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# Enzyme and Nucleic Acid Formation During Synchronous Growth of *Rhodopseudomonas spheroides*

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The synthesis of various cell components was examined during the anaerobic photosynthetic growth of synchronous populations of *Rhodopseudomonas spheroides*. Net deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein increased continuously as did the rate of incorporation of radioactive precursors into protein. The rates of incorporation of radioactive precursors into RNA and DNA were marked by abrupt discontinuities. It is not clear whether these discontinuities represent changes in rates of synthesis or fluctuations in precursor pools. Although the synthesis of bacteriochlorophyll occurred in a continuous manner, those enzymes examined which are involved in the synthesis of tetrapyrroles, i.e., succinyl CoA thiokinase, δ-aminolevulinic acid synthetase, and δ-aminolevulinic acid dehydrase, increased discontinuously. Two other enzymes not involved in tetrapyrrole biosynthesis were examined. Alkaline phosphatase increased in a stepwise manner during the division cycle, whereas the synthesis of ornithine transcarbamylase increased rapidly before leveling off for a period of time until synthesis began again. In each instance of discontinuous enzyme synthesis, increases occurred at regular and characteristic times during the division cycle. Ammonium sulfate precipitation was employed to remove low molecular weight end product inhibitors from enzyme preparations. These studies suggested that the stepwise increases in enzyme activity observed in the present investigation were not affected by periodic end product inhibition. A temporal map of enzyme synthesis during the division cycle was constructed. Both δ-aminolevulinic acid synthetase and δ-aminolevulinic acid dehydrase appeared early in the division cycle, whereas alkaline phosphatase and succinyl CoA thiokinase appeared later on.

The synthesis of many enzymes has been shown to be discontinuous in synchronously dividing populations of bacteria (17, 22-24) and yeast (2, 13, 16, 29). In each instance, changes in the rate of enzyme synthesis have been found to occur at regular times during the division cycle and to result in a characteristic sequence of enzyme synthesis. It has been suggested that the replication cycle is initiated at the same time for all cells during synchronous cell division (8), and, since evidence exists indicating that gene replication is sequential and nonrandom (5, 7, 26), the synthesis of gene products might similarly be expected to occur in a sequential manner. Therefore, it would appear that a temporal map of enzyme synthesis during the division cycle might be similar to an actual genetic map; in fact, Masters and Pardee (24) have shown this to be the case in *Bacillus subtilis*.

The nonsulfur purple bacterium *Rhodopseudomonas spheroides* is capable of growing in either aerobic-dark or anaerobic-light conditions. When grown under the latter conditions, energy is obtained by photosynthesis which requires the participation of the specialized pigment, bacteriochlorophyll. The enzymes succinyl CoA thiokinase, δ-aminolevulinic acid dehydrase, and δ-aminolevulinic acid synthetase are involved in the initial steps of the biosynthesis of bacteriochlorophyll. Lascelles has extensively studied these enzymes and several of the factors involved in controlling their synthesis (18, 19).

In this report, we intend to describe the characteristics of synthesis of these and certain other enzymes as well as to define the general nature of macromolecular synthesis during synchronous growth of *R. spheroides*.

## MATERIALS AND METHODS

*Cultivation of organism. R. spheroides* (strain 2.4.1) was grown in the medium described by Cohen-Bazire et al. (7) with the addition of 0.1% sodium glutamate

and 0.1% sodium acetate. The cultures were grown in either 1-liter Roux bottles or, when larger cultures were required, 2-liter straight-sided reagent bottles. These culture bottles were placed in a thermostatically controlled aquarium water bath, surrounded by tungsten lamps for illumination, and were maintained at 25 C. The light intensity incident on the culture bottles was generally kept at 150 ft-c, and anaerobic conditions were maintained in all experiments by gassing with a mixture of 95% $N_2$-5% $CO_2$.

*Bacterial synchronization.* Cultures were synchronized by the stationary-phase method as described by Cutler and Evans (8). Samples (2 ml) for cell counts were collected in tubes containing 0.05 ml of 40% formaldehyde, and cells were counted routinely in a Petroff-Hausser chamber with a Zeiss phase-contrast microscope. At least 500 to 1,000 cells were counted in each sample.

