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RIBOSOMAL ALTERATIONS DURING ADAPTATION OF THE FACULTATIVE AUTOTROPH THIOBACILLUS NOVELLUS

BY

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ABSTRACT

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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

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TABLE OF CONTENTS

Table of Contents Continued Page

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

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

The GC content of DNA was calculated from the empirical equation (Shildkraut, Marmur and Doty, 1962):

$$\% \text{ GC} = (\rho - 1.660) / 0.00098$$

where ρ= density in g/cc

(B) Spectral Analysis:

The 3 term analysis for native DNA (Felsenfeld and Hirschman, 1966) was used to determine AT concentrations in DNA. Absorbance of DNA in SSC buffer was measured in a Unicam SP 700 spectrophotometer at wavelengths from 235 to 290 mμ in 5 mμ increments. The absorbance readings at each wavelengths were multiplied by the parameters given in Table 2. The values μ_1, μ_2, and μ_3 were calculated from the equations:

$$\mu_1 = \Sigma Ai\ \alpha i$$

$$\mu_2 = \Sigma Ai\ \beta i$$

$$\mu_3 = \Sigma Ai\ \gamma i$$

where Ai = absorbance at wavelengths i

αi, βi and γi = parameters for 3 - term analysis at wavelengths, i, (see Table 2). The AT content is then calculated from the equation

$$\% \text{ AT} = 0.9329\mu_1 + 2.0631\mu_2 + 0.6198\ \mu_3.$$

Chemicals:

The following chemicals were obtained from commercial sources: Sodium deoxycholate from Matheson Coleman and Bell Co., Norwood, Ohio, U.S.A.); Macaloid from Baroid Division National Lead Co.;

TABLE 2

Parameters for three - term analysis of native DNA spectra

λ_i (nm)	α_i	β_i	γ_i
235	−2026	−656	3952
240	−1889	−1251	5031
245	−1390	−1917	6338
250	43	−2830	7480
255	−319	−1807	7616
260	−608	−1141	7307
265	2515	−3379	7052
270	871	−1409	5740
275	−386	−154	4587
280	1159	−1558	3938
285	1797	−2424	3164
290	1187	−2099	2188

Multiply each term by 10^{-7}

$S1$	0·9329	$S4$	0·2792
$S2$	2·0631	$S5$	0·2124
$S3$	0·6198	$S6$	0·8513

*Taken from Felsenfeld (1971)

PEP (phosphoenolpyruvate) from Sigma Chemical Co., Crystalline DNAase from Worthington Biochemical Corp.; C^{14}-phenylalanine from New England Nuclear Corp.; Cesium Chloride from Harshaw Chemical; Dithiothreitol from Biochemical Corp, Cleveland; RNAase from Calbiochem, Los Angeles, Calif. U.S.A.; Aquasol from New England Nuclear, Boston, Mass. U.S.A., Sucrose (density gradient grade, ribonuclease-free) from Mann Research Lab.; Pancreatic ribonuclease from Sigma Chemical Co.

