
tion or with the French press, revealed that the sonic oscillation
procedure more finely homogenized the cell material than the French
press. It seemed possible that cell breakage by the latter pro-
cedure might not provide the conditions for the ATP-MgCl₂ dependent
loss of the synthetase activity seen with sonication. Comparison
of enzyme activity in homogenates, from cells ruptured in buffer
or in buffer plus substrates, after a single treatment in the
French press showed the enzyme activity to be essentially stable in
both treatments; however, the substrates lowered the initial activity
while buffer alone gave higher activity than even sonicated cells
(Table I). The lower enzyme activity of cells in the presence of
substrates in the French press experiments apparently was due to the
slight protection of the permeability barriers by substrates.
Table I

The Activity of Phosphoribosylglycinamide Synthetase in *Chlorella*
Broken by Various Methods in the Presence of Buffer or Buffer plus Substrates

Cells were cultured and harvested as in Fig. 7. These *Chlorella* cells were suspended in buffer or buffer plus substrates and subjected to various methods of cell breakage. The activity of the enzyme was determined.

<table>
<thead>
<tr>
<th>Method of breakage</th>
<th>Solution for breakage</th>
<th>Units enzyme/50 ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze-Thaw</td>
<td>Buffer + substrates</td>
<td>1.16</td>
</tr>
<tr>
<td>Freeze-Thaw + Sonication</td>
<td>Buffer + substrates</td>
<td>1.83</td>
</tr>
<tr>
<td>Sonication</td>
<td>Buffer</td>
<td>1.88</td>
</tr>
<tr>
<td>French Press</td>
<td>Buffer</td>
<td>2.06</td>
</tr>
<tr>
<td>French Press</td>
<td>Buffer + substrates</td>
<td>1.64</td>
</tr>
</tbody>
</table>
During cell cycle experiments, it is convenient to harvest samples of cells and to freeze them until assay at some later time. It was important, therefore, to determine the stability of the enzyme in cells which had been frozen, thawed, and ruptured in the French press. Such an experiment was performed, by passing the cell material in buffer through the French press twice, to ascertain the effect of the treatment on the stability of the enzyme (Fig. 9). The enzyme was stable through both treatments, and did not show the rapid loss in activity observed in whole frozen-thawed cells in buffer (Fig. 7).

To attempt to determine if endogenous compounds or soluble protein concentration was playing a role in stabilizing the enzyme, the homogenate was diluted 5-fold in buffer or in buffer plus substrates after the second consecutive breakage in the French press. There was no significant difference between the treatments, however, the activity was lower than expected (Fig. 9).

Since the enzyme appeared to be stable to repeated treatments in the French press, cells previously frozen in buffer were passed through twice to eliminate variation in breakage. Their enzyme activities were compared to fresh cells receiving the same treatment, and to sonicated fresh cells (Table II). The frozen cells broken twice showed lower activity than fresh cells sonicated or passed through the French press. Frozen cells from the fourth and eleventh hour harvest showed some loss in activity after being passed through the French press for the second time.
Fig. 9. Stability of phosphoribosylglycinamide synthetase was assayed after breaking cells twice in the French press in buffer, and again after diluting 5-fold with buffer or buffer plus all the substrates. Chlorella was cultured and harvested as in Fig. 7. One ml p.c.v. were suspended in buffer, pelleted, frozen for 48 hours at -20°, and suspended in buffer. This suspension was broken in the French press once, O, and then broken again, A, removing 1 ml samples to incubate at 38.5°. After the second time the cells were broken in the French press, samples were taken and diluted 5-fold with buffer, A; or buffer plus all the substrates, O. Samples were taken every 20 min to assay for enzyme activity.
Fig. 9
Table II

The Activity of Phosphoribosylglycinamide Synthetase in Chlorella
Broken by Various Methods at the Fourth and Eleventh
Hours of the Cell Cycle

Cells were cultured as in Fig. 7, and harvested at the
fourth and eleventh hours. Pellets of Chlorella were suspended in
buffer and subjected to the various methods of breakage. The
activity of the enzyme was assayed.