*Bacteriochlorophyll analysis.* The bacteriochlorophyll concentrations in whole cells were determined by the method of Cohen-Bazire et al. (7).

*Preparation of samples for analysis.* Measured samples of the synchronized cells were withdrawn from the main culture bottle at 30-min intervals, quickly cooled in an ice bath, and centrifuged at 4 C for 10 min at 10,000 × *g*. Immediately after decanting the supernatant solution, the pellet of cells was stored in the freezer at −20 C until just before the performance of specific analyses. The pellet of cells was adjusted to a known volume with 0.05 M tris(hydroxymethyl)aminomethane buffer, *p*H 7.5, and the cells were disrupted in an MSE ultrasonic disintegrator. Samples of the cell-free extract equivalent to $10^9$ to $2 \times 10^9$ bacteria were employed in each of the enzyme assays.

*Assay of enzymatic activities.* δ-Aminolevulinic acid synthetase (ALA synthetase) and δ-aminolevulinic acid dehydrase (ALA dehydrase) were measured according to the procedures described by Burnham and Lascelles (4) by use of the color reaction of Mauzerall and Granick (25). One unit of ALA synthetase or ALA dehydrase is defined as the amount of enzyme which catalyzes the formation of 1 mμmole of product per hr under the standard conditions of assay. Succinyl CoA thiokinase was measured according to the procedure of Burnham and Lascelles (4); the color reactions described by Lipman and Tuttle (21) were employed. One unit of succinyl CoA thiokinase is defined as the amount of enzyme which catalyzes the formation of 1 μmole of succino-hydroxamate per hr under the standard conditions of the assay.

Alkaline phosphatase activity was measured according to the method of Garen and Levinthal (11). One unit of alkaline phosphatase is that activity liberating 1 mμmole of *p*-nitrophenol per hr under the standard conditions of assay.

Ornithine transcarbamylase activity was measured by the method of Rogers and Novelli (27). One unit of activity is defined as the amount of enzyme which catalyzes the formation of 1 μmole of citrulline per hr under the standard conditions of assay.

*Incorporation of labeled precursors.* Samples of cell suspension, of a volume to contain a constant cell mass (0.26 mg, dry weight), were incubated under the same light and gassing conditions as the main culture for 2 min with the labeled precursor. The amounts and specific activities of the labeled precursors were: 0.5 μc of $^{14}$C-uracil (0.30 μc/μmole); 2.5 μc of $^{3}$H-thymidine (2 c/mmole); 0.5 μc of $^{14}$C algal protein hydrolysate (1 mc/mg). The incorporation of labeled precursors was stopped by the addition of an equal volume of cold 14% trichloroacetic acid. Precipitates were collected and washed with cold 7% trichloroacetic acid on membrane filters. The amounts of radioactivity on the filters were measured with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

## RESULTS

The generation time of 3.5 hr was consistent in repeated experiments, and the synchrony index (28), which relates time for division and number of cells dividing, varied between 0.40 and 0.60. This indicates that the cultures are in an adequate degree of synchrony. The variations in levels of protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) during induced synchronous growth are shown in Fig. 1. After a slight lag, the cell mass increased in an exponential manner. The level of protein increased sharply initially and then adjusted to a constant rate of formation. The synthesis of RNA was marked by discontinuous adjustments of rate, but these

