THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

TRANSFER RNA METHYLATION AND PROTEIN SYNTHESIS IN *ARTHROBACTER* MORPHOGENESIS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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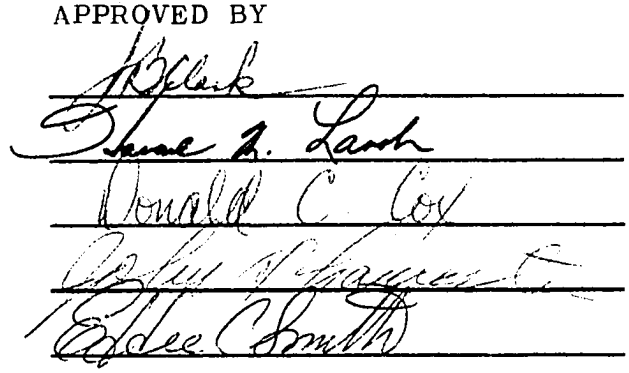
Norman, Oklahoma

1971

TRANSFER RNA METHYLATION AND PROTEIN SYNTHESIS

IN ARTHROBACTER MORPHOGENESIS

APPROVED BY



DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Drs. H. W. Larsh, D. C. Cox, J. H. Lancaster and E. C. Smith for their discussions and critical reading of this dissertation.

Special recognition is given to Dr. J. B. Clark for his guidance and encouragement throughout the work leading to this dissertation.

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CHAPTER I

INTRODUCTION

> The causes and levels of control responsible for morphogenesis are many and complex; their interdependence must be understood in order to interpret their individual roles in the regulation of morphogenesis (56).

I. *Arthrobacter* Morphogenesis

Members of the genus *Arthrobacter* are characterized by the cycle of morphological changes exhibited during growth. Among the various species some difference in the cycle is observed (34, 40, 47, 49). *Arthrobacter crystallopoietes* is characteristically spherical during the stationary phase of growth. The spherical cells elongate into rods during a lag phase that results from inoculation of fresh medium. The rods divide by binary fission while growing exponentially, and gradually shorten into typical spherical cells (13).

The term morphogenesis is commonly used to describe the cycle of morphological variations exhibited by *A. crystallopoietes*. In discussions of eucaryotic cells, the

term morphogenesis refers to the irreversible development of a sequence of events that results in morphological changes (53). In reference to Arthrobacter the term morphogenesis does not exclude reversible phenomena. In this sense the term morphogenesis closely resembles the definition of cellular modulation, the ability to reversibly assume different physiological states (41, 53).

The simple morphological variation and the synchrony of the cycle make A. crystallopoietes a useful tool for studies of the physiological basis of regulatory mechanisms in morphogenesis. A nutritional control of morphogenesis in A. crystallopoietes is reported by Ensign and Wolfe (13). In a chemically defined glucose salts medium, the organism grows and reproduces without undergoing the typical cycle. Upon the addition of a list of apparently unrelated compounds, the organism exhibits the typical morphological variation. Krulwich et. al. (27, 28) report an investigation of the possible correlation between morphogenesis inducing compounds and cell wall composition. Their study reveals a quantitative and qualitative difference in sphere and rod cell walls. Specifically, the rods have a polysaccharide backbone three times longer than the spheres while the spheres show additional amino acid residues in the peptide bridges. Subsequently, Krulwich and Ensign (29) demonstrate that a morphogenesis inducing compound, succinate, inhibits and represses glucose permease. The results also suggest a

direct relationship between nutritional control and cell wall structure. Ferdinandus (14) and Ferdinandus and Clark (15) further correlated morphogenesis with a metabolic role of the inducing compounds. Succinate stimulates the enzymes of lipogenesis in rod formation while the transition from rod to sphere is characterized by an increase in activity of lipolytic enzymes.

II. Transfer RNA Methylation

The detailed analysis of transfer ribonucleic acid (t-RNA) composition reveals the presence of various methylated nitrogenous bases (3). Mandel and Borek (33) show that methionine is the source of methyl groups for the alkylation of nucleic acids *in vitro* and *in vivo*. The immediate methyl donor *in vivo* is shown to be S-adenosylmethionine. Methylation does not occur with the free bases but occurs on the synthesized polynucleotide chain (16, 50).

Studies on the mechanism by which t-RNA methylation affects protein synthesis are based on the model that alteration of the t-RNA conformation affects the function in translation. That t-RNA conformation is altered is suggested by the difference in absorbency (4) and difference in chromatographic mobility (30, 39) shown between methylated and methyl deficient t-RNA.

Some specific functions of t-RNA are shown to be altered by methylation. Several experiments suggest that methylation of t-RNA serves a cognitive role in the

interaction with amino acyl synthetases (36). Escherichia coli amino acyl synthetase charges methylated and methyl deficient t-RNA equally well. On the other hand, yeast amino acyl synthetase shows a reduced ability to charge methyl deficient t-RNA. More recently, Shugart et. al. (43, 44) show the restoration of charging activity of hypomethylated E. coli t-RNA by in vitro methylation. Methylation is also implicated in the ability of the t-RNA to bind to ribosomes (18). Modification of the base adjacent to the anticodon appears to play a role in the attachment of the charged t-RNA to the ribosome.

Qualitative and quantitative changes in the t-RNA methylases are observed in several biological systems which exhibit alteration in regulatory mechanisms. These include metamorphosing insects (1), differentiating lens tissue (25), and the colonizing slime mold (38). In addition, differences in methylase activity exists in a number of malignant tumors as compared with normal tissue (5, 19). These differences include both a change in the capacity to methylate and the specific bases methylated.

More recently, additional evidence is available strengthening the case for methylation of t-RNA serving a regulatory role in morphogenesis. Kerr (25) reports the isolation of a natural inhibitor of t-RNA methylases from adult organs of the rabbit but notes the absence from foetal organs. Similarly, Sharma and Borek (42) report an

inhibitor of t-RNA methylases in the differentiating slime mold Dictyostelium discoideum.

Published work concerning A. crystallopoietes suggests that control mechanisms at different levels are involved in the changes exhibited in the morphogenic cycle. In other biological systems that show shifts in control mechanisms, procaryotic and eucaryotic, the evidence is mounting for t-RNA methylation involvement in regulation of protein synthesis at the translational level. This study was initiated to investigate the occurrence of t-RNA methylation and its possible role in the control processes of A. crystallopoietes morphogenesis.

CHAPTER II

MATERIALS AND METHODS

Growth Conditions

Arthrobacter crystallopoietes (ATCC 15481) was grown in complex and defined media depending on the experiment. The complex medium consisted of the following (w/v): yeast extract, 0.25%; tryptone, 0.5%; glucose, 0.1%; pH 7.0. The defined medium contained (w/v): $(NH_4)_2SO_4$, 0.1%; $MgSO_4.7H_2O$, 0.01%; glucose, 0.5%; potassium phosphate buffer, 0.025 M (pH 7.0), and 1.0% trace salts solution (v/v). The trace salts solution contained (w/v): $CaCl_2.2H_2O$, 0.2%; $MnSO_4.4H_2O$, 0.1%; $FeSO_4.7H_2O$, 0.05% in 0.1 N HCl. Glucose, as a 20% solution, and the trace salts solution were sterilized separately by filtration (13). Cells were routinely grown in two liter flasks containing 500 ml medium. When cells were grown in complex medium, 24 hr broth culture cells were used as the inoculum. Cells grown in defined medium were inoculated with a 48 hr culture of cells grown in defined medium. In all cases cultures were inoculated to 0.04 OD_{485} (Spectronic 20, Bausch and Lomb) and incubated at 30 C while shaking 150 rpm on a New

Brunswick model VS rotory shaker.

Radioactivity Determination

In all experiments employing radioisotopes, the activity was monitored by a Beckmen DPM 100 Liquid Scintillation System. The scintillation fluor contained (w/v): diphenyloxazole, 0.5%; naphthalene, 10%; dioxane, to volume. Ten ml of scintillation fluor was used in each counting vial. The following radioisotopes were purchased from New England Nuclear and had the characteristics listed: L-methionine, ^{14}C-methyl, specific activity 14.7 mc/mM; L-amino acid mixture, ^{14}C uniformly labeled, specific activity depending on the particular amino acid; uridine, 5-^{3}H, specific activity 26.6 c/mM. S-adenosylmethionine, ^{14}C-methyl, specific activity 28.9 mc/mM was purchased from International Chemical and Nuclear Corporation. L-methionine, ^{35}S, specific activity 27 c/mM was purchased from Amersham/Searle Corporation.

Sucrose Gradient Analysis of in vivo Methylation

The method of Starr and Fefferman (45) was used to isolate RNA from A. crystallopoietes harvested at various stages in the growth cycle. Linear 5 to 20 percent sucrose gradients containing Tris buffer, 5×10^{-3}M (pH 7.4), and magnesium acetate, 10^{-4}M, were prepared by using the Buchler Gradient Former. Centrifugation was carried out at 100,000

x g in the SW 39 rotor (Beckman, Spinco Division) for 260 min. Two drop fractions were collected by puncturing a hole in the bottom of the tube. Each aliquot was diluted to 1 ml with distilled water and the optical density at 260 nm determined. An aliquot (0.5 ml) of each fraction was placed in a scintillation vial and the radioactivity determined.

Preparation of Enzyme Solution

Cells were harvested at various stages of the growth cycle by means of the Servall (type KSB-R1) continuous flow centrifuge at 4 C. After washing two times in Tris-HCl buffer, 0.01 M, pH 8.2 containing 30 µg/ml $MgCl_2$, the cells were suspended to a 25% (w/v) solution in the same buffer. The cells were disrupted by sonic treatment (Blackstone model SS-2C) using three 5 min pulses at 4 C. Unbroken cells and cell debris were removed by centrifugation at 30,000 x g for 15 min. The supernatant was subjected to centrifugation at 100,000 x g for 60 min (Beckman model L-2 preparative ultracentrifuge). Aliquots of the supernatant were taken for protein determination (31) and as a source of t-RNA methylase enzymes (22, 38).

Assay of t-RNA Methylase

The methylase activity of the extracts was assayed by measuring the incorporation of the ^{14}C-methyl group from

S-adenosylmethionine into E. coli t-RNA. The reaction mixture contained: tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 100 μmoles (pH 8.2); $MgCl_2$, 30 μmoles; dithiothreitol, 10 μmoles; E. coli B t-RNA, 100μg; ^{14}C-S-adenosylmethionine, 0.2 μc and enzyme in a total volume of 1 ml. For each enzyme concentration a control was prepared which contained on t-RNA. After incubation at 37 C for 10 min, the reaction was terminated by the addition of 5 μmoles of sodium pyrophosphate, 0.05 ml of 0.5% bovine serum albumin and 0.2 ml of 7% ice cold perchloric acid. The tubes were chilled in an ice bath and after approximately 30 min the precipitates were collected by one of two methods. In early experiments the precipitate was collected by centrifugation and washed three times with 2% ice cold perchloric acid containing 2×10^{-3} M sodium pyrophosphate. The pellets were dissolved in 1 ml 0.2 M NH_4OH and the activity of 0.5 ml aliquots was determined. In later experiments the precipitate was collected on HAWP (0.45μ) membrane filters (Millipore Corp., Bedford, Mass.). The filters were washed with 10 ml of the wash solution used above, dried 10 min at 80 C and placed in scintillation vials for counting (16, 22, 38, 42).

