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Relationship between Protein and Nucleic Acid Synthesis in *Pseudomonas azotogensis* Grown in Hexetidine[1]

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Introduction

During the past decade the accumulative evidence in microorganisms has clearly indicated that ribonucleic acid (RNA) is involved in protein synthesis (1, 2). Studies with subcellular systems have shown at least two types of RNA which play a role in protein synthesis. Approximately one-tenth of the RNA is soluble and functions in the activation of amino acids (3). The remainder of the RNA (80–90%) is present in ribonuclear particles which have been postulated as the templates for protein synthesis (4, 5).

In addition the suggestion has been made that protein cannot be formed without the concurrent formation of RNA, i.e., the two processes are mandatorily coupled. Experiments which show a constancy, under a variety of conditions, in the ratio of newly formed protein to newly formed RNA have been used to support the contention of mandatory coupling (6, 7). Conversely, if it were shown that the ratio of Δ protein/Δ RNA were subject to wide variation, then one would have to abandon or modify the concept. One could, for example, assume that protein synthesis is coupled to the formation of a particular fraction of the RNA (soluble RNA or ribonuclear particles) and not to total RNA.

We have undertaken to obtain further information on the protein/RNA ratio in exponentially growing bacteria by employing the more convenient and accurate measurement of Δ protein/Δ nucleic acid. The effect of hexetidine on this ratio has been examined and it has been found that the ratio can be increased by a factor of 2 from that found in control cultures.

Methods and Materials

Growth of Bacteria

Pseudomonas azotogensis was grown in a synthetic medium, consisting of NH_4NO_3, 1 g.; K_2HPO_4, 6.5 g.; $MgSO_4$, 0.5 g.; and glucose, 5 g. in 1 l. water, pH 7.0. The

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medium was shaken at 30°C. Growth was followed at 600 mμ in a Beckman spectrophotometer.

Cell Fractionation and Radioactivity Measurements

For measurements of isotope incorporation, 10-ml. samples were removed at intervals. The cells were washed with nonradioactive synthetic medium and chemically fractionated by a modification of the Schneider method (8). The hot trichloroacetic acid (TCA)-insoluble fraction was resuspended in 10% NH_4OH. Aliquot samples of the various fractions were spread in duplicate on stainless steel planchets and counted with a conventional gas-flow counter. Where necessary, corrections were made for self-adsorption.

Materials

Hexetidine (bis-1,3-β-ethylhexyl-5-methyl-5-aminohexahydropyrimidine) was furnished by the Warner Lambert Pharmaceutical Company. Uniformly labeled glucose (2.4 mc./mmole) was obtained from the Fisher Scientific Company, carrier-free sulfate-S^{35} and phosphate-P^{32} from the Oak Ridge National Laboratory, and DL-phenylalanine-3-C^{14} (2.1 mc./mmole) from the Commissariat à l' Énergie Atomique, France.

Results

Effect of Hexetidine on Growth and Glucose Assimilation

Figure 1 shows the growth of *P. azotogensis* in synthetic medium containing 0.5% radioactive glucose with and without 10 μg./ml. hexetidine. Ra-

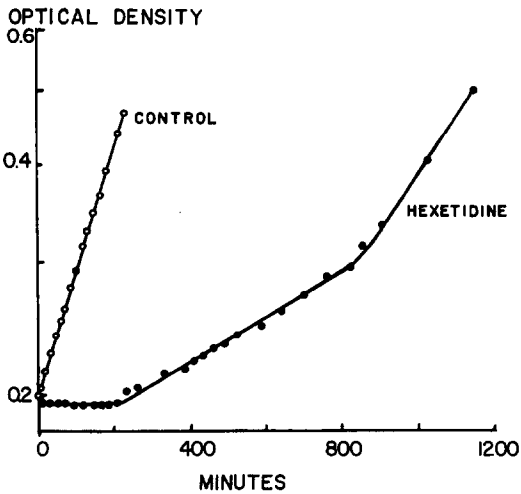

FIG. 1. Effect of hexetidine on the growth of *Pseudomonas azotogensis*. Logarithmically grown cells were centrifuged, washed, and resuspended in synthetic medium (200 ml.) to an O.D. of 0.200. Uniformly labeled glucose (0.5%) was added to the culture, the culture was divided, and hexetidine (10 μg./ml.) was added to one of the flasks. The flasks were shaken aerobically at 30°C., and the density was followed with a Beckman DU spectrophotometer at 600 mμ.