RESULTS

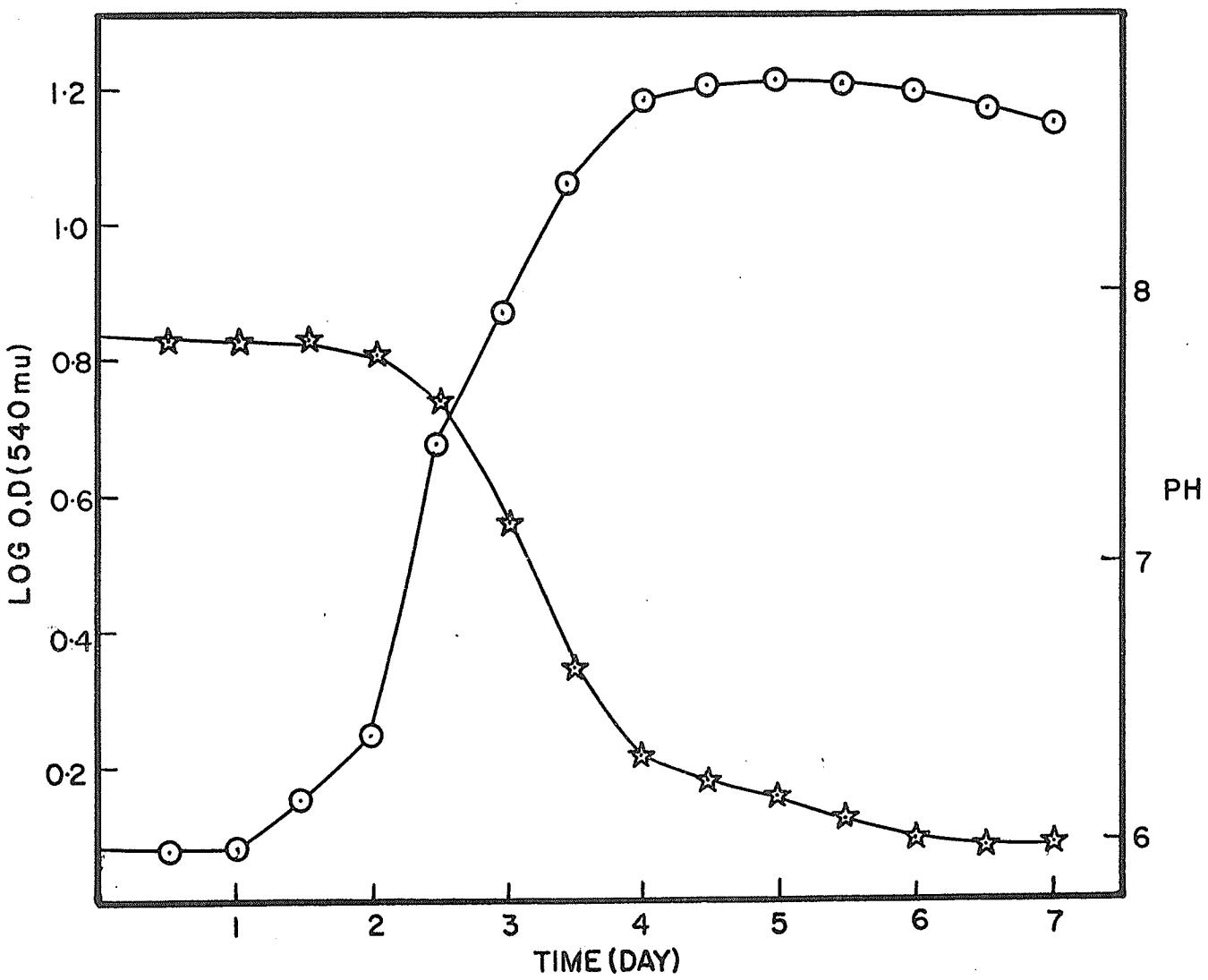
Growth of Organisms

The growth of T. novellus in autotrophic and heterotrophic media was studied in order to determine the time required for cells to enter stationary phase. In both cases inoculum cells were grown in the same medium as used for growth studies. For growth under autotrophic conditions, No. 3 medium of Starkey (1935) with 1% sodium thiosulfate was used and for heterotrophic growth conditions the same medium was employed except sodium glutamate replaced thiosulfate. Details of medium composition are given in Methods.

Approximately 0.1 volume of inoculum was added to culture medium to initiate growth at 28° which was followed as the increase in optical density of the culture as measured at 540 mμ in a Klett-Summerson colorimeter. Figure 1 shows the results obtained when T. novellus was grown autotrophically. Growth proceeded at a slow rate characteristic of autotrophs and did not enter logarithmic growth phase until the end of the first day. Logarithmic growth proceeded for 3 to 4 days at which time the culture entered stationary phase. Aliquots of the culture medium were aseptically removed at ½ day intervals for pH analysis and the results superimposed on the growth curve.

