THE UNIVERSITY OF MANITOBA

RIBOSOMAL ALTERATIONS DURING ADAPTATION OF THE FACULTATIVE AUTOTROPH THIOBACILLUS NOVELLUS

BY

IN KOOK PARK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

WINNIPEG, MANITOBA

October, 1973

ABSTRACT

by IN KOOK PARK

Under the supervision of Dr. P. Y. Maeba

Ribosomal proteins extracted from heterotrophically - and autotrophically - grown T. novellus were studied by disc and split polyacrylamide gel electrophoresis. These studies indicated that there were major differences between ribosomal proteins derived from heterotrophically - and autotrophically - grown T. novellus. Furthermore, the protein content of ribosomes purified from heterotrophically - and autotrophically - grown T. novellus was determined. The results showed that the protein content of T. novellus ribosomes was essentially the same, although the protein content of autotrophically - grown T. novellus was 3.8% greater than those from cells grown heterotrophically. A cell-free system from the heterotrophically - grown T. novellus was found to actively incorporate phenylalanine into hot TCA - precipitable material in the presence of ribosomes, supernatant factors and polyuridylic acid. Phenylalanine incorporation was absolutely dependent on the presence of ribosome and poly U. To determine the genetic affinity between the heterotrophic and autotrophic T. novellus, DNA base composition was determined by cesium chloride density gradient centrifugation

and spectral analysis. The present study reports a significant difference in CsCl buoyant density of DNA observed between heterotrophic and autotrophic T. novellus. The G + C contents in the heterotrophic and autotrophic DNA are 52.1% and 65.4% respectively. The significance of this unexpected difference between heterotrophic and autotrophic DNA base composition suggests they are genetically heterogeneous.

T O M Y P A R E N T S

ACKNOWLEDGEMENTS

The author expresses profound appreciation to Dr. P. Y. Maeba for assistance and guidance throughout the course of this investigation and preparation of the manuscript. For many helpful and stimulating discussions, special thanks are due to Dr. I. Suzuki and Dr. C. T. Chow.

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ABBREVIATIONS

A_{260}	Absorbance at 260 mμ
A.T.C.C.	American Type Culture Collection
DNAase	Deoxyribonuclease
DOC	Sodium deoxycholate
DTT	Dithiothreitol
PEP	Phosphoenolpyruvate
Poly U	Polyuridylic acid
RNAase	Pancreatic ribonuclease
γ-protein	Ribosomal protein
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate (0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0)
TCA	Trichloracetic acid
TK	0.01 M Tris + 0.1 M KCl, pH 7.8
TKM	0.01 M Tris + 0.1 M KCl + 0.01 M Mg acetate, pH 7.8
TM	0.01 M Tris + 0.01 M Mg acetate, pH 7.8
Tris	Tris (hydroxymethyl) aminomethane

INTRODUCTION

Zamenick (1960) first showed the involvement of microsomal fractions in protein synthesis making it possible to study protein synthesis in cell-free systems. As the early work on the structure of the ribosome was proceeding, a general picture of its functional properties had begun to emerge. Recent data indicate that E. coli ribosomes are heterogeneous and there is, at most, one copy of each protein per ribosome (Hardy, Kurland, Voynow, and Mora, 1969). The variation of protein composition in E. coli ribosomes as a function of growth rate was reported by Deusser and Wittman (1972) under different growth conditions. They found that as a result of the condition of cells grown in different media, the relative amounts of ribosomal proteins varied. In this thesis, we have attempted to study this problem using T. novellus system grown heterotrophically and autotrophically. Studies were undertaken to understand the changes in general physical and chemical properties of ribosomes due to different physiological environment. However, the results have not yet led us to the point of being able to understand the changes in ribosome structure on the basis of the indicated scattered group of data.

II. HISTORICAL

In 1887 Winogradsky observed H_2S could serve as electron donor for the respiration of a Beggiatoa species. This was the first in a series of studies which culminated in the discovery of chemoautotrophy, i.e., the ability of some organisms to use inorganic compounds as a sole energy.