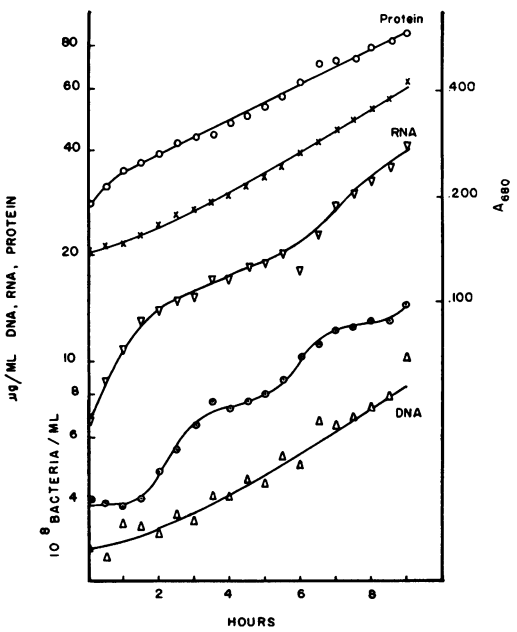


FIG. 1. *Synthesis of DNA, RNA, and protein during synchronous growth. The amounts of RNA, DNA, and protein were determined as previously described (15). Cell mass was determined by the absorbance at 680 mμ (7). Symbols: ○, protein; ×, cell mass; ▽, RNA; ⊙, cell number; △, DNA.*

did not seem to be directly related to the cell division cycle. The variations in rate of RNA and protein synthesis are probably reflections of the conditions employed in the synchronization procedure. DNA is apparently synthesized continuously, since there were no obvious changes in rate during the course of growth.

To further examine the rates of formation of cellular macromolecules, the rates of incorporation of labeled precursors were studied. Figure 2 shows the variation in rate of uptake of $^{14}C$-labeled algal protein hydrolysate during synchronized growth. The variations are very similar to those in total protein. The levels of total RNA and DNA are different from the rates of incorporation of $^{14}C$-uracil and $^{3}H$-thymidine (Fig. 3). The rates of incorporation of precursors into both RNA and DNA underwent discontinuous increases at regular periods in the division cycle. Whether these discontinuous increases are, in fact, variations in the rates of formation of DNA and RNA or merely fluctuations in the rate of uptake of precursors is difficult to determine in the absence of data on the precursor pools. The variance from the constant rate of accumulation of DNA suggests that precursor fluctuations may be the explanation.

The synthesis of bacteriochlorophyll (Fig. 4) during synchronized photosynthetic growth was continuous and unmarked by the stepwise increases that were observed when the rates of incorporation of uracil and thymidine were measured. This finding contrasts with the ob-

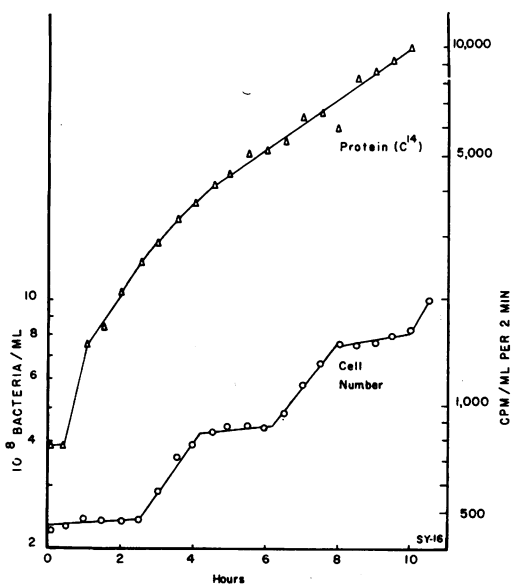


FIG. 2. *Rate of incorporation of $^{14}C$-labeled amino acids during synchronous growth. Symbols: △, counts/min per ml of cell suspension per 2 min of incorporation; ○, cell number.*

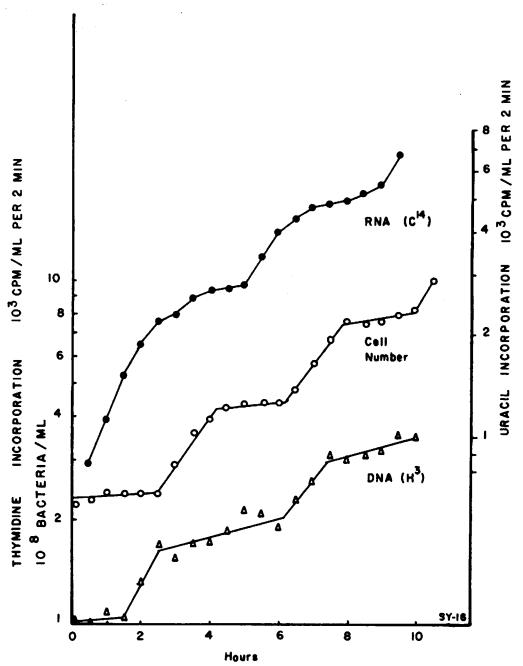


FIG. 3. *Rate of incorporation of labeled precursors into RNA and DNA during synchronous growth. Symbols: ●, $^{14}C$-uracil incorporation, counts/min per ml of cell suspension per 2 min of incubation; △, $^{3}H$-thymidine incorporation, counts/min per ml of cell suspension per 2 min of incubation; ○, cell number.*