As shown in Figure 1, logarithmic growth was accompanied by decreasing pH of the culture medium and in stationary phase the pH levelled off at 6.0, a decrease of 1.6 pH units compared to the

FIGURE 1. Growth of T. novellus in autotrophic medium. Cells were grown in 600 mls Starkey No. 3 medium with 1% thiosulfate (w/v) at 28°, aeration being provided by shaking. Details of the growth conditions are given in Methods. The circles represent the optical density of the cultures measured in Klett units (540 mμ) and the stars represent the pH of the medium.


1·2
1·0
0·8
0·6
0·4
0·2
LOG O.D(540 mu)
8
7
6
PH
1
2
3
4
5
6
7
TIME (DAY)

original pH of the medium. As would be expected, the rate of decrease in pH is greatest during logarithmic growth when cells actively oxidize thiosulfate to H_2SO_4 (Vishniac, 1952). During this phase doubling time(Dawes, 1969) was calculated to be approximately 26 h.

Growth in heterotrophic medium, i.e., with sodium glutamate as carbon and energy source, proceeded at a much faster rate (Fig. 2) and the culture entered into stationary phase 20-22 h after inoculation. The doubling time was 3 h during logarithmic phase. Growth in heterotrophic medium was much faster than in autotrophic medium. Judging by the optical density at stationary phase in autotrophic and heterotrophic media, (110 and 340 Klett unit, respectively), the cell density was 3 fold greater in heterotrophic medium. The increased growth and growth rates indicated that glutamate was used more readily and more efficiently than were CO_2 and thiosulfate as carbon and energy sources, respectively, under autotrophic conditions.

Adaptive Growth

The time taken for autotrophically - grown T. novellus to adapt to heterotrophic medium and vice versa was studied. The adaptation to heterotrophic medium took place readily, requiring little time for cells to adapt. The response of organisms to an organic carbon source is illustrated in Figure 3. In this

FIGURE 2. Growth of T. novellus in 600 ml Starkey medium No. 3 with 1% glutamate at 28°. Growth was followed as described in legend to Figure 1.