Chemoautotrophic sulfur-oxidizing bacteria are placed in a single genus, Thiobacillus, that is divided into two groups (Breed, Murray, and Smith, 1957) - strict and facultative autotrophs. Generally, thiobacilli are gram-negative, aerobic, polarly flagellated rods that use carbon dioxide as a sole source of carbon. Their energy is obtained from the oxidation of sulfur compounds; most species growing best with thiosulfate, although sulfide, sulfur and thiocyanate can be used by certain species. During growth, the sulfur compounds are more completely oxidized, the energy released by the reaction enabling the bacteria to fix CO_2 into cell constituents. As a group, they play an important role in the ecosphere by converting sulfur and partially oxidized sulfur compounds to sulfate that can be incorporated by plants and microorganisms into organic compounds in the reduced form as thiols or disulfide groups (Stanier, Doudoroff and Adelberg, 1970).

The facultative autotrophs differ from the strict autotrophs in their ability to grow on organic compounds, such as glutamate or glucose in the absence of thiosulfate. Characteristically they grow well on nutrient agar, although no acid production from carbohydrates has been reported. Autotrophically, the facultative species can oxidize thiosulfate to sulfate and can utilize CO_2 as the sole source of carbon (Skerman, 1967).

The mode of oxidation of sulfur compounds varies considerably depending upon organism and nature of the substrate. The overall reaction by which T. thioparus oxidizes thiosulfate is:

$2S_2O_3^{=} + O_2 + AMP + 2Pi + 4H^{+} \longrightarrow 2S^{o} + 2SO_4^{=} + ATP + 2H^{+}$

(Peck and Fisher, 1962). On the other hand, T. novellus growing on thiosulfate as a sole energy source oxidizes thiosulfate as follows:

$S_2O_3^{=} + 2O_2 + H_2O \longrightarrow 2SO_4^{=} + 2H^{+}$ (Charles and Suzuki, 1966). T. novellus as stated earlier, are facultative with respect to energy source and are able to switch to either mode of growth. This characteristic is shared with T. caproliticus (Lipman and McLees, 1940) and T. intermedius (London, 1963).

T. novellus was first isolated from the soil and described by Starkey in 1935 during the course of an investigation on thiosulfate-utilizing bacteria. The organism is a small and non-motile rod, about 0.5 to 1 μ wide and 1 to 4 μ long. They grow

best at a pH between 8.0 and 9.0 and somewhat resemble the strict autotroph T. thioparus which also grows at a pH optimum close to neutrality. T. novellus differs from other facultative organisms in this genus in several respects. For instance, T. intermedius can grow heterotrophically only in the presence of thiosulfate indicating that this species still has a requirement for an inorganic energy source although it is able to use an organic carbon source (London and Rittenberg, 1966). Also, T. novellus appears to be unique in not producing tetrathionate ($S_4O_6^{=}$) as an end product (Starkey ab, 1934; Parker and Prisk, 1953). This is a characteristic feature of other thiobacilli growing on thiosulfate (Gleen and Quastel, 1953; Vishniac, 1952; Jones and Happold, 1961; Vishniac and Trudinger, 1962). The process of reversible adaptation to autotrophic and heterotrophic modes of growth has been little studied, although the process may be fundamental to understanding evolution and processes such as differentiation. Since in this mode organisms use CO_2 as a sole carbon source, enzymes such as carboxydismutase and enzymes of the Calvin - Benson - Bassham Cycle; (Calvin and Benson, 1948; Bassham, Benson, and Calvin, 1950; Benson et al, 1950) which are not present during heterotrophic growth, must be formed. In addition, a system to generate reducing power in the form of NADPH + H^+ is required. Some of these biochemical events have been studied and are summarized below.

As a heterotroph, T. novellus can grow on a limited number of organic carbon substrates - glutamate being the best. Surprisingly, they cannot utilize sugars such as glucose, sucrose and lactose nor the dicarboxylic acids succinate, malate and acetate (Santer, Boyer and Santer, 1959). On the other hand, Charles and Suzuki (1965) showed that T. novellus could be adapted to growth on glucose. No explanation was offered to reconcile this difference. Using this organism LeJohn, Van Caeseele and Lees (1967) conducted a series of experiments to determine the relation between heterotrophic carbon sources and ability to oxidize thiosulfate. They concluded that a thiosulfate - oxidizing system could be induced by thiosulfate and that this system was subject to catabolite repression by fermentable carbon sources such as glucose, lactose, ribose, glycerol, lactate and pyruvate. However, carbon compounds such as amino acids and organic acids which are metabolized aerobically caused no repression.