Polyacrylamide Disc Gel Elcetrophoresis

At various stages in the growth cycle, cells were collected by centrifugation and washed three times with

Tris-glycine buffer, 0.05 M pH 8.91. The washed cells were suspended to 25% (w/v) solution in the same buffer and disrupted by sonication. After removing cell debris by centrifugation at 30,000 x g for 30 min the supernatant was dialyzed overnight against three changes of Tris-glycine buffer (21). Protein was determined by the method of Lowry (32). After samples were diluted to contain the same concentration of protein, equal aliquots were fractionated using the anionic system according to Buchler instruments manual (8). The gels were stained with amido black for 1 hr and electrophoretically destained with 7% acetic acid. Qualitative data was obtained by scanning the gels using the linear transport attachment to the Gilford recording spectrophotometer.

Microbiological Assay

Methionine was determined quantitatively using *E. coli* Hfr AB 311 Met^-. Ten ml glucose minimal medium was supplemented with various concentrations of methionine. Washed cells grown overnight and adapted for growth on methionine were used as inoculum. The density of the cultures were determined at 485 nm on the Beckman DU spectrophotometer. Pool methionine was determined by making dilutions of the cell free extract such that the growth response fell on the standard curve that was prepared (55).

RNase Assay

RNase activity was measured by the method of Kalnitsky *et. al.* (23).

CHAPTER III

RESULTS

I. T-RNA Methylation Studies

1. In Vivo

Preliminary to a more detailed study of t-RNA methylation, a pilot experiment was designed to determine if methylation of t-RNA occurred in A. crystallopoietes. A late logarithmic culture of short rod shaped cells was exposed to ^{14}C-methyl methionine, which was reported to be the methyl donor for methylation of nucleic acids found in other organisms (33). Nucleic acids were extracted, treated with DNase and fractionated on a linear 5 to 20% sucrose gradient. As indicated in Figure 1, the ^{14}C radioactivity corresponds very well to the characteristic peaks of ribosomal (fractions 10-16) and transfer RNA (fractions 18-24) and suggests that Arthrobacter RNA is methylated. Since the label employed was an amino acid, there existed the possibility that the RNA fractions were contaminated with protein or polypeptide that had incorporated methionine. To investigate this possibility, a similar culture was exposed to a mixture of ^{14}C-L-amino

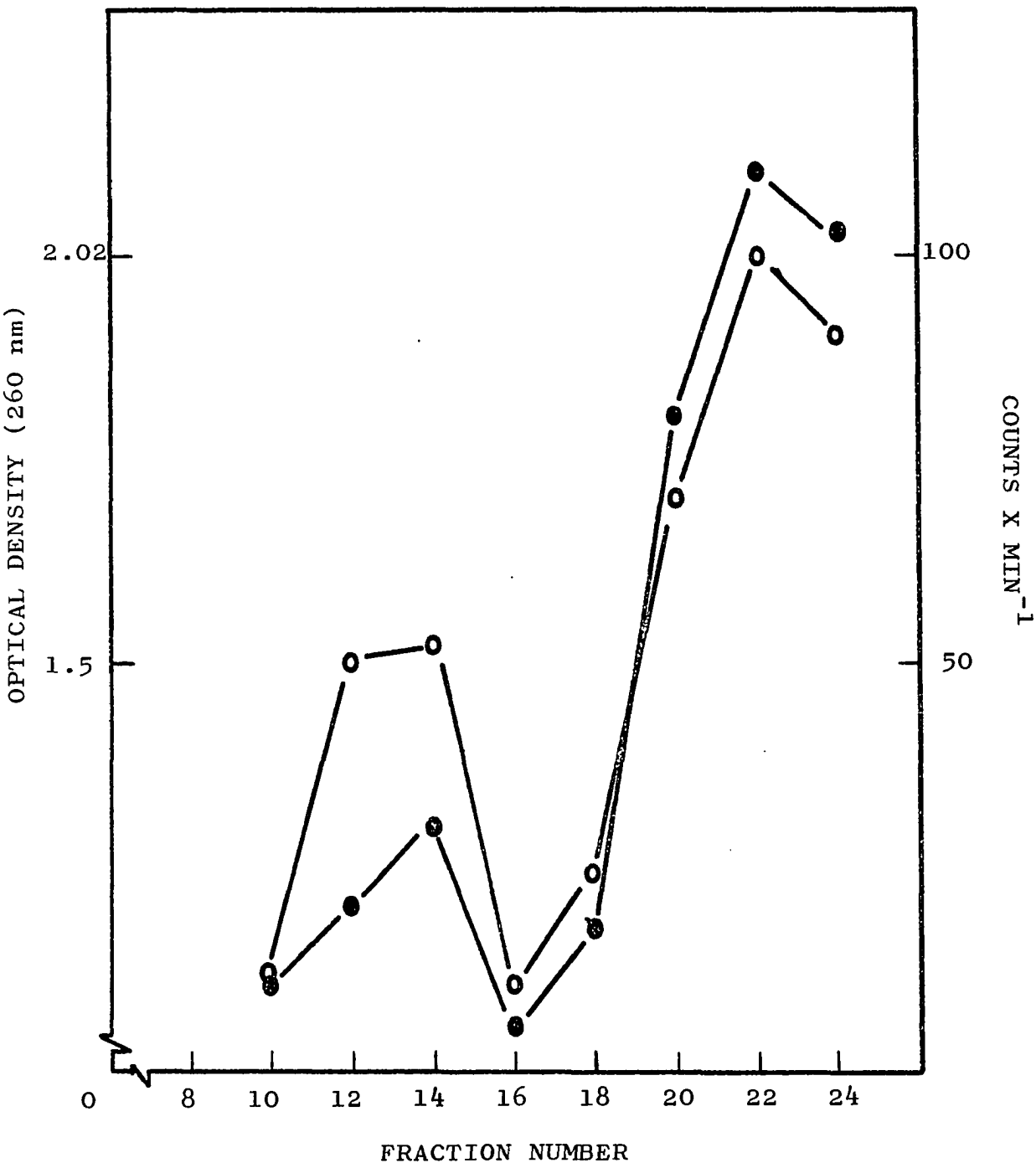

Fig. 1.--Incorporation of the ^{14}C-methyl group of S-adenosyl methionine into Arthrobacter RNA. Cultures were pulse labeled for 30 min at 30 C. After extraction with phenol, RNA was fractionated on a linear 5-20% sucrose gradient (100,000 x g for 260 min.). Optical density —o—o; counts x min^{-1} —●—●—

acids containing no methionine, extracted and fractionated as previously. The results plotted in Figure 2 indicate essentially no contamination of the t-RNA peak and very little contamination in the ribosomal RNA peak. These results suggested that, as in other systems methionine serves as the methyl donor for RNA methylation in A. crystallopoietes.

If serving as a control mechanism of morphogenesis, the methylation of t-RNA should change in some characteristic manner in relation to the growth cycle. At least two parameters can be considered in the investigation of a change in methylation; the rate of methylation and the extent of methylation. Based on previous reports concerning the functional changes on t-RNA by methylation, it appears most significant to demonstrate a change in the extent of methylation.

The determination of the extent of methylation appeared possible by relating the rate of t-RNA methylation to the rate of t-RNA synthesis. That is, the ratio of t-RNA methylation to t-RNA synthesis would show, at least relatively, a change in the extent of methylation at any particular time. Experimentally this involved double labeling cells from various stages of the growth cycle. Incorporation of the ^{14}C-methyl group of L-methionine per unit time per unit t-RNA was used to indicate the rate of t-RNA methylation and incorporation of ^{3}H-uridine per unit time

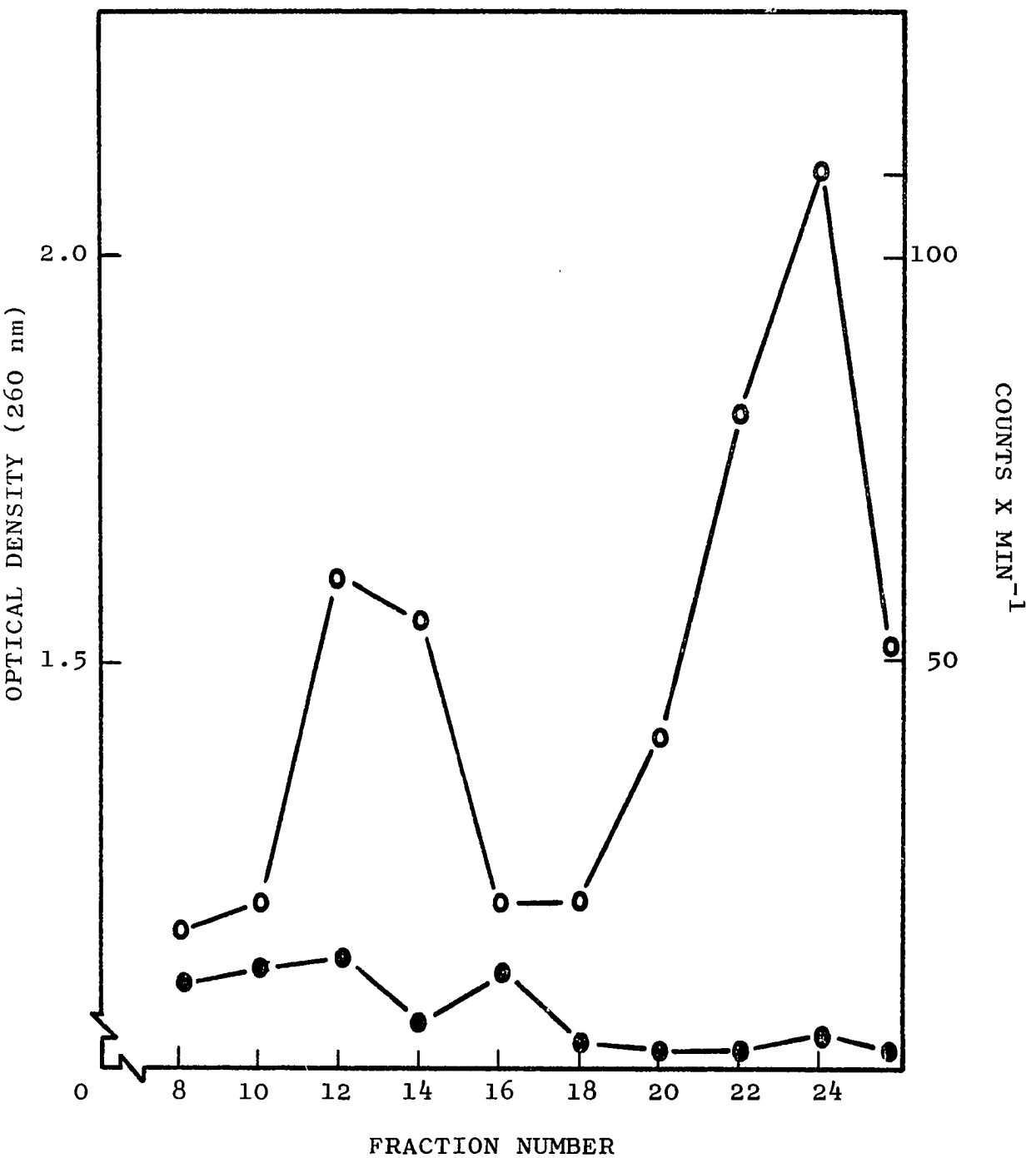

Fig. 2.--Fractionation of Arthrobacter RNA after exposure to a mixture of ^{14}C-L-amino acids. Cultures were labeled for 30 min while shaking at 30 C. RNA was extracted with phenol and fractionated on a linear 5-20% sucrose gradient (100,000 x g for 260 min). Optical density —O—O— ; counts x min^{-1} —●—●—

per unit t-RNA was used to indicate the rate of t-RNA synthesis. The results, Table 1, demonstrate that the individual rates of incorporation of uridine and methionine decrease during rod formation and increase during the transition from rods to spheres, but they do not change at the same rate. The ratio $^{14}C/^{3}H$, and therefore the extent of methylation decreases during rod formation and increases during the rod-sphere transition.