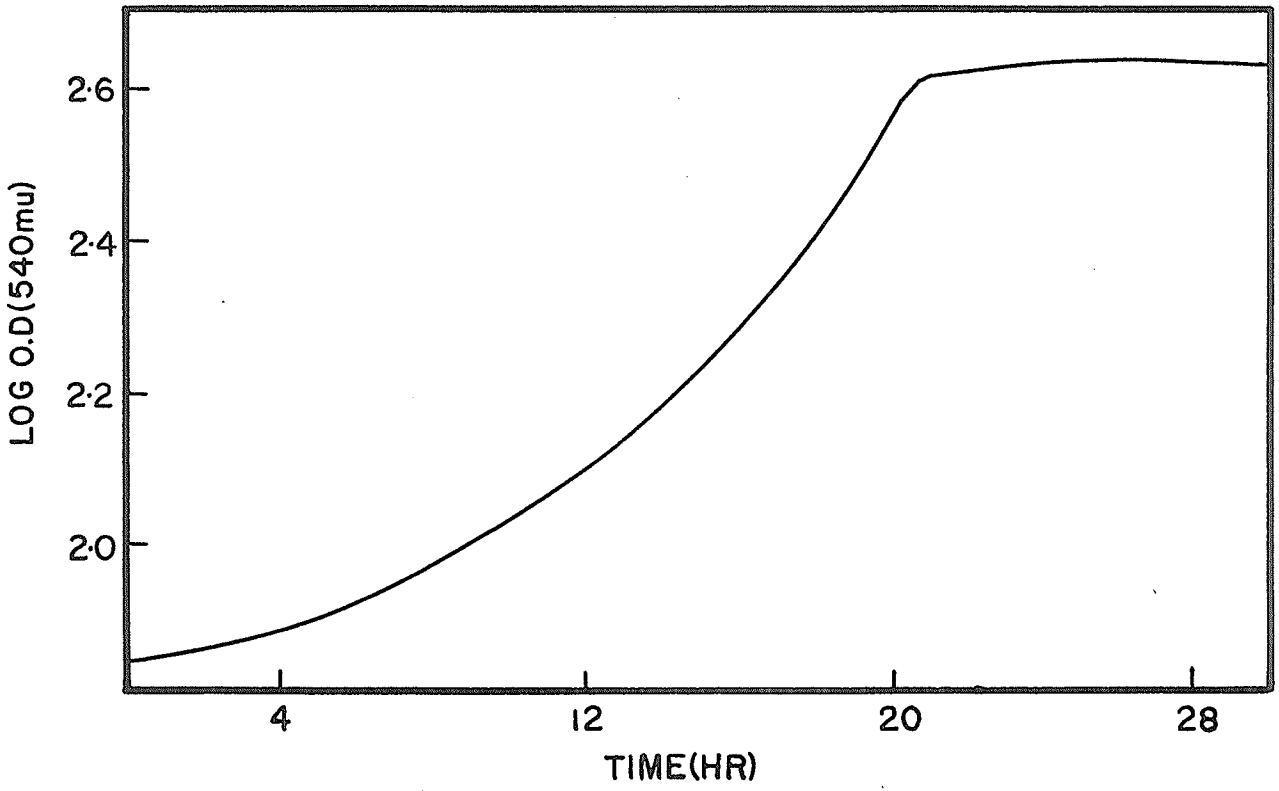
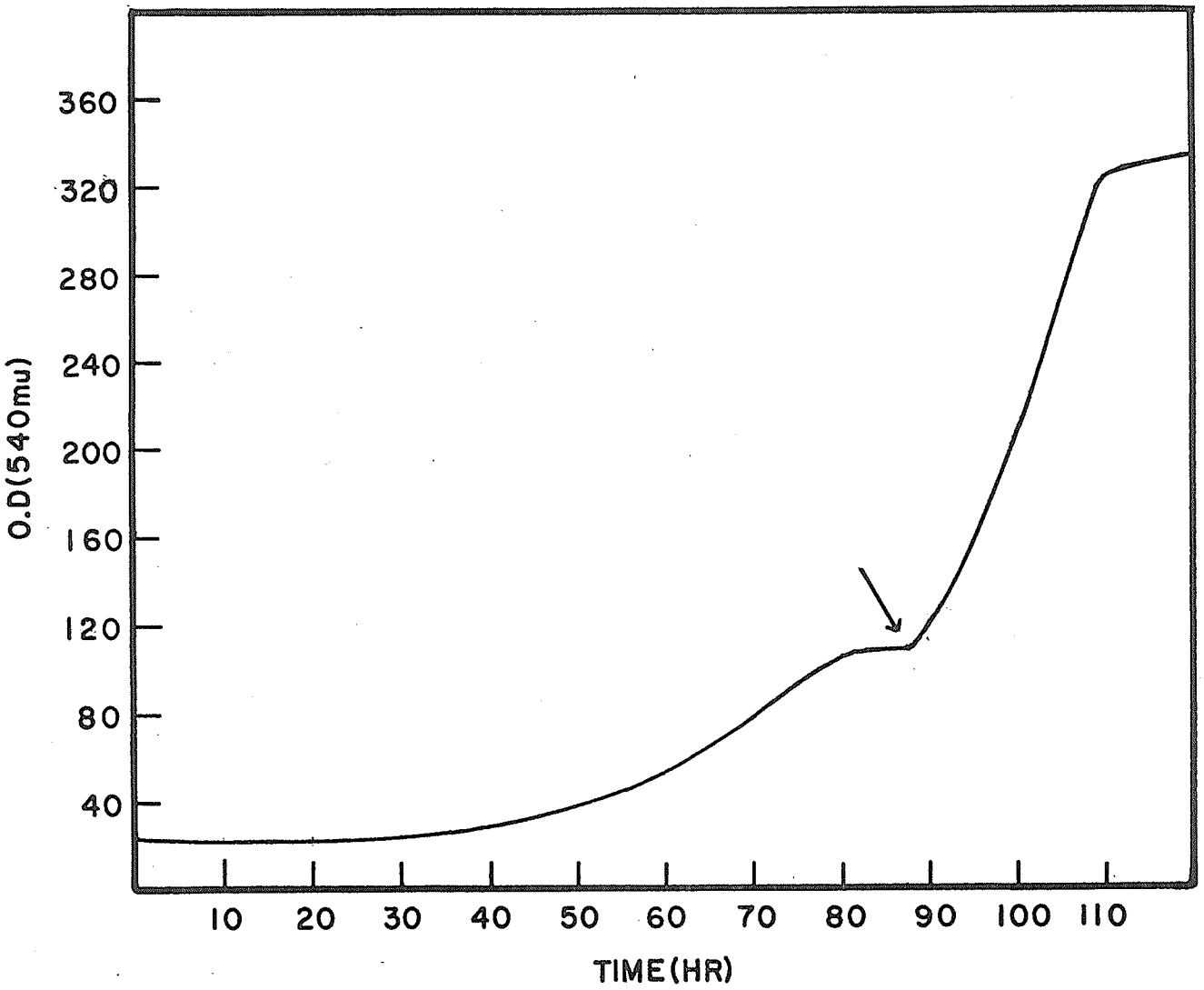