Although growth on organic medium occurs readily (Santer et al, 1959; Charles et al, 1965; LeJohn et al, 1967), the conversion from heterotrophy to autotrophy is a time-consuming process. To adapt T. novellus to autotrophy, Charles and Suzuki (1965) subjected the organism to repeated transfers into mineral salts medium with decreasing concentrations of glucose and increasing concentrations

of thiosulfate. Four transfers were required before autotrophic characteristics were acquired. This may, in part, be attributed to "glucose" repression of thiosulfate oxidizing enzymes (LéJohn et al, 1967). The pathway by which this proceeds has been elucidated by Charles and Suzuki (1965, 1966a, 1966b) and required enzymes for thiosulfate cleavage, and sulfur oxidation as well as the sulfite and cytochrome oxidases. Energy is generated in this system via oxidative phosphorylation coupled to the oxidation of sulfite by sulfite oxidase and the cytochrome system. It is apparent that organisms growing autotrophically and heterotrophically represent two physiological states of T. novellus.

The altered growth rates, the time required for adapting to autotrophy and the requirement for different enzymes indicate that many concomitant alterations may occur during this transition. The physiological responses to altered environment may be related to the rate and type of protein synthesis taking place on the ribosomes. Therefore studies towards an understanding of the structure and function of ribosomes of the T. novellus growing in 2 physiological conditions was undertaken.

The ribosome is an exceedingly complex organelle that is essential to protein synthesis in both prokaryotes and eukaryotes and is composed of two subunits, sixty or so distinct species of proteins, and at least three species of rRNA (Spirin and Gavrilova,

1969). The ribosomes of bacteria and blue green algae possess a molecular weight of about 2.8 to 3 x 10^6 daltons and a sedimentation coefficient of about 70 S; the dimensions of the dry 70 S ribosomes are 200 x 170 x 170 A^o. In animals, higher plants, fungi and algae they are somewhat larger, and are characterized by a molecular weight of 4 to 5 x 10^6, a sedimentation coefficient of about 80 S, and dimensions in the dry state of 240 x 200 x 200 A^o. A universal structural feature of all ribosomes is that they are constructed from two unequal subparticles - the 50 S and 30 S subunits. The 80 S ribosome of eukaryotes is analogously subdivided into 60 S and 40 S subparticles.

The structure of ribosomal particles from E. coli was initially studied by Tissières, Watson and their collaborators (1959). The 30 S ribosomal subunit contains one 16 S RNA molecule (0.55 x 10^6) whereas the 50 S subunit contains one 23 S RNA molecule (1.1 x 10^6) and one 5 S RNA molecule (4 x 10^4). In contrast to the RNA components, the protein composition of ribosomes is complex. Waller (1964) first demonstrated that many different proteins are present in bacterial ribosomes by fractionating ribosomal proteins by electrophoresis on starch gel and by chromatography on carboxymethyl cellulose. He concluded that there are at least 24 separable protein components in ribosomes that are not

artifacts caused by the aggregation of a smaller number of proteins. Waller also showed that 30 S and 50 S ribosomal subunits contain characteristically different proteins, and that ribosomal proteins from different species of bacteria are unique. The heterogeneity of ribosomal proteins was also indicated by many bands observed after starch gel or polyacrylamide gel electrophoresis of the proteins (Traub, Hosokawa, Craven, Nomura, 1967; Gesteland and Staehelin, 1967). They provided support for the view that the apparent electrophoretic complexity of ribosomal proteins reflected a real protein heterogeneity.

The possibility of common sequences or homologies among the different proteins has also been investigated by immunological methods (Fogel and Sypherd, 1968). Each of eight pure 30 S proteins was tested for cross reaction with each of the seven others, using the antiserum made against total 30 S protein. Of the 28 pairs tested, 26 showed a clear reaction of nonidentity. Only two pairs of proteins gave reactions of partial identity. The results indicated that extensive homologies do not exist among these proteins.