These results suggested that changing patterns of t-RNA methylation may be correlated with distinct changes in the growth cycle of A. crystallopoietes. That these results were not conclusive was suggested by a further experiment regarding permeability.

Similar results regarding incorporation of radioactive label into t-RNA might also be produced if the cell permeability to the respective labels changed during the growth cycle. To determine if a permeability change could account for the data, separate aliquots of cells from various stages in the cycle were pulse labeled with ^{3}H-uridine and ^{14}C-L-methionine. The results, shown in Figure 3, indicate that, in fact, there was a change in permeability to the compounds tested. Particularly, there is a decrease in the ability of the compounds to be incorporated into whole cells. A similar pattern has been shown for glucose (14) and this period of decreased permeability corresponds to the time of highest endogenous metabolism

TABLE 1

INCORPORATION OF ^{3}H-URIDINE AND ^{14}C-METHIONINE INTO ARTHROBACTER T-RNA IN VIVO

CULTURE AGE (Hr)	^{3}H (CPM)	^{14}C (CPM)	$^{14}C/^{3}H$ *
4	806	766	0.95
12	264	123	0.55
24	633	386	0.61

*CPM as ^{14}C/CPM as ^{3}H

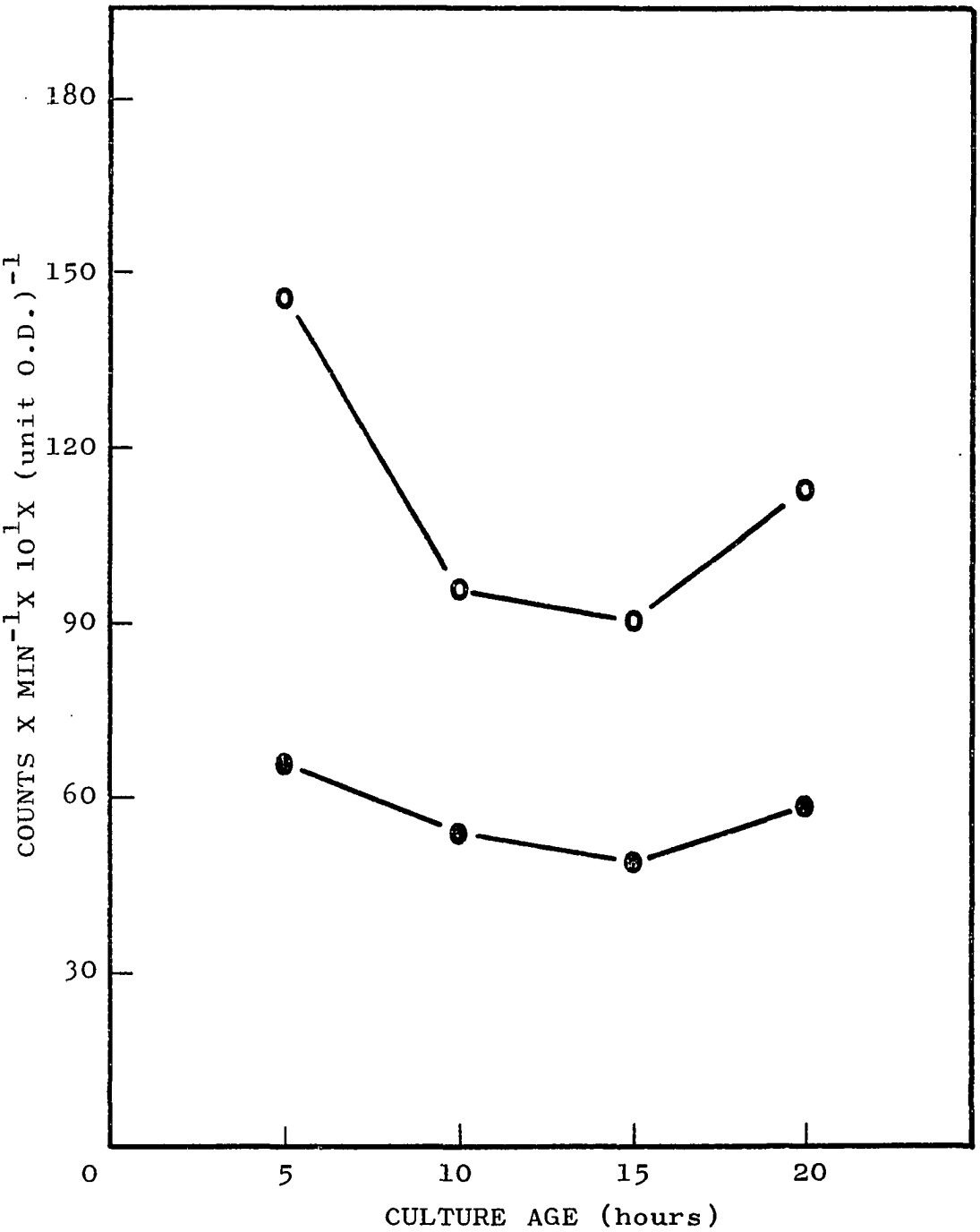

Fig. 3.--Incorporation of radioactive labels at specific stages of the growth cycle of Arthrobacter. At the culture ages indicated, aliquots were pulse labeled for 10 min. The reaction was stopped by collecting cells on millipore filters. After washing with sterile medium, the filters were dried and counted. ^{3}H-uridine -o-o- ; ^{14}C-methionine -●-●-

(14, 15).

The resolution of the question could have been approached in at least two ways--through the use of surface active agents to alter the cell permeability or by means of *in vitro* studies. Surface active agents have been used by other workers (7) to obtain uniform labeling kinetics. The artificiality imposed on the *in vivo* system as a result of surface active agents appears to nullify any advantage over the use of an *in vitro* system. Although extrapolation of results from an *in vitro* study to the *in vivo* system is often objectionable, it appeared the better choice since it should be more reproducible and more easily standardized.

2. In Vitro

Since no published work was available concerning the methylase reaction in *Arthrobacter*, a study was undertaken to determine some of the characteristics. To study the incorporation of methyl groups with respect to time, a tube containing the equivalent components of five assays (Materials and Methods) was prepared in addition to a similar control tube which contained no *E. coli* t-RNA. Duplicate 1 ml aliquots were removed at varying times for determination of radioactivity. The reaction (Figure 4) appears linear for at least the first 10 min and then gradually reaches a plateau, indicating no further label incorporation with time. In Figure 5, the incorporation of methyl groups is demonstrated with respect to varying

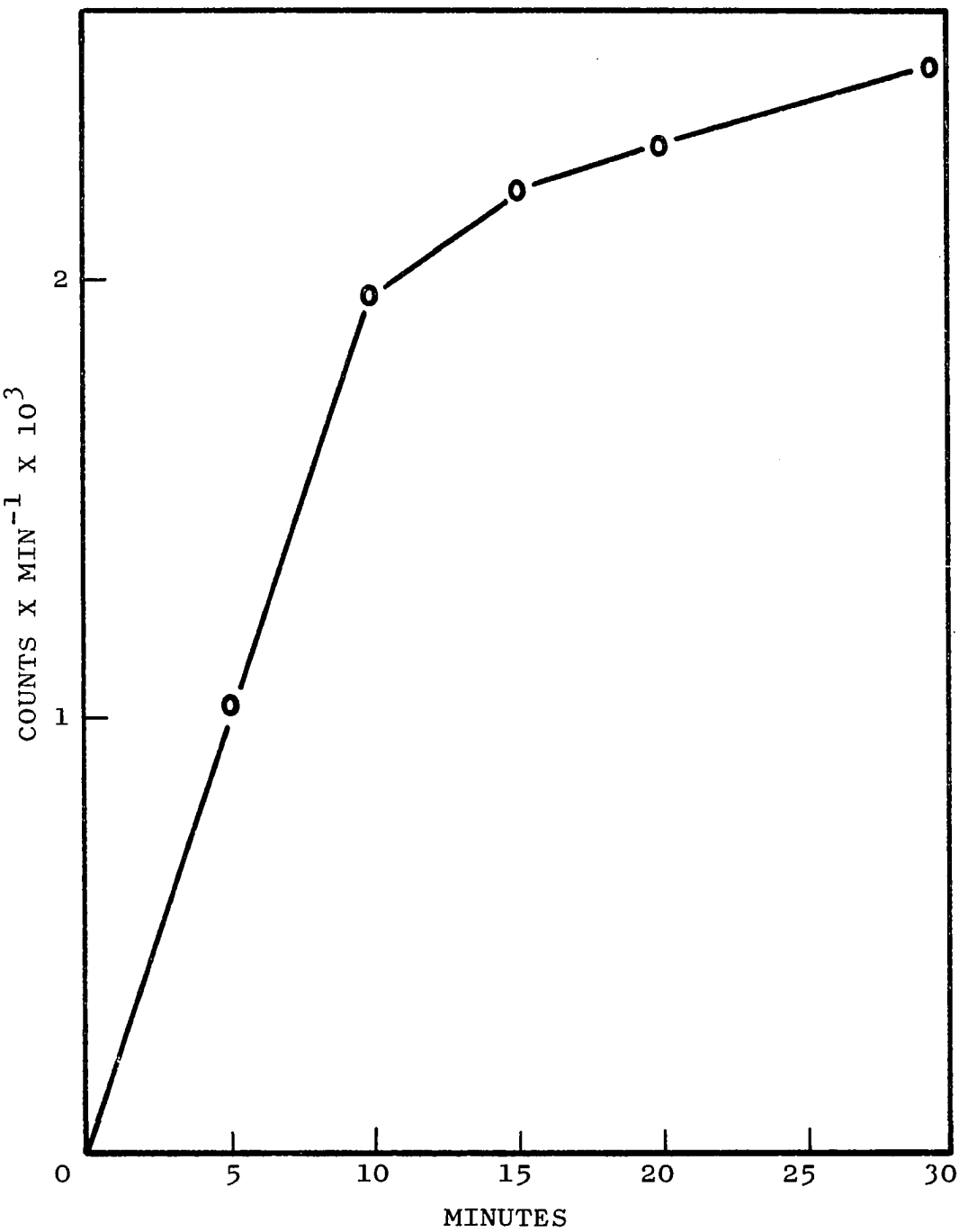

Fig. 4.--Incorporation of ^{14}C-methyl group of S-adenosyl methionine into t-RNA as a function of time. A tube containing the equivalent components of five assays (Materials and methods) was prepared. The control tube contained no E. coli t-RNA. At the times indicated 1 ml aliquots were collected on millipore filters and the radioactivity determined. Data points represent incorporation above control values.