LOG O.D(540mu)
2·6
2·4
2·2
2·0
4
12
20
28
TIME(HR)

FIGURE 3. Adaptation of T. novellus from autotrophic to heterotrophic growth. Cells were grown at 28° in Starkey No. 3 medium with 1% thiosulfate. After reaching stationary phase, the medium was made to 1% glutamate (arrow). Growth was measured at 540 mμ with a Klett-Summerson colorimeter.


360
320
280
240
200
160
120
80
40
O.D(540mu)
10
20
30
40
50
60
70
80
90
100
110
TIME(HR)

experiment, cells were in Starkey No. 3 medium with 1% sodium thiosulfate until the cells entered into stationary phase, at which time sterile 10% sodium glutamate was added to the culture medium to a final concentration of 1%. As shown in Figure 3, growth resumed immediately and continued for another 20 h. This indicates that T. novellus has little difficulty in adapting to heterotrophic growth.

On the other hand, T. novellus cells that had been grown under heterotrophic conditions with glutamate did not adapt readily to growth in autotrophic medium. The protocol of Charles and Suzuki (1965) was followed in order to adapt cells to autotrophic growth conditions. Basically, the method consisted of culturing cells by repeated transfers to medium with progressively decreasing glutamate concentrations and increasing thiosulfate concentrations. An outline of the medium used is shown in Table 3. Cells were allowed to grow to late log in each medium then 0.1 vol was inoculated into the succeeding medium. It took 4 to 5 weeks for the cells to adapt to a completely autotrophic environment.

The results indicated that whereas adaptation to heterotrophy proceeds readily, that to autotrophy was a slow process, suggesting time may be required to acquire functions for CO_2 fixation and thiosulfate oxidation. To determine whether adaptive response may involve more basic changes such as protein synthesizing

TABLE 3

Media used for adapting heterotrophically - grown *T. novellus* to autotrophy.

Number of Transfer	Basic medium with % Na glutamate (w/v)	% thiosulfate (w/v)
0	1.0%	0%
1	0.8%	0.2%
2	0.6%	0.4%
3	0.4%	0.6%
4	0.2%	0.8%
5	0	1.0%

Legend

Cells were grown in basic medium No. 3 of Starkey (Methods) containing 1% glutamate originally then with each transfer, as indicated in column 1, transferred to medium containing less glutamate and more thiosulfate.

components, a study of ribosomes of autotrophically - grown and heterotrophically - grown T. novellus was undertaken.