dioactive glucose and hexetidine were added at an initial optical density (O.D.) of 0.200. Following the addition of hexetidine, growth abruptly ceased and some cell-clumping occurred. After a lag period, exponential growth is resumed but at a decreased rate (division time 1060 min. compared to 170 min. in the control). The extent of the lag period and the rate of exponential growth achieved are a function of the concentration of hexetidine employed. The cells eventually appear to overcome in part the inhibition by hexetidine. In subsequent experiments, unless otherwise indicated, the analyses were restricted to the earlier portion of the growth curve (namely the inhibited period). Upon hexetidine addition, the number of viable centers dramatically decreases probably due in part to the formation of cell clumps. However, the validity of employing increases in optical density as a measure of cellular division is shown in Fig. 2. The Δ viable count/Δ O.D. of the control and hexetidine cultures were 2.6×11^9.

Throughout the experiment represented by Fig. 2, cells were removed and fractionated, and the radioactive carbon content of the various frac-

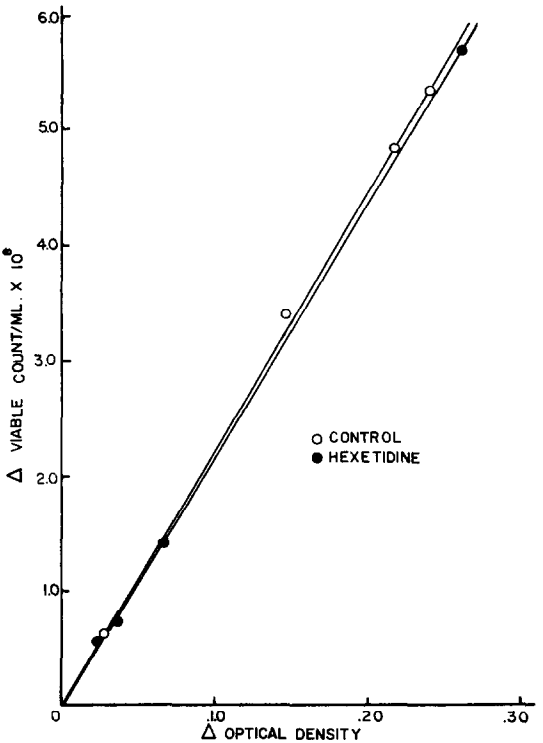

FIG. 2. Relationship between optical density and viable cells. See Fig. 1 for details.

TABLE I

Effect of Hexetidine on Glucose Assimilation

At intervals, 10-ml. samples were removed from the cultures described in Fig. 1. The cultures were fractionated as described under Methods, the μmoles C/ml. of culture for each fraction determined, and the differential rates (Δ μmoles C/Δ O.D.) calculated.

Fraction	Control Δ μmoles C/ Δ O.D.	Δ cells/ Δ O.D.	Hexetidine Δ μmoles C/ Δ O.D.	Δ cells/ Δ O.D.
Cold TCA-soluble	2.58		2.42	
Hot TCA-soluble	10.40		9.50	
Hot TCA-insoluble	17.2		35.5	
Total cells	31.9	2.6×10^9	48.2	2.5×10^9
Per cent recovery	95		99	

tions was determined. From the specific activity of the initial glucose, the differential rate of carbon uptake (Δ μmoles C/Δ O.D.) to the various fractions was calculated. The results shown in Table I indicate that although the ratio of Δ cells/Δ O.D. is the same in the two cultures, the mass as represented by the C content is about 50% higher in the hexetidine culture (48.2 μmoles C/Δ O.D. compared to 31.9 μmoles C/Δ O.D.). That is, the mass/unit O.D. and hence the mass/cell is increased in the presence of hexetidine, and this increase is entirely accounted for by the material associated with the hot TCA-insoluble fraction. Despite the difference in mass/cell, microscopic observation revealed no apparent differences in cell size in the control and inhibited culture.