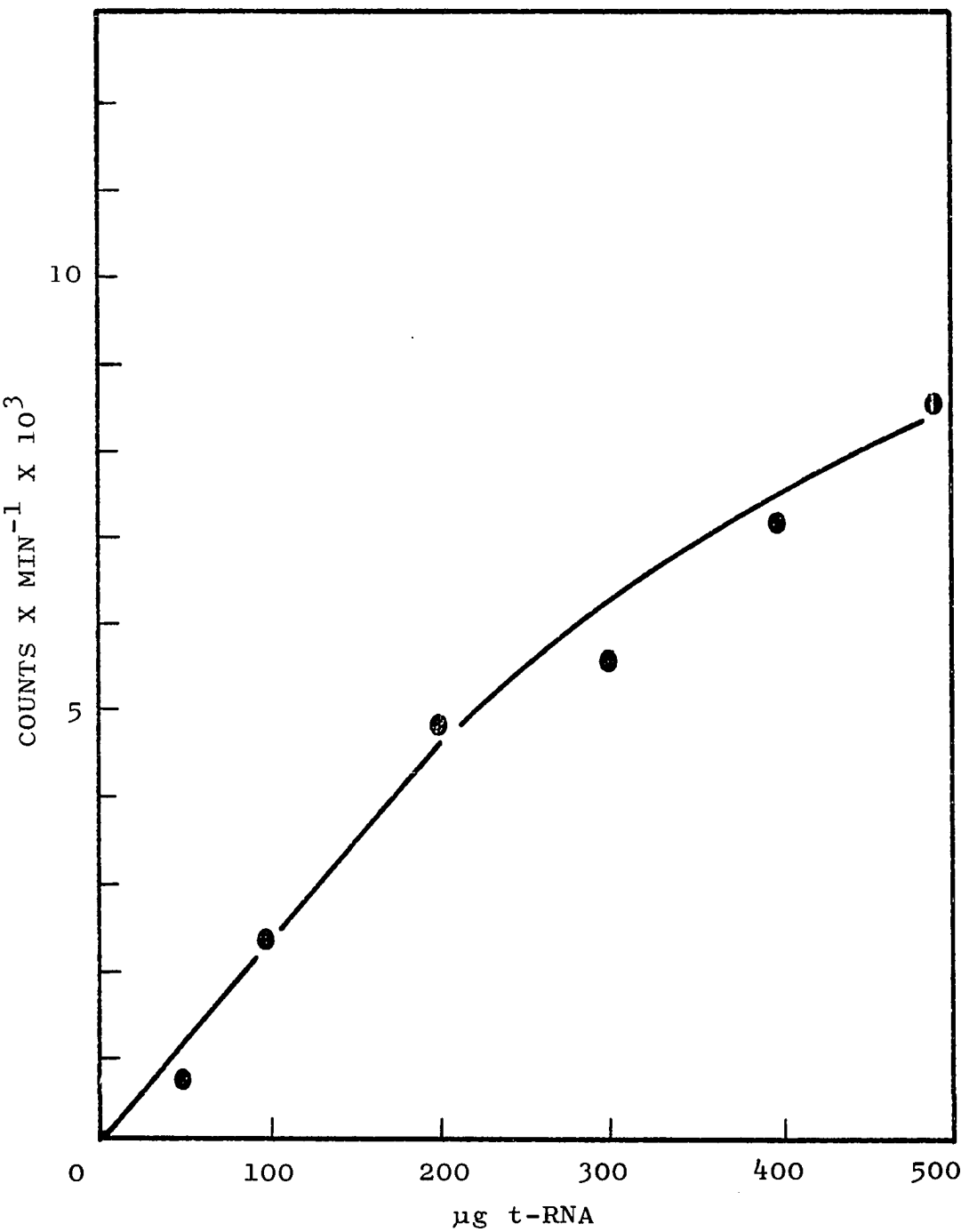

Fig. 5.--Incorporation of ^{14}C-methyl group of S-adenosyl methionine into t-RNA as a function of increasing t-RNA concentration. Assay was as in materials and methods. Data points represent incorporation above control values.

amounts of E. coli t-RNA. The reaction mixture contained varying amounts of E. coli t-RNA and 0.5 ml of the 100,000 x g supernatant fraction. The results indicate that enzyme activity is linear up to approximately 200 µg RNA. Methyl incorporation with respect to varying pH is indicated in Figure 6. The reaction mixture was routine except that in each assay 100 µmoles of Tris buffer at varying pH was introduced. The data indicate a pH optimum of about 8.0. Since the routine assay was performed at pH 8.2, the value used by other workers (38, 42), and since only full pH values were assayed, it was felt to be sufficiently close to the optimum to avoid changing.

Based on the studies of some of the characteristics of the methylase reaction, the conditions were established for A. crystallopoietes. The various values determined do not differ significantly from the values reported by other workers (19, 22, 25, 38). Higher incorporation values could possibly be attained using larger concentration of t-RNA. Since the activities obtained were usable, and in favor of economy, the lower concentration of t-RNA was retained in the routine assay mixture.

Using the standard assay derived from the above experiments, a study was initiated to determine if a correlation existed between t-RNA methylation and the growth cycle of A. crystallopoietes by means of the in vitro methylase assay. At various stages in the growth cycle,

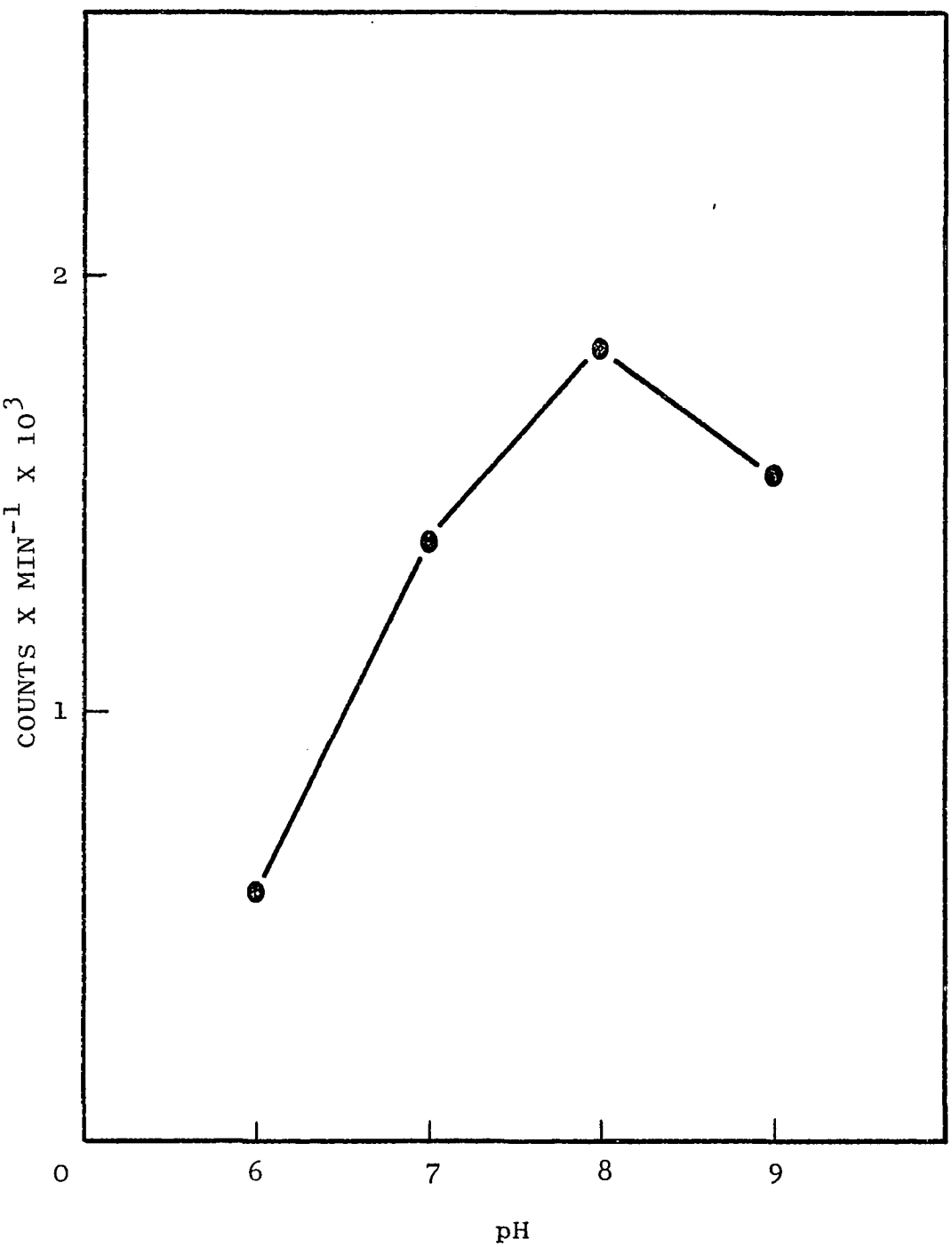

Fig. 6.--Incorporation of ^{14}C-methyl group of S-adenosyl methionine into t-RNA as a function of pH. Assay as in materials and methods. Data points represent incorporation above control values.

cell free extracts of A. crystallopoietes were tested for the ability to incorporate the ^{14}C methyl group of S-adenosylmethionine into E. coli t-RNA. The results are plotted in Figure 7. ^{14}C methyl incorporation above endogenous levels is plotted as counts per minute x 10^2 per 100 µg E. coli t-RNA. Enzyme concentration is plotted as mg Lowry protein in the volume of cell free extract tested. The height of the curves represents the extent of methylation. This decreases from 4 to 12 hours and then increases to a maximum at 24 hrs. This data is better correlated with the different morphological stages in Figure 8. ^{14}C methyl incorporation at enzyme saturation level is plotted against the stage in the morphogenic cycle. The extent of methylation by the t-RNA methylases decreases during rod formation and then increases during the transition from rod to sphere shaped cell.

Figure 7 also demonstrates a reduction of incorporation of methyl groups beyond enzyme saturation level. The pattern was present in all stages of cells tested. This type of pattern has suggested to others the presence of a t-RNA methylase inhibitor, the effect of which is dependent on the absolute concentration (25, 38, 42).

II. Protein Studies

A study was initiated to determine some of the characteristics of protein synthesis during the growth cycle of

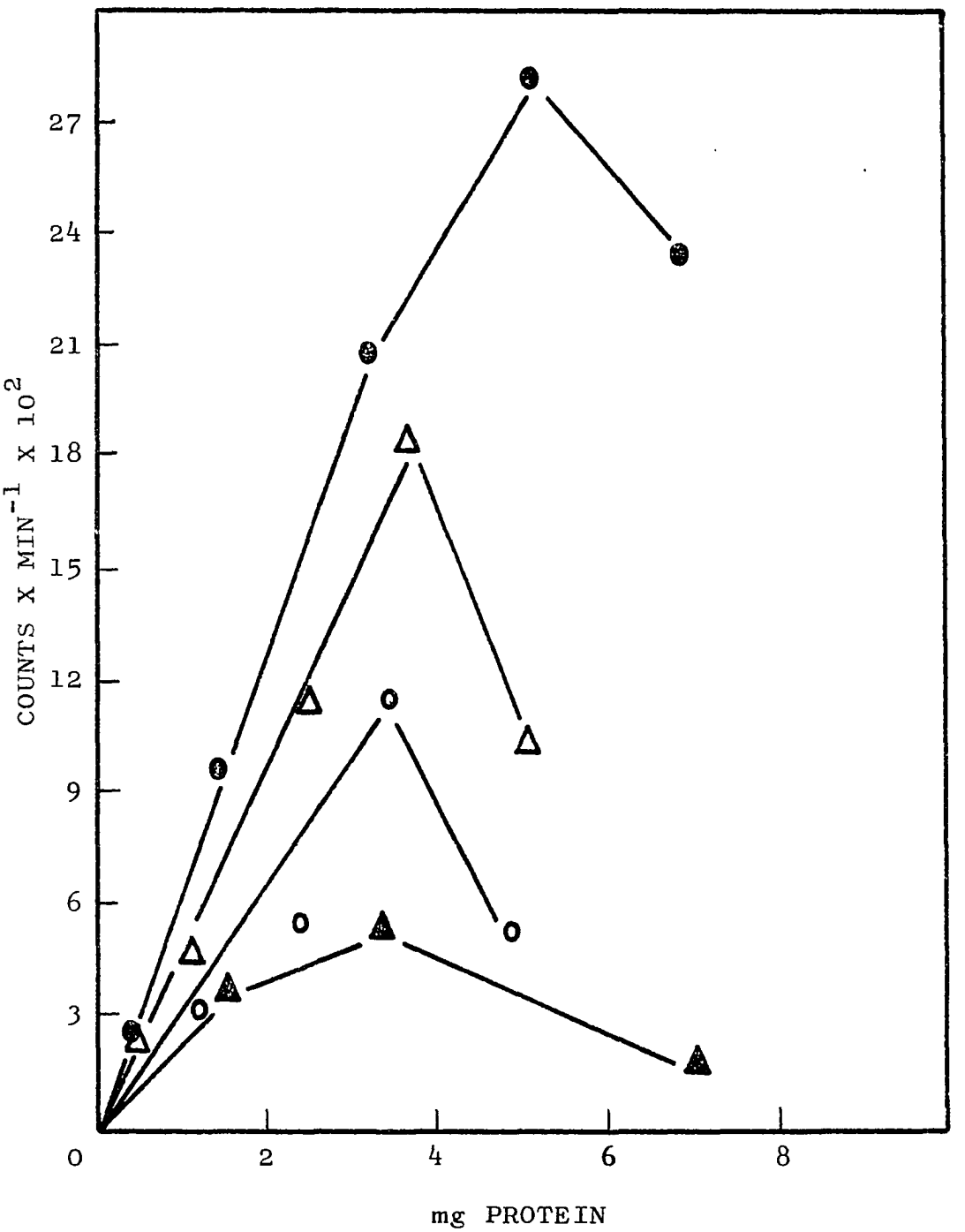

Fig. 7.--Incorporation of ^{14}C-methyl groups of S-adenosyl methionine into t-RNA as a function of protein concentration. At various stages of the life cycle Arthrobacter methylase (expressed as Lowry protein) was extracted and assayed as in Materials and Methods. 4 hr -Δ- ; 10 hr -o- ; 12 hr -▲- ; 24 hr -●-.