Recent work from several laboratories has shown that there are about 20 - 21 different proteins in the 30 S subunit and 34 in the 50 S subunit (Traut, Moore, Delius, Noller, and Tissières, 1967; Kaltschmidt, Dzionara, Donner, and Wittman, 1967; Fogel and Sypherd, 1968). Ribosomal proteins of the 30 S subunit were separated

into 20 different proteins all of which were characterized. Each 30 S subunit contains a single 16 S RNA with a molecular weight between 530,000 and 560,000 daltons (Kurland, 1960; Midgely, 1965; Stanley and Bock, 1965) and is 30-33% protein corresponding to 230,000 to 280,000 daltons of protein per 30 S subunit. The total mass of protein in a particle that has one copy of each of the twenty purified proteins would be 410,000 daltons. The sum of molecular weights for twenty 30 S proteins exceeds by 130,000 - 180,000 daltons the average mass of protein per 30 S subunit. Thus the subunit is not sufficiently large to accomodate 1 copy of each protein. This indicated that not only are ribosomal proteins heterogeneous but also the ribosomes themselves are heterogeneous with respect to protein complement. Stoichiometric data indicated only 6 proteins were present in amounts close to one copy per 30 S subunit (the unit proteins); an equal number were present in amounts much less than one copy per ribosome (the fractional proteins) (Kurland, Voynow, Hardy, Randall and Lutter, 1969; Voynow and Kurland, 1971).

There are two interpretations that could explain the heterogeneity of 30 S subunits (Traub et al, 1968). The static model suggests that the functional specializations of subclasses of ribosomes are permanently fixed by stable association of specific fractional proteins with a common core of unit proteins. The steady-state model, alternatively, suggests that the fractional

proteins exchange from one ribosome to another in an orderly cycle. In this case, each phase of protein synthesis - initiation, propagation, termination, and so on - is mediated by the same 30 S subunit but each functional mode is associated with a different set of exchangeable fractional proteins. The presence or absence of proteins therefore determines the functional state of the 30 S ribosomal subunits. The 30 S subunit can be fractionated into different structural classes with somewhat different functional properties. This data has appeared to support the structural heterogeneity of 30 S subunit (Dzionara, Kaltschmidt and Wittmann, 1970; Wittmann, 1972).

Apirion and Schlessinger (1968) studied ribosomes extracted from aerobically and anaerobically grown E. coli and compared their capacities for in vitro polypeptide synthesis. Apirion et al (1968) showed that the 50 S subunit of anaerobic ribosomes was the main component responsible for the inability of t-RNA to stimulate polylysine formation directed by polyadenylic acid. It has long been known that some bacterial strains have altered growth requirements when grown anaerobically (Wilson and Miles, 1946); anaerobiosis markedly reduces the antibacterial effect of streptomycin (Bondi, Dietz, and Spaulding, 1946; Stern, Barner, and Cohen, 1966), an antibiotic known to effect the ribosomes.

The ribosomes of mammalian cells are known to occur in two classes, free and membrane-bound. The proportion of ribosomes associated with membranes varies widely in different types of cells (Palade, 1955). It has been proposed that the membrane-bound ribosomes are involved in the synthesis of proteins for secretion, whereas free ribosomes are thought to synthesize proteins destined for intracellular use (Palade, 1966; Takagi and Ogata 1968). Supporting this contention is membrane-bound ribosomes synthesize proteins different from those synthesized by free polyribosomes (Dautrevaux, Boulanger, Han, and Biserte, 1969; Air, Thompson, Richardson and Sharman, 1971). Karadjova and Genov (1970) also suggested that membrane proteins are synthesized by membrane-bound ribosomes.