Relatively little change is observed following hexetidine addition to either the cold TCA-soluble or hot TCA-soluble fractions. The over-all recovery of carbon in the control and hexetidine-grown cells was 95 and 99%, respectively. In a parallel experiment, hexetidine was observed to have no effect on the differential rate of synthesis of the alcohol-soluble fraction.

Nucleic Acid and Protein Synthesis in the Presence of Hexetidine

The results of Table I indicate that the ratio of Δ hot TCA-insoluble C/Δ hot TCA-soluble C is increased in the presence of hexetidine. It will be assumed for the moment that these are represented as protein and nucleic acid, respectively. Confirmatory experiments to support this will be described below. The effect of hexetidine is brought out more clearly by plotting Δ protein/Δ nucleic acid for the two cultures (Fig. 3). The results of such a plot show that in each culture the Δ protein/Δ nucleic acid is constant for the duration of the experiment and that in the presence of hexetidine twice as much protein is synthesized per unit of newly formed nucleic acid.

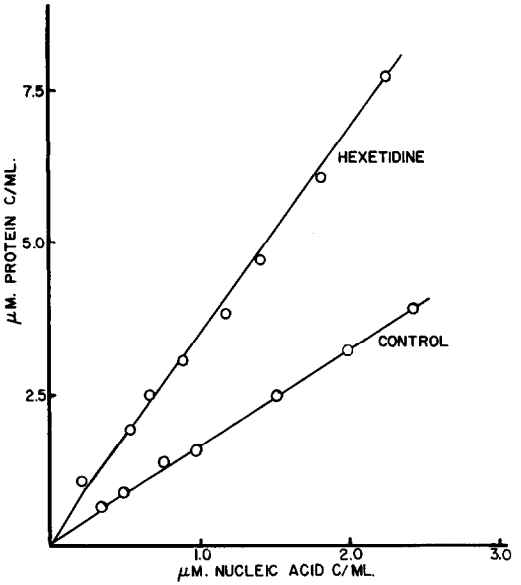

FIG. 3. Relationship between protein and nucleic acid synthesis. See Table I for details. The increments in $\Delta\mu$M C in the protein fraction (hot TCA-insoluble) are plotted against the $\Delta\mu$M C in the nucleic acid fraction (hot TCA-soluble) for the cultures grown in the presence and absence of hexetidine.

The effect of hexetidine on the incorporation of a nucleic acid precursor $P^{32}O_4^{---}$, into whole cells and into nucleic acids is shown in Fig. 4. Although hexetidine increases by 30 % the differential rate of total P uptake, the differential rate of nucleic acid synthesis is unaffected. These later findings are consistent with the failure of hexetidine to influence the carbon flow to the hot TCA-soluble fraction (Table I). The increased P content/bacterium in the presence of hexetidine was found to be largely attributed to the intracellular precursor P pool.

Since *P. azotogensis* grows well in the synthetic medium containing sulfate as the sole source of sulfur, it is convenient to use the radioactive isotope of sulfur, S^{35}, as a labeling atom for measuring protein synthesis. The assimilated sulfur in bacteria is found essentially in cyst(e)ine, methionine, and glutathione (9). Sulfur metabolism therefore provides a relatively simple measure of protein synthesis. The influence of hexetidine on sulfur incorporation into the TCA-insoluble fraction was examined in the following experiment. To an exponentially growing culture of bacteria (O.D. = 0.140), $S^{35}O_4^{--}$ was added to a final activity of 1.77×10^6 counts/min./ml. The culture was immediately divided, and hexetidine (6 μg./ml.) was added to one flask. Figure 5 shows the differential rate of S^{35} incorporation into