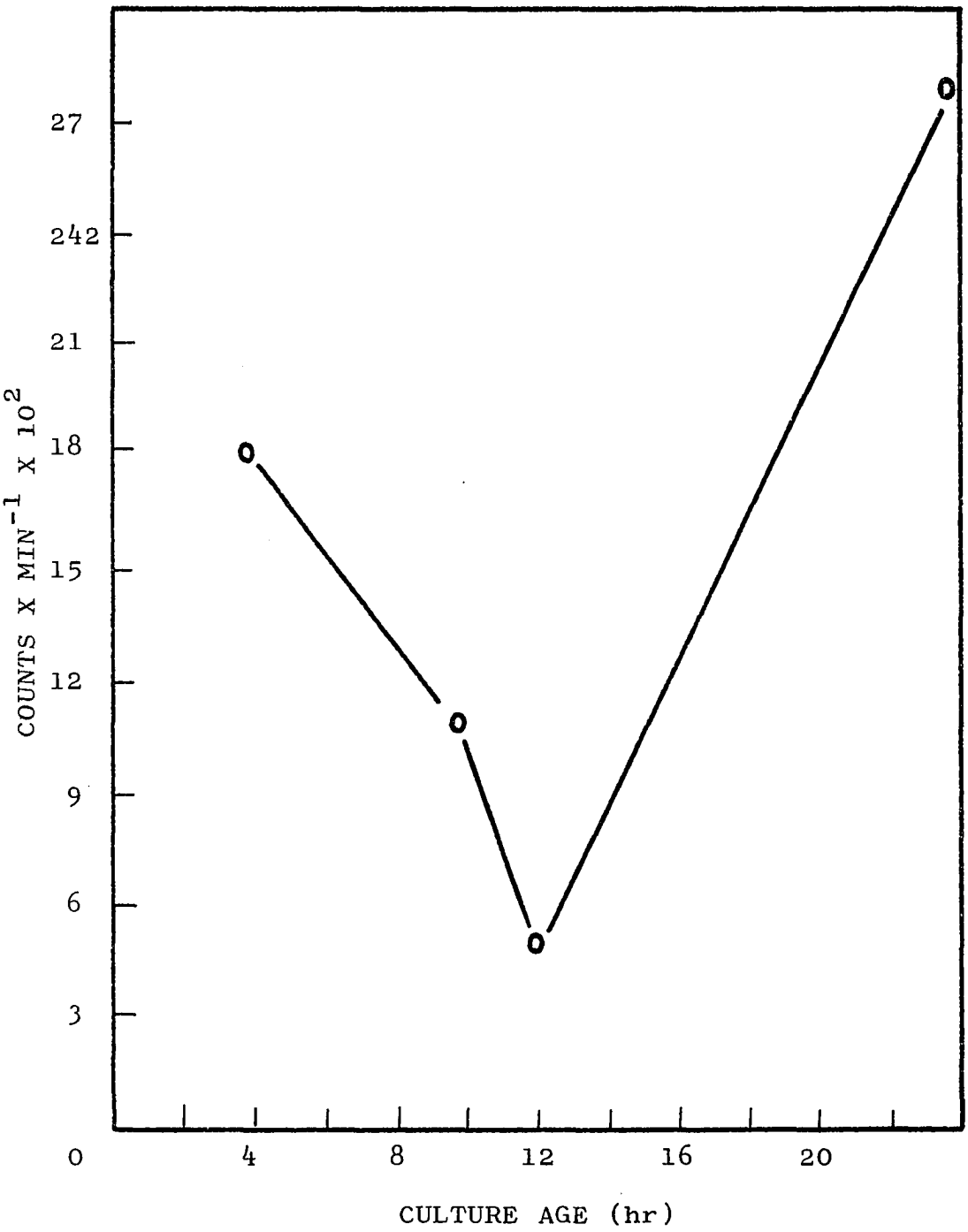

Fig. 8.--Incorporation of ^{14}C-methyl groups of S-adenosyl methionine into t-RNA at enzyme saturation as a function of culture age. Data points represent the methylase saturation value as indicated in Figure 7.

A. crystallopoietes. Changes in protein synthesis as a result of regulatory mechanisms could be expressed either as a change in the rate of synthesis of all or particular proteins and/or a change in the quantitative or qualitative pattern of proteins synthesized.

1. Rate of Protein Synthesis

Previous studies indicated that amino acids were not incorporated by the cell to the same extent for all morphological stages. Preliminary to the study of the rate of protein synthesis than it became necessary to determine the characteristics of the amino acid pool from which proteins are synthesized in vivo. The definition of the amino acid pool that was adopted is that proposed by Britten and McClure (6). Briefly stated this is, the amino acids that may be extracted from the cell with 5% TCA. The conditions are such that macromolecules are not degraded.

To determine if this definition could be correctly used for Arthrobacter, an experiment was designed to determine the extractability of the amino acids using 5% TCA. A culture of cells was labeled with a mixture of ^{14}C-L-amino acids. A very short time for equilibration of internal and external pools of the labeled amino acids was assumed. This assumption was based on the data from other systems demonstrating very short equilibration times (6, 10). Following incubation, the cells were harvested by centrifugation, washed and suspended in a known volume of medium. Duplicate

aliquots were collected on millipore filters. These represented total incorporation of label. The remainder of the cells were again collected by centrifugation and suspended in ice cold 5% TCA to the same concentration as above. At 10 min intervals, duplicate aliquots were centrifuged. A known volume of the supernatant was placed in a scintillation vial for counting and indicated 5% TCA soluble label. The corresponding TCA insoluble material was collected on millipore filters and washed with ice cold 5% TCA. The filters were placed in vials and counted as the TCA insoluble label. In addition, an aliquot of cells representing the maximum exposure time to TCA was sonicated for 10 min, centrifuged and assayed for TCA soluble and insoluble label. The results listed in Table 2 indicate that with Arthrobacter the amino acid pool may be extracted with 5% TCA and that essentially all of the pool is extracted within the first ten min.

With the applicability of the Britten and McClure definition of amino acid pool demonstrated for Arthrobacter, a modification of the procedure for extracting the amino acid pool was devised and tested. This study concerned the feasibility of extraction of the amino acid pool while the cells were collected on millipore filters. Cells were labeled as above and duplicate aliquots collected directly on millipore filters. The amount of fresh medium necessary to remove residual nonincorporated label was determined.

TABLE 2

TIME REQUIRED FOR TOTAL EXTRACTION OF AMINO ACID POOL WITH 5% TCA

TIME (min)	CONTROL (SONOCATED) (CPM)	EXPERIMENTAL (WHOLE CELLS) (CPM)
10	---	600
20	---	600
30	---	650
40	650	650

Figure 9 shows that after 15 ml of wash medium, essentially no label appears in the filtrate, indicating that nonspecifically bound label was removed from the cells and filter.

Next, it was determined if the amino acid pool could be completely extracted by washing the cells already collected on a millipore filter. Cells suspended to 0.5 OD_{485} were labeled, equal collected on millipore filters and washed to remove residual label as in previous experiment. After washing with known volumes of ice cold 5% TCA, aliquots of the filtrate were counted. A control representing 100% of the TCA extracted label was prepared by the method of Britten and McClure. Figure 10 represents the results obtained. Compared to the control extraction, essentially 100% of the amino acid pool can be extracted by this procedure using 6 ml ice cold 5% TCA. The results presented establish the standard procedure that was used in all subsequent experiments concerning amino acid pool and the rate of protein synthesis.

The size and specific activity of the amino acid pool was determined by the incorporation of ^{35}S-methionine and microbiological assay of the pool methionine. At various stages, cells were collected, washed and resuspended to OD_{485} 0.5 in GS medium containing 20 µg/ml chloramphenicol. ^{35}S-methionine (20 µmoles/ml) was added and the cultures incubated for 10 min. Incorporation was halted by collecting the cells on millipore filters. The cells were washed