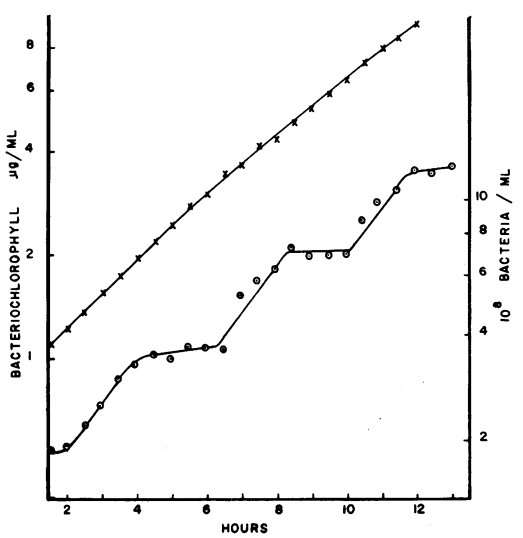


FIG. 4. *Formation of bacteriochlorophyll during synchronous growth. Symbols: ×, bacteriochlorophyll; ⊙, cell number.*

served course of synthesis of three of the enzymes involved in the production of this photopigment. Figure 5 shows the variations in activity of the first three enzymes in the metabolic pathway that culminates in bacteriochlorophyll formation: succinyl CoA thiokinase, ALA synthetase, and ALA dehydrase. The activity of each of these enzymes underwent stepwise increases at characteristic intervals in the division cycle followed by periods of little or no net increase.

To determine whether these discontinuous increases in enzyme activity were restricted to enzymes involved in tetrapyrrole synthesis, two other enzymes not involved in this pathway were examined (Fig. 6). Alkaline phosphatase showed stepwise increases at regular intervals during the division cycle, whereas ornithine transcarbamylase increased very rapidly and its synthesis was not obviously stepwise. After two division cycles, the level of ornithine transcarbamylase activity began to level off and then appeared to begin to increase in a discontinuous manner. The initial rapid synthesis of ornithine transcarbamylase may be a response to the re-establishment of growth following a stationary phase.

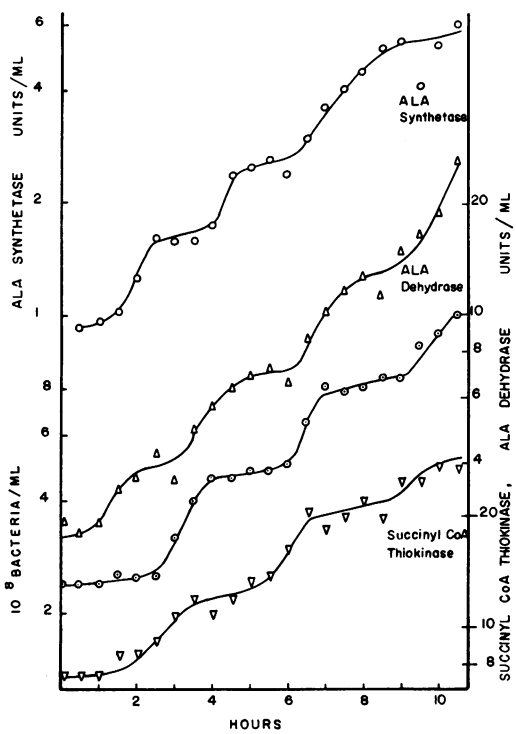


FIG. 5. *Variations in levels of ALA synthetase, ALA dehydrase, and succinyl CoA thiokinase during synchronous growth. Symbols:* ○, *ALA synthetase;* △, *ALA dehydrase;* ▽, *succinyl CoA thiokinase;* ⊙, *cell number.*