To test heterogeneity of ribosomal subunits Deusser and Wittman (1972) studied the protein composition of ribosomes synthesized by E. coli grown in rich (yeast extract and peptone) or in minimal media, labelling the cells with different isotopes (^{14}C or ^{3}H - amino acids). Ribosomal proteins from ^{14}C and ^{3}H labelled cells were extracted together and identified by two dimensional gel electrophoresis. The ratio of ^{3}H and ^{14}C radioactivity associated with each protein was calculated. On this basis, the proteins were divided into 4 groups: about two-thirds of all proteins gave ratios between 0.90 and 1.10. A rather large group (S1, S10, S11, S19, S20 from 30 S subunits and L8 + L9, L17, L27, L32, L33 from 50 S) had ratios

which were slightly but not always reproducibly different from those of the first group. This shows that most ribosomal proteins are found in the same amounts whether cells were grown in rich or minimal medium. For proteins L7 and L16 the ratios were 0.8. This represents a decrease of these proteins in rich medium and an increase in minimal medium. The ratios for three proteins, i.e., 3.1 for L12; 2.5 for S6; and 2.4 for S21, differed considerably from the others. This showed the increase of three proteins (L12, S6, and S21) in rich medium. It was suggested that when cells are grown in different media, the rates of synthesis of macromolecules are substantially changed resulting in heterogeneous ribosomes.

III. MATERIALS AND METHODS

Organism:

T. novellus (ATCC #8093) was used in this investigation. The cells were grown in 500 ml Erlenmeyer flasks on a rotary shaker in 200 ml medium or, for larger preparations, in 15 litre aerated carboys with the same medium. The composition of this medium (Starkey, 1935) per litre is as follows:

10.0 g	$Na_2S_2O_3.5H_2O$
4.0 g	K_2HPO_4
1.5 g	KH_2PO_4
0.02 g	$CaCl_2.2H_2O$
0.1 g	$MgSO_4.7H_2O$
0.3 g	$(NH_4)_2SO4$
0.02 g	$MnSO_4.2H_2O$
0.02 g	$FeCl_3.6H_2O$

A small amount of 0.2% phenol red was added to the medium as a pH indicator. The pH adjusted to 7.8 before sterilization and was maintained at this pH during cell growth by addition of 10% sterilized Na_2CO_3 or K_2CO_3. After 5 - 7 days at 28^{o} the autotrophic culture reached stationary phase and were harvested with a Sharples centrifuge then stored at - 70^{o} until used.

Heterotrophic growth medium was the same as above except thiosulfate was omitted and 1% glucose or glutamate served as the carbon and energy source. In this medium the culture entered late log phase around 20-22 h. Cells grown in heterotrophic or autotrophic medium were aerated by shaking or by air forced through bubbling tubes. Cells were harvested and stored in the same manner as autotrophically-grown cells. Growth was followed by measuring the optical density of cell suspensions at 540 mμ in a Klett-Summerson colorimeter.

Preparation of Ribosomes:

Ribosomes were prepared from 20 g frozen cells by a modification of the method of Kurland (1966). Cells were disrupted by 1 passage through an Aminco French pressure cell in TKM buffer containing 6 mM mercaptoethanol, 0.1 ml DNAase (1 mg/ml) and 2 g macaloid. The cell debris was removed by centrifugation at 15,000 r.p.m. for 30 min in a Sorvall RC 2-B. The supernatant was subjected to 2 successive ammonium sulfate fractionations; the final pellet being dissolved in TM buffer containing 0.6 M $(NH_4)_2SO_4$. The ribosomes were washed 2 times in this buffer by pelleting at 87,500 x g for 3½ h, resuspended in fresh buffer, frozen in 1.0 ml portions in liquid nitrogen, then stored at - 70° at a concentration of 300 A_{260} units/ml.

Sedimentation Analysis:

Sedimentation analysis was performed using E. coli 70 S ribosomes as markers. Ribosomes were dialyzed in different buffers at 4° for 5-6 h prior to analysis. Approximately 2 A_{260} units of 70 S ribosomes were applied to 5 ml of a 5-20% linear RNAase-free sucrose gradient made in the same buffer used for dialysis and centrifuged at 50,000 r.p.m. for 1 3/4 h in a Spinco SW 50.1 rotor. Gradients were analyzed with a flow-through cell in a Gilford Model 2000 recording spectrophotometer at 260 mμ.