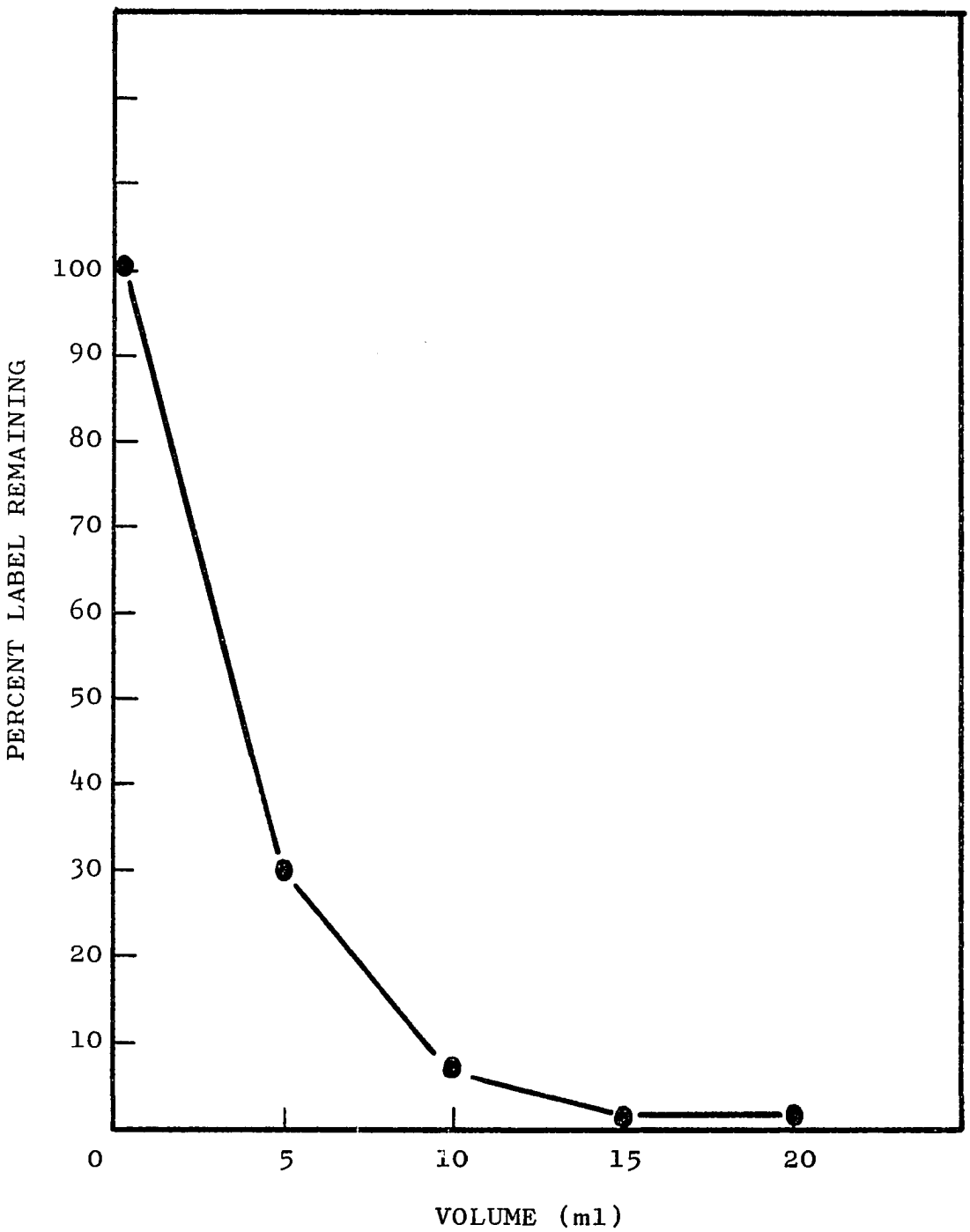

Fig. 9.--Volume of wash solution required to remove residual nonincorporated label. Cells labeled for 10 min with a mixture of ^{14}C-L-amino acids were collected on millipore filters. Each volume of wash solution was collected separately and the radioactivity determined.

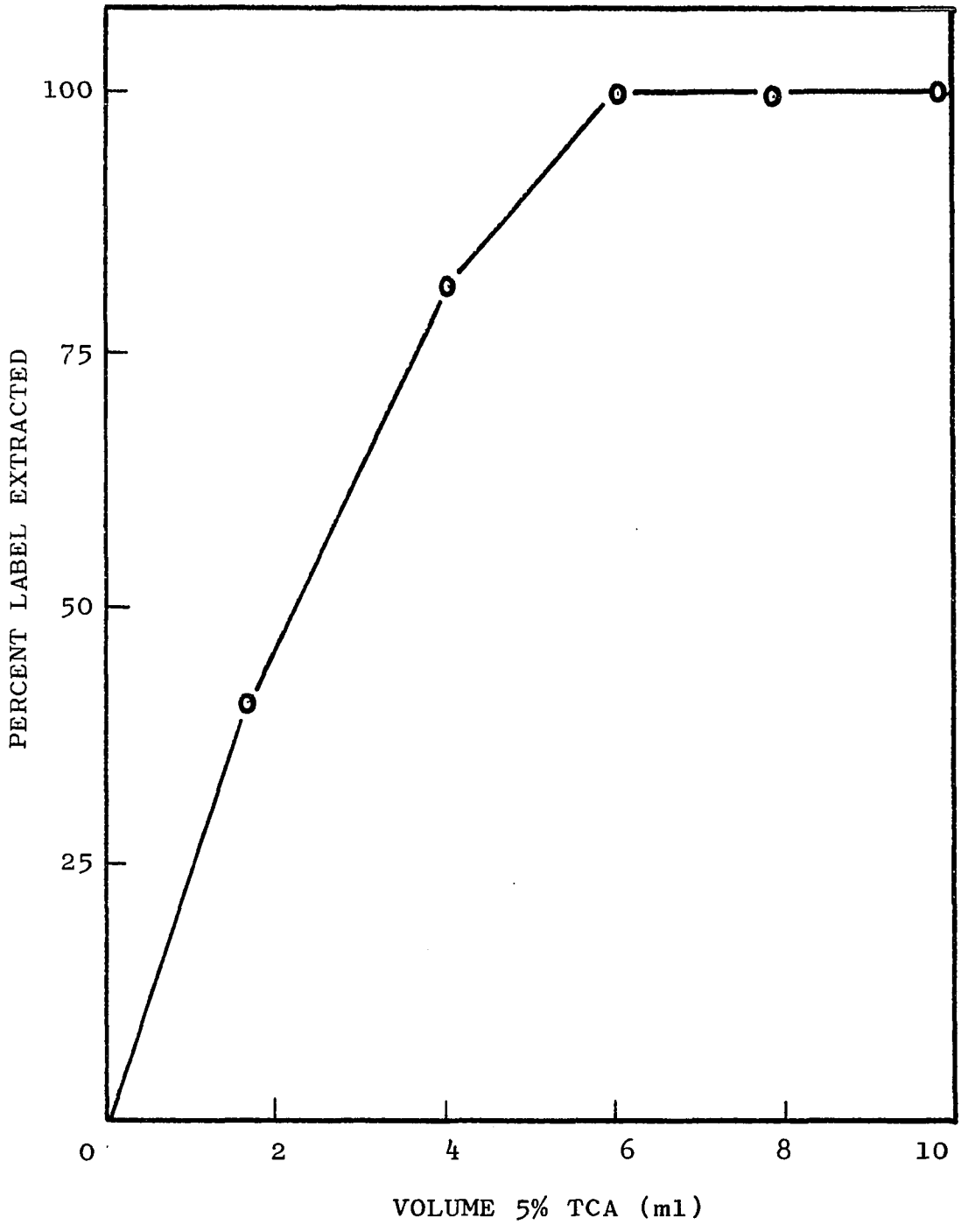

Fig. 10.--Volume of 5% TCA required to extract total amino acid pool by the millipore filter technique. Each volume of TCA was collected separately and the radioactivity determined. The control representing 100% extraction was prepared by the method of Britten and McClure (6).

and the amino acid pool extracted. The radioactivity and the amount of methionine in the pool was determined (Table 3). The size of the methionine pool does not change significantly throughout the growth cycle of A. crystallopoietes. On the other hand, the specific activity of the pool decreases with the stage of the cells.

With knowledge of the methionine pool size and the result that this did not change significantly during the growth cycle, the rate of ^{35}S-methionine incorporation into TCA insoluble material was calculated by the method of Fry and Gross (17). The mathematical statement of the rate of incorporation that was developed is as follows:

$$R = \frac{I_T(k_1-k_2)}{(k_1-k_2)T + P \log[\frac{P}{P + (k_1-k_2)T}]}$$

where

R = rate of incorporation

I_T = total label incorporated over pulse time T

P = normal endogenous methionine pool size

The constants k_1 and k_2 are characteristic of each stage of the growth cycle and were determined as follows. Cells from various stages in the growth cycle were collected, washed and resuspended in minimal medium containing glucose to OD_{485} 0.5. ^{35}S-methionine was added and at various time intervals two one ml aliquots were

TABLE 3

SIZE AND SPECIFIC ACTIVITY OF AMINO ACID POOL IN ARTHROBACTER

CULTURE AGE (hr)	POOL SIZE[a]	SPECIFIC ACTIVITY[b]
4	2.49	8452
12	2.73	5524
24	3.04	4865

[a] μmoles/unit O.D.

[b] cpm/μmole

collected on millipore filters. Filters were washed with 15 ml minimal medium containing methionine to remove residual label from filter and cells. One filter was dried and counted to indicate total label uptake. The second filter was extracted with 6 ml of 5% TCA. This was then dried and counted to indicate label incorporation into TCA insoluble material. The data for three stages are shown in Figure 11. This figure indicates varying rates of both uptake and incorporation. These variations appear to be due to changes in specific activity of the pool since upon calculation of the rate of incorporation using the Fry and Gross equation, there is essentially no difference (Table 4).

2. Studies of Protein Electrophoretic Patterns

During the growth cycle of A. crystallopoietes various enzyme activities have been reported to change. For some enzymes it was reported that no activity could be detected at some stages but appeared at others (14). In the cases where the enzyme activity changed, the mechanism for the change in activity was found to be due to metabolite feedback inhibition (15). For those enzymes that could not be detected, similar feedback mechanisms may control the activity or the synthesis of the enzyme may be limited to only a specific stage in the cycle. In the latter situation one explanation is the repression of synthesis at the transcription level. The t-RNA molecule can be imagined to be ideally suited to serve as a repressor molecule. In

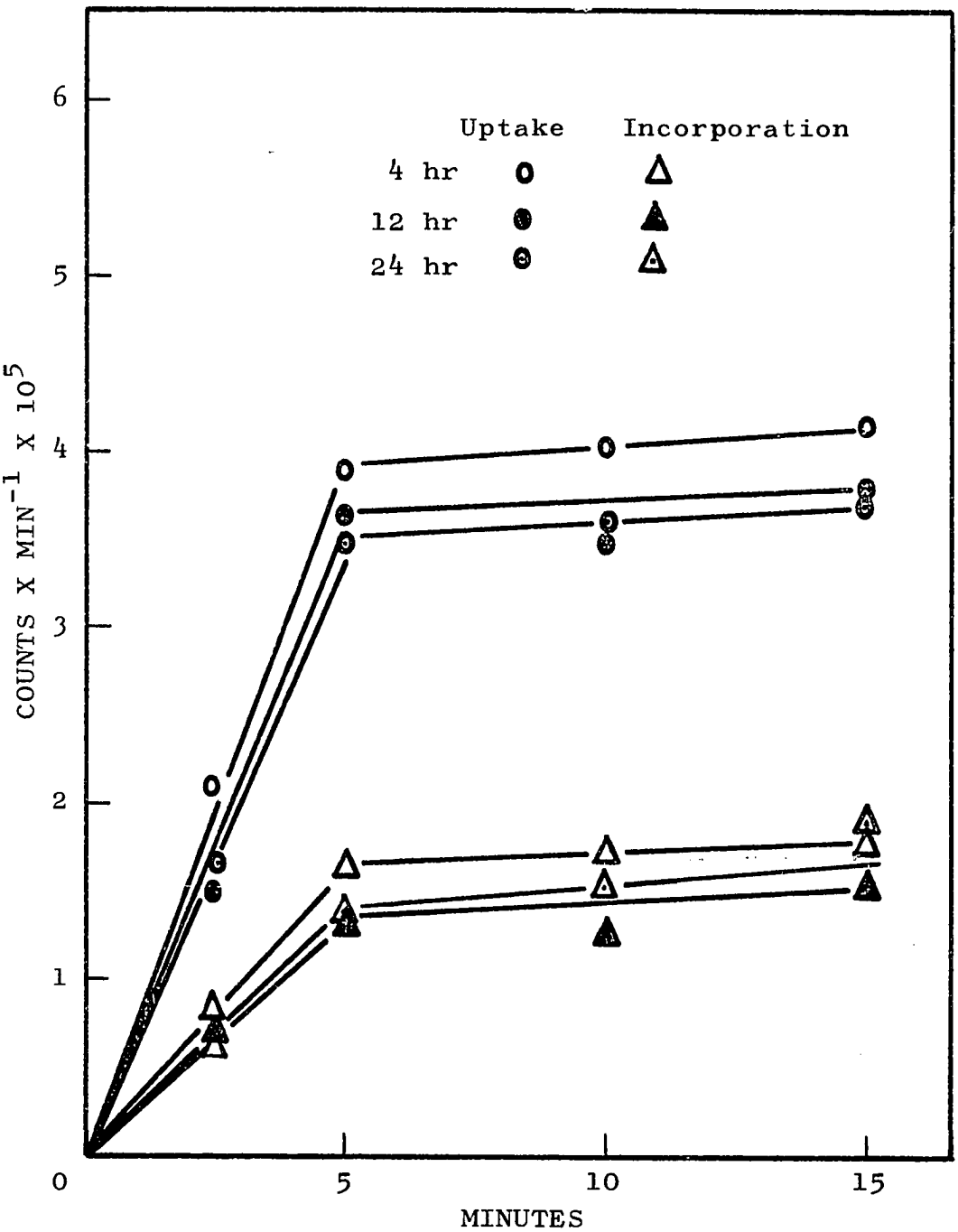

Fig. 11.--Rate of uptake and TCA insoluble incorporation of ^{35}S-methionine into Arthrobacter as a function of stage in growth cycle. At various culture ages cells were suspended to 0.5 O.D.$_{485}$. Label was added and at the times indicated two aliquots were collected on millipore filters. After removal of residual label one filter was counted to indicate uptake of label. The second was extracted with 5% TCA and indicated TCA insoluble incorporated label.