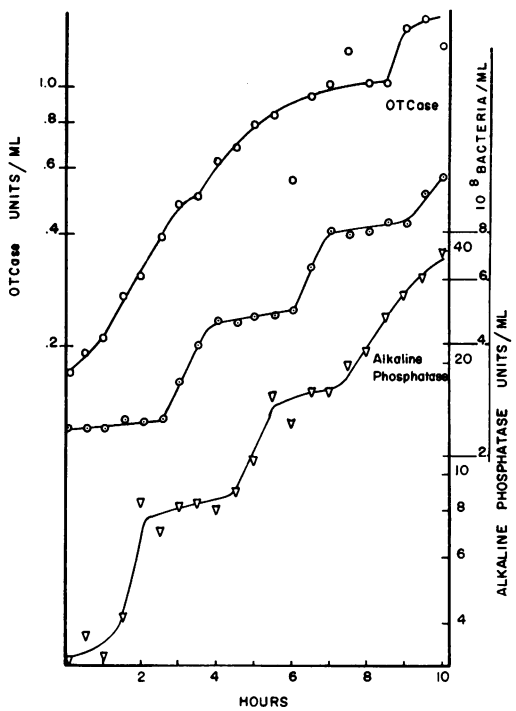


FIG. 6. *Variation in levels of activity of ornithine transcarbamylase (OTCase) and alkaline phosphatase during synchronous growth. Symbols:* ○, *ornithine transcarbamylase;* ▽, *alkaline phosphatase;* ⊙, *cell number.*

In many microbial systems, the synthesis of ornithine transcarbamylase has been shown to be regulated by the concentration of the end product arginine (12, 20). To examine the effect of this corepressor on the pattern of enzyme synthesis during synchronous growth, an experiment was conducted in the presence of 0.5 mM arginine. In the absence of arginine (Fig. 7), enzyme synthesis proceeded in a nonstepwise manner as previously described. However, when arginine was present from the beginning of synchronous growth, the initially rapid increase in enzyme activity did not occur and fairly regular fluctuations were apparent as enzyme synthesis increased in a nearly continuous manner. Thus, the presence of arginine apparently altered the manner in which enzyme activity increased during synchronous division, though it did not result in a characteristic stepwise increase during each division cycle.

The stepwise increases in enzyme activity observed in synchronous cell cultures might be due to variations in the extent of end product inhibition rather than to fluctuations in enzyme formation. An examination of this possibility required

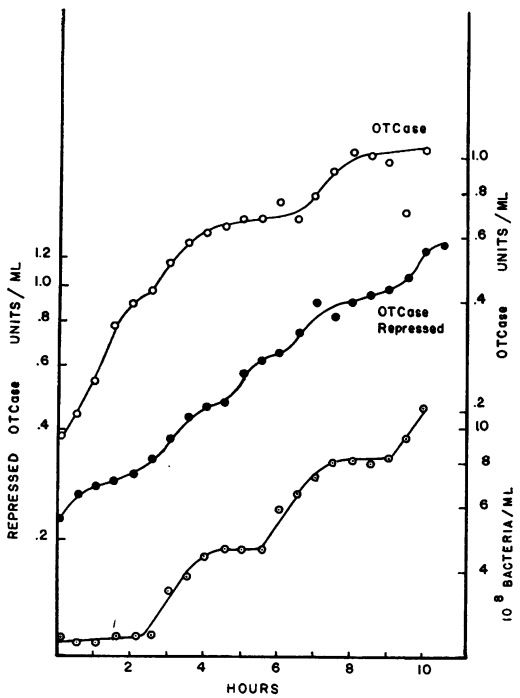


FIG. 7. *Effect of arginine on the formation of ornithine transcarbamylase (OTCase) during synchronous growth. Symbols:* ○, *ornithine transcarbamylase in control culture;* ●, *ornithine transcarbamylase in culture growing in presence of 0.5 mM arginine;* ⊙, *cell number.*