Preparation of Ribosomal Subunits:

50 and 30 S ribosomal subunits were isolated from ribosomes by sucrose gradient centrifugation. Ribosomes in TKM buffer were dialyzed against TK buffer containing 1.0 mM Mg acetate for 5-6 h prior to centrifugation. Samples containing a total of 200-300 A_{260} units of ribosomes were layered on a 60 ml 5-20% linear RNAase-free sucrose gradient in TK buffer containing 1 mM Mg acetate. The tubes were centrifuged at 22,000 r.p.m. for 12 h in a Spinco SW 25.2 rotor. Fractions (1-2 ml) were collected and suitably diluted aliquots were assayed at 260 mμ in DB Beckman spectrophotometer. The peak fractions corresponding to 50 and 30 S ribosomal subunits were pooled and precipitated with 0.7 volume of ethanol (Staehelin, Maglott and Monroe, 1969) after increasing the magnesium concentration to 0.01 M. The pooled fractions were allowed to stand at -20°

overnight. The precipitated subunits were collected by centrifugation at 10,000 x g for 30 min then dissolved in TKM buffer and dialyzed against the same buffer for 5-6 h. The subunits were stored at -70° in 0.5 ml quantities at 100 A_{260} units/ml.

Preparation of Ribosomal Protein and RNA:

Ribosomal proteins were extracted from 70 S ribosomes with 67% cold glacial acetic acid according to the method of Waller and Harris (1961). The magnesium concentration of the ribosome suspension was raised to 0.1 M, then 2 volumes of glacial acetic acid were added. The sample was left on ice for 60 min and mixed occasionally with a vortex mixer. After low speed centrifugation (20 min at 10,000 x g), proteins remained in the supernatant and RNA sedimented as a pellet. The protein fraction was dialyzed against Tris-Urea buffer (0.1 M Tris-HCl, 8.0 M urea, pH 8.0) containing 0.001 M DTT overnight, then stored at -20°. The ribosomal RNA fraction was lyophilized to remove the acetate, then stored at -70°.

Protein Determination:

Protein content of the sample was determined by the phenol method of Lowry, Rosebrough, Farr, and Randall (1951) using crystalline bovine serum albumin (Sigma Chemical Co.) as a standard.

Polyacrylamide Gel Electrophoresis:

Electrophoresis was performed as described by Leboy, Cox, and Flaks (1964) at pH 4.5. The separating gel contained 12.5% acrylamide and 0.75% bisacrylamide in 8.0 M urea. Electrophoretic runs were made in glass tubes, 10.5 mm long and with an inner diameter of 0.6 mm. The volume of the mixture for the lower gel was 1.0 ml, for the spacer gel, 0.1 to 0.2 ml, and for the sample gel, 0.15 to 0.2 ml. Usually fifty to 100 µg of ribosomal proteins in a volume of 10 to 20 µl was mixed with the sample gel before polymerization. Electrophoresis was carried out at 4^{o} with β-alanine-acetic acid buffer at a constant current of 3 ma/gel. A trace of 0.1% pyronine red was applied to the top of the sample gel and served as the tracking dye. When the tracking dye reached the bottom of the gels, electrophoresis was stopped, and gels were removed from the glass tubes by rimming with water. Gels were fixed in 12.5% TCA at 37^{o} for 1 h, and stained for at least 1 h in 0.05% Coomassie Brilliant Blue (Sigma) in 12.5% TCA. The gels were destained in 10.0% TCA at room temperature.

The split gel technique (Leboy, Cox and Flaks, 1964) was used to electrophorese two different samples in a single gel by including a liquid-tight plastic divider that splits the sample and spacer gels longitudinally into two compartments.

Different protein samples were placed on either side of the insert and the electrophoretic run was carried out as described above. Under these conditions the separation of ribosomal proteins takes place in about 3-4 h.

Components for *in vitro* Polypeptide Synthesis:

The procedure for preparation of cell-free extracts was based on that developed by Nirenberg (1964) for *E. coli*. Cells washed with TM buffer were disrupted with the French press at 20,000 p.s.i., and centrifuged in a Sorvall RC 2-B at 20,000 x g for 20 min. The supernatant fluid was recentrifuged in Spinco 50 rotor at 30,000 x g for 30 min. The upper 4/5 of this supernatant was removed by a syringe, and portions were quickly frozen in liquid nitrogen were stored at -70^{o} until needed. This fraction was labelled S-30.