<table>
<thead>
<tr>
<th>Method of Breakage</th>
<th>Units Enzyme/50 ul</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh Cells (4th Hr)</td>
</tr>
<tr>
<td>Sonication</td>
<td>1.42</td>
</tr>
<tr>
<td>Freeze-Thaw</td>
<td>--</td>
</tr>
<tr>
<td>French Press #1</td>
<td>1.33</td>
</tr>
<tr>
<td>French Press #2</td>
<td>1.45</td>
</tr>
</tbody>
</table>
To determine if enzyme activity was proportional to cell concentration, frozen and fresh cells which had been broken twice in the French press in the previous experiment, were diluted and assayed (Fig. 10). The activity of both preparations was proportional to cell concentration down to 0.5 ml p.c.v. Although the diluted activity was stable, the activity was lower than anticipated (Fig. 9), indicating that some activator may have been diluted out. For all subsequent experiments the cell material that was homogenized in 15 ml was maintained over 0.5 ml p.c.v.

The highest enzyme activity could be achieved by breaking fresh cells in the French press twice. To examine this procedure at different times in the cell cycle, cells were harvested at the fourth and eleventh hours and passed through the French press three times in the presence of buffer or buffer plus substrates. These activities were compared to cells sonicated in buffer (Table III, A and B). Cells passed through the French press two or three times in the presence of buffer plus substrates showed the highest activity. Therefore, in cell cycle experiments, samples were suspended in buffer plus substrates, passed through the French press twice, and assayed.

The microenvironment of the enzyme must be considered in explaining the differences in activity and stability of phosphoribosyl-glycinamide synthetase in the different treatments. In cells subjected to freeze-thawing, low molecular weight molecules can pass
Fig. 10. The activity of phosphoribosylglycinamide was assayed in crude enzyme preparations from cells diluted to various concentrations. *Chlorella* was cultured and harvested as in Fig. 7. Cells were suspended in buffer and broken in the French press twice, O. Another sample was pelleted, frozen, thawed, and resuspended in buffer. These frozen-thawed cells were broken in the French press twice, △. Samples from both of these treatments were diluted 2-, 4-, and 8-fold, and assayed for enzyme activity.
Fig. 10
Table III

The Activity of Phosphoribosylglycinamide Synthetase in Fresh Chlorella Cells Broken by Various Treatments in the Presence of Buffer and Buffers plus Substrates

Cells were cultured and harvested as in Fig. 7, and Table II. These Chlorella cells were suspended in buffer and buffer plus substrates, and subjected to various breakage treatments. The enzyme activity was assayed.

<table>
<thead>
<tr>
<th>Method of Breakage</th>
<th>Buffer</th>
<th>Buffer + Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td>1.16</td>
<td>----</td>
</tr>
<tr>
<td>French Press #1</td>
<td>1.18</td>
<td>1.20</td>
</tr>
<tr>
<td>French Press #2</td>
<td>1.20</td>
<td>1.37</td>
</tr>
<tr>
<td>French Press #3</td>
<td>1.29</td>
<td>1.42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method of Breakage</th>
<th>Buffer</th>
<th>Buffer + Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonication</td>
<td>1.02</td>
<td>----</td>
</tr>
<tr>
<td>French Press #1</td>
<td>1.06</td>
<td>0.94</td>
</tr>
<tr>
<td>French Press #2</td>
<td>1.01</td>
<td>1.08</td>
</tr>
<tr>
<td>French Press #3</td>
<td>1.02</td>
<td>1.10</td>
</tr>
</tbody>
</table>
through the disrupted membrane barriers while high molecular weight molecules are retained (26,27). If these cells or fresh cells are broken in the French press or sonicated, all barriers to permeability are removed and the soluble protein concentrations may be elevated with respect to the environment of the enzyme. This elevated protein concentration may stabilize the enzyme without the presence of substrates. Another possibility is the enzyme may be loosely bound to a membrane or other protein in vivo and in freeze-thawed cells. If this loose association is disrupted by breaking the cells with the French press or by sonic oscillation, a conformational change of the enzyme may occur, making it no longer dependent on substrate for stability.

Phosphoribosylglycinamidase Synthetase Activity during the Cell Cycle - Phosphoribosylglycinamidase synthetase activity showed an exponential increase during the cell cycle of Chlorella cultured under conditions to give a division number of 4 (Fig. 11). The enzyme activity paralleled total protein during most of the cell cycle, and followed essentially the same pattern in cells selected directly from continuously-lighted cultures or from cultures at the end of a dark period. Total DNA and cell number remained constant until the eighth and ninth hours, respectively, when they increased in a stepwise manner.
Fig. 11. The pattern of phosphoribosylglycinamide synthetase was determined in synchronous Chlorella with a division number of 4. The cells were harvested every hour and resuspended in buffer plus all of the substrates (ATP-MgCl₂, 10.95 umoles/50 ul; PRA (ribose-5-P, NH₄OH), 3.44 umoles/50 ul; glycine, 3.125 umoles/50 ul). These cell suspensions were then broken in the French press twice. Experiment 1: cells selected from the end of a dark period, Δ, ○, △, ◊, levels of enzyme, total protein, total DNA, and cell number, respectively. The initial levels of these parameters per ml of culture were 1.74 × 10⁻³ units, 328 ug, 8.43 ug, and 145 × 10⁶ cells. Experiment 2: Cells selected from continuous light, ▽, levels of enzyme.
Fig. 11
The enzyme trend during cell cycles examined by Molloy (15) using freeze-thawed cells and cells broken by sonication showed a sharp increase in activity during the first two hours of the cycle, a plateau between the second and fifth hours, and a peak at the sixth hour. Then the enzyme activity increased in a continuous exponential fashion into the next cycle. The difference between the work presented here and in Molloy's work was probably due to the instability of the enzyme as assayed by Molloy.