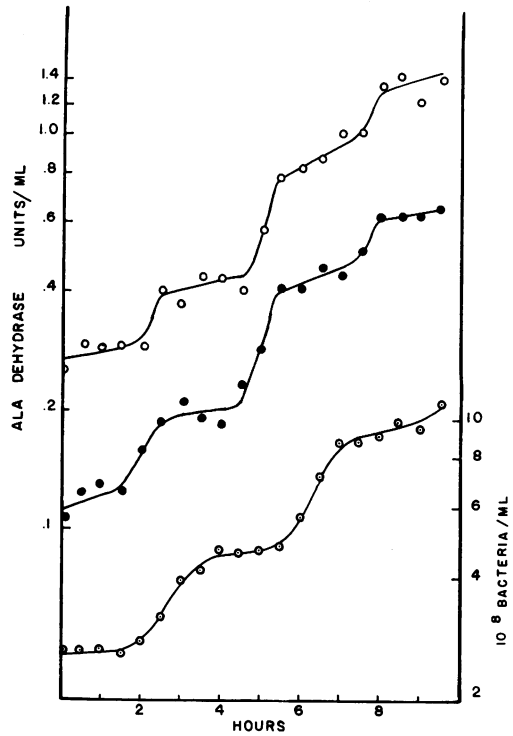


FIG. 8. *Effect of ammonium sulfate precipitation on levels of ALA dehydrase. Symbols:* ○, *ALA dehydrase control;* ●, *ALA dehydrase levels in ammonium sulfate precipitated samples;* ⊙, *cell number.*

the removal from the enzyme of any low molecular weight allosteric effectors which might cause such an inhibition of enzyme activity. To obtain such a separation, ammonium sulfate precipitation was employed. Cell-free extracts were obtained by ultrasonic disruption of samples taken during the time course of a sychrony experiment. Each sample was divided into two equal fractions; one was subjected to ammonium sulfate precipitation and the other served as a control to be employed in the subsequent enzyme assays. The fraction being precipitated was made 80% with respect to ammonium sulfate and was allowed to remain at 4 C for 4 hr. After centrifugation and removal of the supernatant solution, the precipitated protein was dissolved in 0.05 M phosphate buffer, *p*H 7.0. Enzyme assays were then performed on both the ammonium sulfate precipitated and the control samples; an analysis of ALA dehydrase is shown in Fig. 8. Although the precipitation procedure lowered the activity in all samples, a remarkable similarity in the patterns of enzyme activities exists in the control and ammonium sulfate precipitated fractions. The timing and extent of activity increase are virtually identical in both cases. Therefore, it would appear that, in the present study, stepwise increases in enzyme activity were not effected by periodic end product inhibition.

If the points in the cell division cycle in which the stepwise increases of enzyme activity occur are plotted, a linear map can be constructed (Fig. 9). Each marker represents the time of enzyme increase in separate division cycles, and it is apparent that the markers are clustered in different positions for each enzyme. A rapid formation of both ALA synthetase and ALA dehydrase takes place early in the division cycle, whereas alkaline phosphatase and succinic thiokinase are formed later in the division cycle. This suggests that ALA dehydrase and ALA synthetase may be closely positioned to each other on an actual genetic map, as may be expected of enzymes involved in the same pathway.

## DISCUSSION

The synthesis of RNA, DNA, and protein during synchronous growth occurs in a more or less continuous manner. The variations in the rate of synthesis that do occur are attributable to

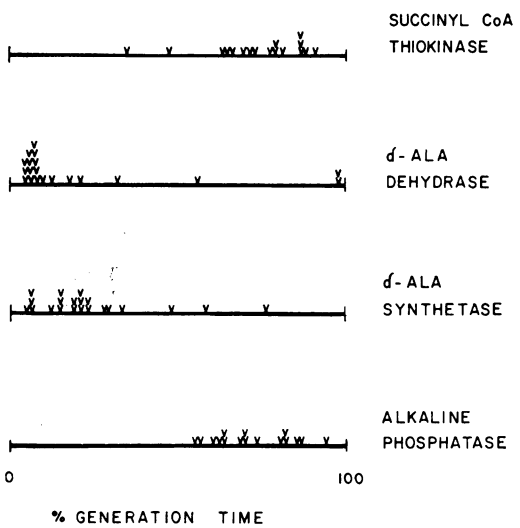


FIG. 9. *Temporal map of enzyme formation during synchronous growth. Points represent midpoints of stepwise enzyme increases. Generation time was measured from midpoints of increases in cell number.*