TABLE 4

CALCULATED RATE OF ^{35}S-METHIONINE INCORPORATION INTO TCA INSOLUBLE MATERIAL*

CULTURE AGE (hr)	RATE (μmoles/min)
4	3.7×10^{-7}
12	4.6×10^{-7}
24	4.1×10^{-7}

*Calculated by method of Fry and Gross (17).

fact, the synthesis of a particular phage protein has been shown to depend on the presence of a particular t-RNA (9). If such a mechanism could be shown to control the synthesis of an enzyme, there would be no question that t-RNA served a regulatory function (5). The role of methylation might then be determined by analyzing the particular t-RNA's in reference to the new function. Proof of such a mechanism would be very difficult. As a very crude preliminary, a study was initiated to determine if indeed some proteins were synthesized only at certain stages of the growth cycle.

The electrophoretic patterns of the soluble proteins at various morphological stages of Arthrobacter are presented in Figure 12. This data represents the average Rf value of twelve gels, six from each of two preparations for each stage reported. The minimum and maximum Rf value observed for each band is indicated by the limiting brackets. For those bands which had overlapping limits no interpretation was persued in relation to Rf vs stage. Although the patterns are very similar for each age studied, there are a few differences that are noteworthy. Qualitatively, three bands are significant. Band A is only in the 16 and 24 hr cells. Band D is present in all cells except the 4 hr cells while band B is absent at 8 hr. Quantitative interpretation of the gel patterns is based on the amount of dye taken up by the protein. Theoretically there is a direct relationship between dye fixed and protein concentration. This

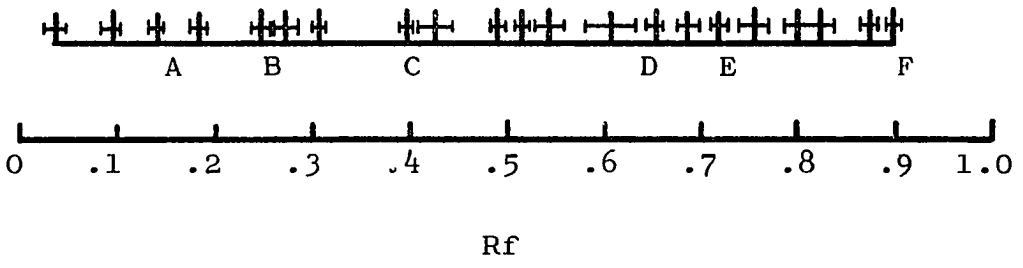

Fig. 12.--Disc gel electrophoretic pattern of Arthrobacter proteins. Each band represents the average Rf determined from 12 gels. The minimum and maximum Rf value observed are indicated by brackets.

relationship has been reported to be based on charge factors and therefore would only hold valid for each protein. Consequently, quantitative interpretation of the various gel patterns is possible only between the corresponding band on each gel and not between the different bands on a single gel (20). With this point in mind, only three bands seemed to yield a pattern and significant differences. Band C increased from 4 to 24 hours. Bands E and F increased from 4 to 16 hours.

CHAPTER IV

DISCUSSION

The investigations of t-RNA methylation in A. crystallopoietes have suggested that the mechanism of the methylation reaction in this organism is the same as in others studied. The in vivo studies indicated that exogenously supplied L-methionine served as the methyl donor. That the immediate donor of methyl groups is S-adenosylmethionine is shown by the in vitro studies. The overall reaction has been summarized by Hancock (19).

ATP + L-methionine $\longrightarrow$SAM + pyrophosphate + monophosphate

t-RNA + SAM $\longrightarrow$methylated t-RNA + S-adenosylhomocysteine

The parameters of the in vitro t-RNA methylation reaction in Arthrobacter also have been shown to be similar to published data (19, 42, 38). It was found that E. coli t-RNA served as a good substrate for methylation studies. The reaction rate was linear for a 10 min period and incorporation of methyl groups was dependent on the amount of E. coli t-RNA. A small amount of endogenous activity was present in all enzyme preparations. The endogenous activity was probably due to the presence of Arthrobacter t-RNA in the crude enzyme preparations. The question of pH effects

is complicated by the fact that the methylase reaction tested represents the combined effect of several enzymes. At least six different enzymes have been found that methylate specific sites on t-RNA (3). The exact pH requirements for each base specific enzyme may be different. The data indicates, however, the optimum activity for the combined enzymes with respect to pH.

In relation to the growth cycle of A. crystallopoietes, t-RNA methylation has been shown to vary. The in vivo studies suggest that t-RNA may be hypomethylated at a time corresponding to early log phase. Examination of the enzyme saturation curves from the in vitro analysis can support this concept. The enzyme saturation curve has been shown to be an invariable constant for those organisms that show no gross differentiation during their growth cycle (38). The mechanism of the saturation phenomenon is not fully understood but Pillenger and Borek suggest that it represents a cooperative effect by the various base specific methylases at their optimum concentration. However, the methylases are not unique in this respect among enzymes that interact with t-RNA. The extent of charging of t-RNA is also a function of the total amount of the acylating enzyme (50).

As mentioned previously, the reduction in methylase activity after saturation has suggested to others the presence of an inhibitor. Inhibitors of the t-RNA methylases

have been observed in other biological systems in addition to those associated with developing systems as mentioned in the introduction. In induced lysogenic microorganisms a specific inhibitor of the uracil t-RNA methylase accumulated (51).

Because of its potential significance in implicating t-RNA methylation with a control mechanism, attempts were made to isolate an inhibitor from Arthrobacter. Kerr (25) and Sharma and Borek (42) have isolated natural methylase inhibitors simply by changing the pH. The enzymes were found to be precipitated while the inhibitor remained in solution. Similar attempts on the crude preparation from Arthrobacter proved negative in all cases. After fractional precipitation and subsequent neutralization, no activity could be detected in either fraction. The results suggested irreversible inactivation of the methylases. Another explanation for the reduction in activity could have been the presence of RNase in the assay mixture. This possibility was tested and found not to be the answer. The question of a methylase inhibitor in Arthrobacter therefore remains unresolved.

A great deal of work and speculation concerning the role of t-RNA methylation has centered around its possible function in the control of protein biosynthesis. Control of protein biosynthesis, in turn, has been an integral feature of hypotheses dealing with regulatory mechanisms of

morphogenesis.

The problem of determining *in vivo* rates of protein synthesis is very complex. Complicating factors generally stem from changes in permeability that occur during cultural growth. It is generally accepted that amino acids and not peptides serve as precursors for protein synthesis, but the actual role of the amino acid pool within the cell is not clear. The assumption that the amino acid pool represents the precursor pool for protein synthesis has been questioned by findings in *Dictyostelium* (54, 55), mammaliam cells (26) and higher plants (49). These studies suggest that only a small portion of the soluble amino acid pool is available for protein synthesis. In contrast, other workers have shown that protein synthesis occurs at the direct expense of the soluble amino acid pool (17, 24).

Despite the disagreement concerning the role of the amino acid pool, it is generally agreed that the calculation of *in vivo* rates must account for differential rates of uptake. In the study of rate of protein synthesis in *Arthrobacter*, use was made of the equation developed by Fry and Gross (17). To make use of this equation several conditions were necessary. Over short pulse times it is necessary to show that the size of the amino acid pool does not change and that both incorporation and uptake of label is constant. These conditions have been demonstrated for *Arthrobacter*. Of the assumptions built into the equation,

the most important, conversion of labeled amino acid into other compounds, was eliminated by using ^{35}S labeled methionine. The calculated rate of incorporation of labeled methionine then, should be a valid indication of the rate of protein synthesis. Other workers have carried the calculations further and computed the absolute rate of protein synthesis. Since this would involve further determinations, for instance, the percentage of methionine in newly synthesized protein, and assumptions such as turnover rate, this calculation was omitted here.

At the beginning of this work, it was hoped that an indirect correlation could be made between protein biosynthesis and t-RNA methylation and that this relationship, in turn, could be related to the growth cycle of Arthrobacter. Two aspects of protein synthesis were considered. First, the quantitative problem of how much protein is being synthesized and whether this synthesis is dramatically changed at some stage. The rates that have been determined suggest that the answer to this question is negative. In light of previous data (48) and the definition of morphogenesis used here, the results are not too surprising. Stevenson (48) has suggested the similarity between the cycle of Arthrobacter and the occurrences observed during a shift up experiment. In such experiments it is generally found that upon transfer to the fresh medium, there is a lag in protein synthesis. This lag is usually of short

duration and the rate of synthesis of protein characteristic of the new medium is attained prior to the end of the cultural lag period. This rate is maintained throughout the log phase of growth. Only after the nutritional value of the medium has been depleted does the rate shift to a lower value.

The second question considered involved the specific proteins synthesized. The results of the disc gel electrophoresis studies suggests that there is at least a minor shift in the pattern of proteins synthesized. These results fit within the model proposed by Ferdinandus (14) suggesting a shift from lipogenesis to lipolysis during the latter stages of growth.

The question of whether methylation of t-RNA can serve a regulatory function has been asked elsewhere (52). The results presented here suggest that changes in t-RNA methylation do not directly relate to gross changes in the rate of protein synthesis. That the response of t-RNA to messenger codons can be altered by methylation and produce more than one functional t-RNA from the same primary sequence of nucleotides has been amply demonstrated in some limited cases (31, 37). Whether a similar mechanism can account for the synthesis of new functional enzymes is still a matter of question. In Arthrobacter at least, there appears to be a change in methylation concomitant with a change in the pattern of proteins synthesized.

This study has been concerned with specific and relatively isolated aspects of what in reality is a complex phenomenon. During the growth cycle of A. crystallopoietes many biochemical changes might be expected to occur. Results often depend on the particular base of reference that is chosen. To properly evaluate any results requires interpretation within the total framework of the system under investigation.

In Arthrobacter, the transition from lag phase to exponentially growing cells is suggestive of a not too well controlled shift up experiment (48). The relationship between cell size and growth rate reported by Donachie (12) has been shown to explain several phenomena associated with shift up experiments. When applied to the cycle of Arthrobacter Donachie's work could be interpreted to explain the changes observed in morphology.

Further, the results obtained for t-RNA methylation in Arthrobacter lend themselves to an alternate interpretation when considered in reference to the macromolecular changes and controls that occur during a shift up. During a shift up many enzymes become repressed while the genes for ribosomal proteins, as well as ribosomal and t-RNA, become derepressed. Consequently, the differential rate of synthesis of most enzymes might be expected to decrease during a shift up. Since the methylase enzyme studied in Arthrobacter was only a crude preparation, it seems possible that,

although total protein concentration increased in the assay mixture, the specific activity of the methylase may have been lower at some stages. Such a situation appears most likely during the lag phase and early log phase, the times observed to have the lowest methylase activity.