Because the enzyme increases in an exponential manner before and during the period of DNA replication, the change in gene dosage does not appear to effect the rate of synthesis of the enzyme. This lack of gene dose effect and the parallel increase of the enzyme to total protein and RNA (29), suggests that the rate of translation may be limiting the rate of synthesis of the enzyme. Alternately, because RNA is synthesized at an exponential rate, the end products of the purine biosynthetic pathway might be decreasing in a similar manner resulting in an exponential derepression of the structural gene of this enzyme, overshadowing the change in gene dosage.

Mitchison suggested in a recent review (30), that there may be a tendency for catabolic enzymes to increase in a continuous manner while biosynthetic enzymes increase in steps during the cell cycle of eucaryotes. Because phosphoribosylglycinamide synthetase
increases in a continuous exponential manner in the cell cycle of *Chlorella*, it would appear that the proposal that biosynthetic enzymes are always stepwise does not hold for all eucaryotes.
SUMMARY

The blue-green alga, *S. lividus*, was isolated free from contaminating bacteria by selecting colonies off agar plates which had been maintained in an atmosphere of 4% CO$_2$-air. Attempts were made to synchronize the algae by intermittent illumination, isopycnic centrifugation, and differential centrifugation. Cells selected by differential centrifugation were the only ones to give acceptable synchrony with cell number increasing in a short division time and a high cross-wall index (85%).

Because the density of the green alga *C. sorokiniana* appears to increase during the cell cycle, synchronous cultures of this organism were successfully selected with isopycnic centrifugation.

The enzyme phosphoribosylglycinamide synthetase was found to be stable in *Chlorella* cells frozen and thawed once, provided substrates were present in the buffer. When frozen-thawed cells or fresh cells were broken in the French press or by a sonic oscillator, the enzyme was no longer dependent on substrates for stability. However, cells broken by sonication showed an ATP-MgCl$_2$ dependent linear loss of enzyme activity on incubation at 38.5°C. Breaking fresh cells twice in the French press in the presence of substrates, yielded an enzyme preparation with the highest activity and stability.

This enzyme increased in a continuous exponential fashion, paralleling the increase in total protein, during the cell cycle of cells selected by the isopycnic procedure. Because the enzyme

- **hb** -
continued to increase at the same exponential rate during the period of DNA replication, the increase in gene dosage did not appear to affect the increase in activity of the enzyme during the cell cycle.
BIBLIOGRAPHY


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DENSITY GRADIENT PROCEDURES FOR THE SELECTION OF SYNCHRONOUS CELLS OF SYNECHOCOCUS LIVIDUS AND CHLORELLA SOROKINIANA, AND THE APPLICATION OF THE ISOPYCNIC TECHNIQUE TO THE STUDY OF THE PATTERNS OF PHOSPHORIBOSYLGLYCINAMIDE SYNTHETASE DURING THE CELL CYCLE OF CHLORELLA

Thomas Orr Sitz

Abstract

The blue-green alga, Synechococcus lividus, was isolated free from contaminating bacteria by selecting colonies off agar plates which had been maintained in an atmosphere of 4% CO₂-air. Attempts were made to synchronize the algae by intermittent illumination, isopycnic centrifugation, and differential centrifugation. Cells selected by differential centrifugation were the only ones to give acceptable synchrony with cell number increasing in a short division time and a high cross-wall index (85%).

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This enzyme increased in a continuous exponential fashion, paralleling the increase in total protein, during the cell cycle of cells selected by the isopycnic procedure. Because the enzyme continued to increase at the same exponential rate during the period of DNA replication, the increase in gene dosage did not appear to affect the increase in activity of the enzyme during the cell cycle.