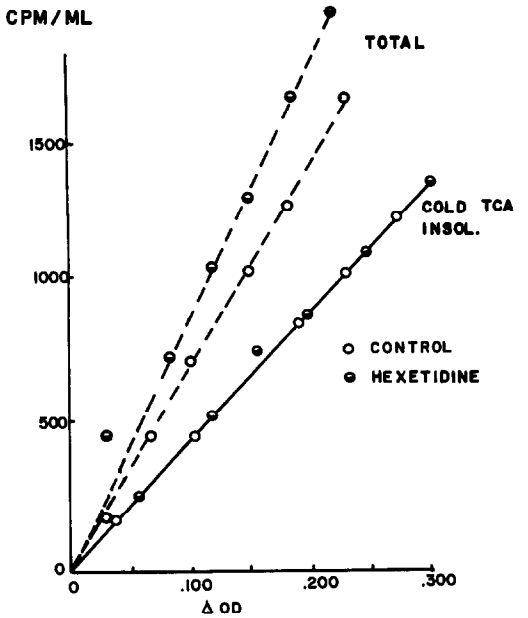

FIG. 4. Effect of hexetidine on phosphate incorporation. In an experiment similar to that described in Fig. 1, $P^{32}O_4^{--}$ and hexetidine (6 μg./ml.) were added to an exponentially growing culture at an O.D. of 0.210. Cells were fractionated at intervals as previously described.

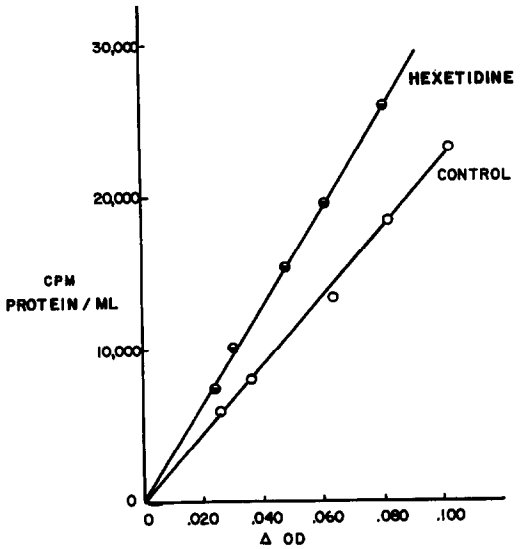

FIG. 5. Stimulation of $S^{35}O_4^{--}$ incorporation by hexetidine. To an exponentially growing culture (O.D. of 0.140), $S^{35}O_4^{--}$ and hexetidine (6 μg./ml.) were added. Radioactivities were determined on the cold TCA-insoluble fraction at intervals during growth.

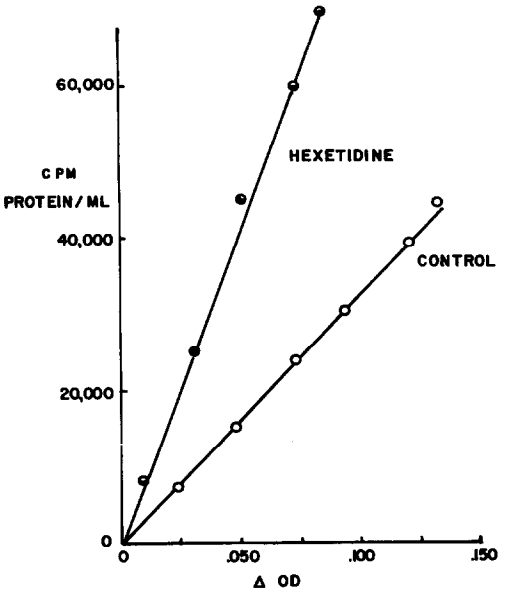

FIG. 6. Stimulation of phenylalanine incorporation by hexetidine. Experiment was identical to that described in Fig. 5, except DL-phenylalanine-3-C^{14} was used as the labeling material.

the TCA-insoluble fraction. The addition of hexetidine increases by 45 % the incorporation of sulfate-S^{35} into the proteins of *P. azotogensis* (2.25×10^5 counts/min./Δ O.D. compared to 3.26×10^5 counts/min./Δ O.D.).