CHAPTER V

SUMMARY

Arthrobacter crystallopoietes exhibits a sphere-rod morphogenesis which can be controlled by the nutritional environment. The relatively simple morphological variation and the nutritional control available make this organism ideal for studying possible regulatory mechanisms during the growth cycle.

In the past few years a great deal of work has been reported on the nucleic acid methylases, and there have been reported several examples of shifts in the methylating activity of the t-RNA methylases in differentiating systems. Such changes in the enzyme activity of t-RNA methylases and the role of t-RNA in protein synthesis has suggested t-RNA methylation as a possible regulatory control mechanism in morphogenesis.

This investigation demonstrates that in *A. crystallopoietes*, as in other organisms studied, t-RNA is methylated with methionine serving as the methyl donor. The relative rates and patterns of synthesis of proteins have been determined throughout the growth cycle.

The activity of the t-RNA methylases are shown to change during the growth cycle but no correlation was found to exist with the rate of protein synthesis. Minor changes in the pattern of proteins synthesized is reported.

Finally, the phenomenon of Arthrobacter morphogenesis and the results reported for the t-RNA methylases were interpreted within the overall framework of biochemical changes that result from bacterial growth in different media.

BIBLIOGRAPHY

1. Baliga, B. S., P. R. Srinivasan, and E. Borek. 1965. Changes in the t-RNA methylating enzymes during insect metamorphosis. Nature. 208:555-557.

2. Bidwell, R. G. S., R. A. Barr, and F. C. Steward. 1964. Protein synthesis and turnover in cultured plant tissue: sources of carbon for synthesis and the fate of the protein breakdown products. Nature. 203:367-373.

3. Borek, E., and P. R. Srinivasan. 1966. The methylation of nucleic acids, p. 275-298. In P. D. Boyer ed., Annual Review of Biochemistry, vol. 35. Annual Reviews, Inc., Palo Alto.

4. Borek, E., and J. Christman. 1965. A decrease in absorbency in t-RNA produced by enzymatic methylation. Federation Proc. 24:292.

5. Borek, E. 1969. Methylation reactions as possible control factors in protein synthesis: a personal essay, p. 163-190. In Exploitable molecular mechanisms and neoplasis: a collection of papers presented at the twenty-second annual symposium on fundamental cancer research. Williams and Wilkins, Baltimore.

6. Britten, R. J., and F. T. McClure. 1962. The amino acid pool in Escherichia coli. Bact. Rev. 26:292-335.

7. Brown, O. R., and S. Reda. 1967. Enqyme and permeability changes during morphogenesis of Nocardia corallina. J. Gen. Microbiol. 47:199-205.

8. Buchler Instruments Inc. An analytical temperature regulated disc electrophoresis manual. 1-9. 1967.

9. Capecchi, M. R., and G. N. Gussin. 1965. Suppression in vitro. Identification of a serine-sRNA as a "nonsense" suppressor. Science. 149:417-422.

10. Cowie, D. B. 1962. Metabolic pool and the biosynthesis of protein, p. 633-645. In J. T. Holden, ed., Amino Acid Pools. Elsevier Publishing Co., Amsterdam.

11. Dische, Z. 1955. In E. Chargaff and J. N. Davidson, eds., p. The nucleic Acids, vol. 1. Academic Press, New York.

12. Donachie, W. D. 1968. Relationship between cell size and time of initiation of DNA replication. Nature. 219:1077-1079.

13. Ensign, J. C., and R. S. Wolfe. 1964. Nutritional control of morphogenesis in Arthrobacter crystallopoietes. J. Bact. 87:924-032.

14. Ferdinandus, J. A. 1969. Enzyme control of sphere-rod morphogenesis in Arthrobacter crystallopoietes. Ph.D. Dissertation. The University of Oklahoma. Norman, Oklahoma.

15. Ferdinandus, J. A., and J. B. Clark. 1969. Selective inhibition of bacterial enzymes by free fatty acids. J. Bact. 98:1109-1113.

16. Fleissner, E., and E. Borek. 1962. A new enzyme of RNA synthesis: RNA methylase. Proc. Nat. Acad. Sci., U. S. 48:1199-1203.

17. Fry, B. J., and P. R. Gross. 1970. Patterns and rates of protein synthesis in sea urchin embryos II. The calculation of absolute rates. Dev. Biol. 21:125-146.

18. Gefter, M. L., and R. L. Russell. 1969. Role of modification in tyrosine t-RNA: a modified base affecting ribosome binding. J. Molec. Biol. 39:145-157.

19. Hancock, R. L. 1967. Utilization of L-methionine and S-adenosyl-methionine for methylation of soluble RNA by mouse liver and hepatoma extracts. Cancer res. 27:646-653.

20. Hansl, Jr., R. 1964. Review of some new applications for disc electrophoresis and on the criteria for quantitative densitometry. In H. E. Whipple ed., Annals of the New York Academy of Sciences. 121:391-403.

21. Hoyem, T., S. Rodenberg, H. A. Douthit, and H. O. Halverson. 1968. Changes in the patterns of proteins synthesized during outgrowth and microcycle of Bacillus cereus T. Arch. Biochem. Biophys. 125: 964-974.

22. Hurwitz, J., and M. Gold. 1966. t-RNA methylases from Escherichia coli. In G. L. Cantoni and D. R. Davies, eds., Procedures in Nucleic Acid Research. Harper and Row Publishers, New York.

23. Kalnitsky, G., J. D. Hymmel, and C. Dierks. 1959. Some factors which affect the enzymatic digestion of ribonucleic acid. J. Biol. Chem. 234:1512-1516.

24. Kemp, J. D., and D. W. Sutton. 1971. Protein metabolism in cultured plant tissues. Calculation of an absolute rate of protein synthesis, accumulation, and degradation in tobacco Callus in vivo. Biochem. 10:81-88.

25. Kerr, S. J. 1970. Natural inhibitors of the t-RNA methylases. Biochem. 9:690-695.

26. Kipnis, D. M., E. Reiss, and E. Helmreich. 1961. Functional heterogeneity of the intracellular amino acid pool in mammaliam cells. Biochem. Biophys. Acta. 51:519-524.

27. Krulwich, T. A., J. C. Ensign, D. J. Tipper, and J. L. Strominger. 1967. Sphere-rod morphogenesis in A. crystallopoietes I. Cell wall composition and polysaccharides of the peptidoglycan, J. Bact. 94: 734-740.

28. Krulwich, T. A., J. C. Ensign, D. J. Tipper, and J. L. Strominger. 1967. Sphere-rod morphogenesis in A. crystallopoietes II. Peptides of the cell wall peptidoglycan. J. Bact. 94:741-750.

29. Krulwich, T. A., and J. C. Ensign. 1969. Alteration of glucose metabolism of Arthrobacter crystallopoietes by compounds which induce sphere to rod morphogenesis. J. Bact. 97:526-534.

30. Lazzarini, R. A., and A. Peterkofsky. 1965. The characterization of a new species of leucyl-sRNA formed during methionine deprivation of Escherichia coli with relaxed control. Proc. Nat. Acad. Sci., U. S. 53:549-556.

31. Littauer, U. Z., M. Revel, and R. Stern. 1966. Coding properties of methyl deficient phenylalanine t-RNA. Cold Spring Harbor Symp. 31:501-514.

32. Lowry, O. H., J. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265-275.

33. Mandel, L. R., and E. Borek. 1963. The biosynthesis of methylated bases in Ribonucleic acid. Biochem. 2:555-560.

34. Mullakhanbhai, M. F., and J. V. Bhat. 1967. Morphogenesis in Arthrobacter species. Proc. Ind. Acad. Sci. 65:321-327.

35. Neidhardt, F. C., and D. G. Fraenkel. 1961. Metabolic regulation of RNA synthesis in bacteria. Cold Spring Harbor Symp. 26:63-74.

36. Peterkofsky, A. 1964. A role for methylated bases in the amino acid acceptor function of soluble ribonucleic acid. Proc. Nat. Acad. Sci., U. S. 52:1233-1238.

37. Peterkofsky, A., C. Jensensky, and J. D. Capra. 1966. The role of methylated bases in the biological activity of E. coli: Leucine t-RNA. Cold Spring Harbor Symp. 31:515-524.

38. Pillinger, D., and E. Borek. 1969. t-RNA methylases during morphogenesis in the cellular slime mold. Proc. Nat. Acad. Sci., U. S. 62:1145-1150.

39. Revel, M., and U. Z. Littauer. 1965. The isolation of methyl deficient phenylalanyl transfer RNA from E. coli. Biochem. Biophys. Res. Commun. 20:187-194.

40. Sacks, L. E. 1954. Observations on the morphogenesis of Arthrobacter citreus spec. nov. J. Bact. 67:342-345.

41. Schaechter, M. 1961. Patterns of cellular control during unbalanced growth. Cold Spring Harbor Symp. 26:53-62.

42. Sharma, O. K., and E. Borek. 1970. Inhibitor of t-RNA methylases in the differentiating slime mold D. discoideum. J. Bact. 101:705-708.

43. Shugart, L., B. H. Chastine, G. D. Novelli, and M. P. Stulberg. 1968. Restoration of amino acylation activity of undermethylated t-RNA by *in vitro* methylation. Biochem. Biophys. Res. Commun. 31: 404-409.

44. Shurgart, L., G. D. Novelli, and M. P. Stulberg. 1968. Isolation and properties of undermethylated phenylalanine transfer ribonucleic acids from a relaxed mutant of *Escherichia coli*. Biochem. Biophys. Acta. 157:83-90.

45. Srinivansan, P. R., and E. Borek. 1963. The species variation of RNA methylase. Proc. Nat. Acad. Sci., U. S. 49:529-533.

46. Starr, J. L., and R. Fefferman. 1964. The occurrence of methylated bases in ribosomal ribonucleic acid of *Escherichia coli* K 12 W6. J. Biol. Chem. 239: 3457-3461.

47. Stevenson, I. L. 1961. Growth studies on *Arthrobacter globiformis*. Can. J. Microbiol. 7:569-575.

48. Stevenson, I. L. 1962. Growth studies on *Arthrobacter globiformis* II. Changes in macromolecular levels during growth. Can. J. Microbiol. 8:655-661.

49. Sundman, V. 1958. Morphological comparison of some *Arthrobacter* species. Can J. Microbiol. 4:221-224.

50. Svensson, I., H. G. Boman, K. G. Eriksson, and K. Kjellin. 1963. Studies on microbial RNA I. Transfer of methyl groups from methionine to soluble RNA from *Escherichia coli*. J. Molec. Biol. 7:254-271.

51. Wainfan, E., P. R. Srinivasan, and E. Borek. 1966. Inhibition of t-RNA methylases in lysogenic organisms after induction by ultraviolet irradiation or by heat. J. Molec. Biol. 22:349-353.

52. Wainfan, E., P. R. Srinvasan, and E. Borek. 1966. Can Methylation of t-RNA serve a regulatory function. Cold Spring Harbor Symp. 31:525.

53. Weiss, P. A. 1968. The problem of cellular differentiation. In Dynamics of Development: Experiments and inferences. Academic Press. New York.

54. Wright, B. E., and M. I. Anderson. 1960. Protein and amino acid turnover during differentiation in the slime mold I. Utilization of endogenous amino acids and proteins. Biochem. Biophys. Acta. 43: 62-66.

55. Wright, B. E., and M. I. Anderson. 1960. Protein and amino acid turnover during differentiation in slime mold II. Incorporation of ^{35}S-methionine into the amino acid pool and into protein. Biochem. Biophys. Acta. 43:67-78.

56. Wright, B. E. 1966. Multiple causes and controls in differentiation. Science. 153:830-837.