S-100 fraction was prepared from S-30 extracts by centrifugation at 105,000 x g for 2 h in a Spinco Model L preparative ultracentrifuge at 4^{o}. The upper 4/5 of the supernatant was labelled S-100 and stored at -70^{o}.

In vitro Polypeptide Synthesis:

The assay system described by Nirenberg (1964) was used to measure incorporation of ^{14}C-phenylalanine into TCA-insoluble

polyphenylalanine directed by polyuridylic acid which served as messenger RNA. The components and their concentrations in the reaction mixture are listed in Table 1. To the reaction mixture was added the S-30 fraction or S-100 fraction and ribosomes. After incubation at 28° for 30 min the reaction was terminated by the addition of 4 ml of cold 10% TCA then was heated at 90° to 95° for 20 min. After cooling at 0° for 30 min, the precipitated material was collected by centrifugation at 17,300 x g for 10 min. The pellet was dissolved in 0.1 N NaOH then reprecipitated with 6 ml of 10% TCA. The precipitate was collected on a membrane filter (HA, 0.45 µm, Millipore Corp.), washed four times with 5 ml of 5% TCA, transferred to scintillation vial. The radioactivity of samples was measured in a Tri-Carb liquid scintillation in Aquasol (New England Nuclear). All samples were assayed in duplicate.

Isolation of DNA:

The method of Marmur (1961) was used to prepare DNA from T. novellus. Five g of cells were lysed with 100 µg/ml of lysozyme and deproteinized with 1 M NaCl. DNA was precipitated from the solution with 2 vol ice-cold ethanol, dissolved in 5 ml SSC then treated with 10 µg/ml of DNAase-free RNAase for 30 min at 37°. After further deproteinization with 1.0 M NaCl and 3

TABLE 1

Components of ^{14}C-phenylalanine incorporating mixture*.

Component	Concentration per ml
Tris - HCl (pH 7.6)	10.0 μ moles
KCl	60.0 μ moles
NH_4Cl	50.0 μ moles
Mg acetate	20.0 μ moles
Spermidine	5.0 μ moles
2-Mercaptoethanol	6.0 μ moles
ATP	2.5 μ moles
GTP	0.25 μ moles
Na phosphoenolpyruvate	20.0 μ moles
Pyruvate kinase	40.0 μg
^{14}C-phenylalanine (4 Ci/mole)	0.05 μ moles
19 other ^{12}C-amino acids	0.05 μ moles
Polyuridylic acid	100.0 μg

* The final volume of each assay was 0.5 ml.

extractions with an equal volume of chloroform-isoamyl alcohol (24:1; v/v), DNA was precipitated with ethanol then dissolved in SSC buffer. To remove residual RNA and polysaccharides, DNA was precipitated from solution with 0.54 vol isopropanol. After dissolving in SSC buffer, denatured material was removed by centrifugation at 10,000 x g. DNA was stored frozen until used.

DNA Analysis:

(A) Buoyant Density Analysis:

Approximately 1 A_{260} unit of DNA was added to 4.5 ml CsCl solution (130 g CsCl in 70 ml 0.02 M Tris-HCl, pH 8.5) and the solution was adjusted with the same buffer to give a refractive index near 1.4. The DNA was centrifuged at 40,000 r.p.m. for 44 h in a Spinco SW 50.1 rotor at 25°. After the run, tubes were punctured and 7 drop fractions ($\simeq$ 0.15 ml) were collected. The refractive index of each fraction was measured with an Abbe-3L refractometer set at 25°. The absorbance of each fraction was measured at 260 mμ, so that the refractive index of the fraction containing the DNA peak could be determined.

The density of the peak fraction was determined by the equation (Ifft, Voet and Vinograd, 1961):

$$\rho = (10.8601 \times R.\ I.) - 13.4974$$

where ρ = density in g/cc

R. I. = refractive index