As further confirmation, the effect of hexetidine on the incorporation of an exogenous amino acid into the newly synthesized proteins was examined. In an experiment analogous to that described with $S^{35}O_4^{--}$, the incorporation of phenylalanine-3-C^{14} (2.5×10^{-5} *M*) was followed in the presence and absence of 6 μg./ml. hexetidine. In the presence of the inhibitor the incorporation of phenylalanine into protein was increased 2.6-fold (8.6×10^5 counts/min./ml./Δ O.D.) compared to 3.3×10^5 counts/min./ml./Δ O.D.

DISCUSSION

Variations in the ratio of Δ protein/Δ nucleic acid in bacteria have been previously reported in the nonsteady-state conditions existing during the transition between two different growth rates (6, 10, 11). The results reported here show that these ratios are altered in *P. azotogensis* at least by a factor of two in the steady-state conditions of exponential growth obtained in the presence of hexetidine. The inhibitor leads to a 50 % increase in mass per cell which is entirely accounted for by increased protein, while the

nucleic acid per cell is unaltered (Table I). The properties of the protein synthesized in the presence of hexetidine will be reported elsewhere.

Before considering the implications of this finding, it will be useful to summarize some of the intermediate stages in protein synthesis which have been recently defined. The initial step in protein synthesis is the enzymic formation of aminoacyl adenylates from amino acids and adenosine triphosphate (12). These "activated" amino acids are then transferred to soluble RNA (3) and finally to the ribonuclear particles (4, 5). The last step in protein synthesis is the polymerization of these amino acids and the removal of the protein molecule from the ribonuclear particle. It has further been proposed that the same aminoacyl nucleotides are intermediates for the synthesis of both RNA and protein (13). If this were correct one might expect that any condition which affected the synthesis or function of the intermediates would influence the rates of synthesis of RNA and protein proportionately. Therefore proportionate depression of RNA and protein synthesis produced by ultraviolet light (14), purine or pyrimidine analogs (7), or amino acid starvation (15, 11) does not necessarily indicate a mandatory coupling. Similar effects at a more trivial level are produced when a restriction is placed on the supply of N, C, or energy. The present findings show clearly that the quantitative relationships between RNA and protein synthesis are not fixed and therefore weaken the case for a model involving rigid mandatory coupling.

Similar conclusions can be derived from studies on enzyme biosynthesis where the kinetics suggest that the enzyme is being formed without concurrent formation of a specific RNA. Thus the differential rate of synthesis of induced β-galactosidase (17) and β-glucosidase (18) is constant and maximal from the moment of addition of the inducer. The implication is that the specific enzyme-forming system, that is, the specific RNA particle, exists prior to the addition of the inducer and that the inducer serves only in stimulating its function.

It is obvious that there must be certain interdependence of protein and nucleic acid synthesis since the latter depends on (*a*) the production of those enzymes needed to make RNA precursors and (*b*) cosynthesis of the structural protein which is a major component of stable ribonuclear particles (19). The conclusion of these findings is that apart from this the rate of protein synthesis is regulated by the function and not by the formation of specific RNA particles.

Acknowledgments

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Summary

The ratio of Δ protein/Δ nucleic acid has been studied in exponentially growing cultures of *Pseudomonas azotogensis*. In the presence of hexetidine, this ratio is doubled, resulting in cells containing 50% more material (carbon) and twice as much protein as cells of a control culture. The implications of these findings in relation to current theories of protein synthesis are discussed.

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