the conditions involved in the synchronization. It seems unlikely that the discontinuous nature of uracil uptake actually represents fluctuation in RNA polymerase activity. The explanation probably involves the periodic formation of some rate-limiting enzyme controlling the entrance of uracil into the cell or its conversion to a nucleotide.

Clark and Maaløe (6) observed discontinuous increases in the rate of thymidine uptake in synchronously growing populations of *Escherichia coli*; they interpreted these discontinuous increases as increases in the rate of DNA synthesis. Similar findings in the present experiments, however, do not seem to be reflected in the rate of formation of total DNA. It would appear that, at least in the case of *R. spheroides*, the discontinuous increases in the rate of thymidine incorporation are not related to the actual rate of DNA synthesis. Studies of fluctuations in the precursor pools would be necessary to resolve this point.

The synthesis of several enzymes in *R. spheroides* was discontinuous during the division cycle in a similar manner to that described for other microorganisms. The pattern of synthesis of ornithine transcarbamylase, which did not show regular stepwise increases, was markedly affected by the presence of arginine. The repressive effects of arginine on ornithine transcarbamylase synthesis were not as pronounced in *R. spheroides* as in other systems. In the presence of this amino acid, however, enzyme formation appeared to show discontinuities, although these were not as regular nor as evident as those of the other enzymes studied. It has been suggested (22) that one of the factors involved in the control of stepwise enzyme synthesis is the cyclic variation in repressor level. This may be the case in *R. spheroides*, and further experiments regarding this point will be reported elsewhere (10).

We considered the possibility that periodic end product inhibition might be responsible for periodic enzyme increases in the present experiments. However, it was shown that the discontinuous pattern of enzyme increases during the division cycle were similar in both the crude cell extract as well as in the fraction that had been subjected to ammonium sulfate precipitation. The possibility that high salt concentrations enhanced hydrophobic interactions between the low molecular weight inhibitor and its corresponding receptor site on the enzyme molecule cannot be excluded. However, since the precipitated enzyme fractions were dissolved in a rather large quantity of low ionic strength buffer before enzyme assay, it was thought that suitable conditions were allowed for a dissociation of the enzyme molecule and any low molecular weight inhibitor which might be present. The present studies, although not conclusive, strongly suggest that discontinuous enzyme synthesis during the synchronous growth of *R. spheroides* is not affected by periodic end product inhibition.

Masters and Pardee (24) have shown that, in *B. subtilis*, the positions of enzyme increase in a temporal map correspond to the relative positions on a genetic map. Although the temporal enzyme map constructed for *R. spheroides* may represent sequential genic expression, this could not be established since methods have not yet been developed for the genetic analysis of photosynthetic bacteria. The construction of a temporal map of enzyme synthesis should prove useful in those systems where genetic exchange has not been possible. The relative positions of enzymes, although reasonably constant, are strongly affected by changes in repressor concentration (22). The cultural conditions must be carefully controlled if a temporal map is to approximate the genic order of the enzymes involved. A similar report of apparent genomic mapping of a microorganism which is not subject to direct genetic analysis has recently been described by Altenbern (1).

In many instances, ALA synthetase was found to be inactivated more rapidly than ALA dehydrase. Similar observations have been made by Bull and Lascelles (3), who showed that ALA synthetase is rapidly destroyed or inactivated when *R. spheroides* is incubated under conditions which prevent protein synthesis, and by Goto et al. (14), who indicated that the rate of turnover

of ALA synthetase was considerably higher than that of ALA dehydrase. In view of these findings, Donachie's suggestion (9) that enzyme instability may contribute to periodic enzyme synthesis cannot be discounted.

ACKNOWLEDGMENTS

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