Isolation of Ribosomes

The isolation of ribosomes from heterotrophically - grown T. novellus is described in "Methods". The procedure yielded 4500 A_{260} units of ribosomes from 40 g cells which is comparable to yields obtained from E. coli cells (Kurland, 1960). Judging by the appearance of ribosomes in solution and chemical composition (see Results) the ribosomes were free of nonspecifically bound proteins.

Ribosome isolation from autotrophically grown cells did not proceed as smoothly. The cells tended to clump together and could not be dispersed into a homogeneous mixture for disruption in the French pressure cell. Using the same protocol for preparing ribosomes from heterotrophically - grown cells, the yield was 5 times lower. Various methods for breaking cells such as the use of alumina powder and anionic detergents did not markedly improve the yield. The observations indicated that there was a major difference between the physical structure of the cells and or ribosome content in the cells.

The final procedure formulated for extraction of ribosomes from cells grown autotrophically involved freezing and thawing the cells twice before disruption by 2 passages through a French

pressure cell at 20,000 p.s.i. During disruption 0.1% sodium deoxycholate was added to the cell suspension to facilitate removal of ribosomes from any membranous material. Thereafter, the procedure described in "Methods" was followed. The ribosome yield was 250 A_{260} units from 10 g cells. The final product could not be separated from a yellow translucent material by differential centrifugation or repeated washings in 0.5 M $(NH_4)_2SO_4$ or 1.0 M NH_4Cl. It could not be determined whether the yellow material was a structural component of these ribosomes or a tightly-bound artifact.

Protein Content of Ribosomes

The protein content of ribosomes purified from heterotrophically - and autotrophically - grown T. novellus was carried out. Protein determinations by the method of Lowry et al (1961) using bovine serum albumin as a standard (Methods). Analyses were made on intact ribosomes solubilized in 0.1 N NaOH. The amount of ribosomes used in the assays was determined on the basis that bacterial ribosomes have an extinction of 65 µg per optical density unit measured at 260 mµ (Kurland, 1960). It was predetermined that the presence of RNA did not interfere with the colorimetric assay for protein.

The results summarized in Table 4 show that the protein

TABLE 4

Protein analysis of T. novellus ribosomes

Growth Condition	mg ribosomes assayed	mg protein	% protein
Heterotrophic	3.705	1.593	
	3.87	1.35	42.2 ± 0.5
	4.43	1.84	
Autotrophic	3.78	1.813	
	3.94	1.832	46.4 ± 1.0
	4.35	1.948	

Legend

Ribosomes purified from heterotrophically - and autotrophically grown T. novellus were assayed for protein content by method of Lowry et al (1961). Details are given in "Results".

content of T. novellus ribosomes are essentially the same even though the cells from which they were derived were grown under different conditions, i.e., 1% glutamate and 1% thiosulfate. The results agree closely with that of Tissières, Watson, Schlessinger and Hollingworth (1959) who reported a protein content of 37% for E. coli ribosomes. Although similar, the protein content of autotrophically - grown T. novellus is 4.2% greater than those from cells grown heterotrophically.

Sedimentation Studies

It is well known that bacterial ribosomes are very sensitive to Mg^{++} concentrations and that they dissociate into large (50 S) and small (30 S) subunits at critical Mg^{++} levels (Tissières et al, 1959). To test whether ribosomes isolated from autotrophically - and heterotrophically - grown T. novellus behave identically to different concentrations of Mg^{++}, the studies described below were carried out. Purified ribosomes isolated from the organism grown under 2 cultural conditions were dialyzed in buffers with varying amounts of Mg^{++} and analyzed in sucrose gradients made in the same buffer by sedimentation velocity centrifugation. E. coli 50 and 30 S ribosomal subunits were centrifuged in parallel gradients and served as markers to which sedimentation rates of T. novellus ribosomes and subunits could be compared.

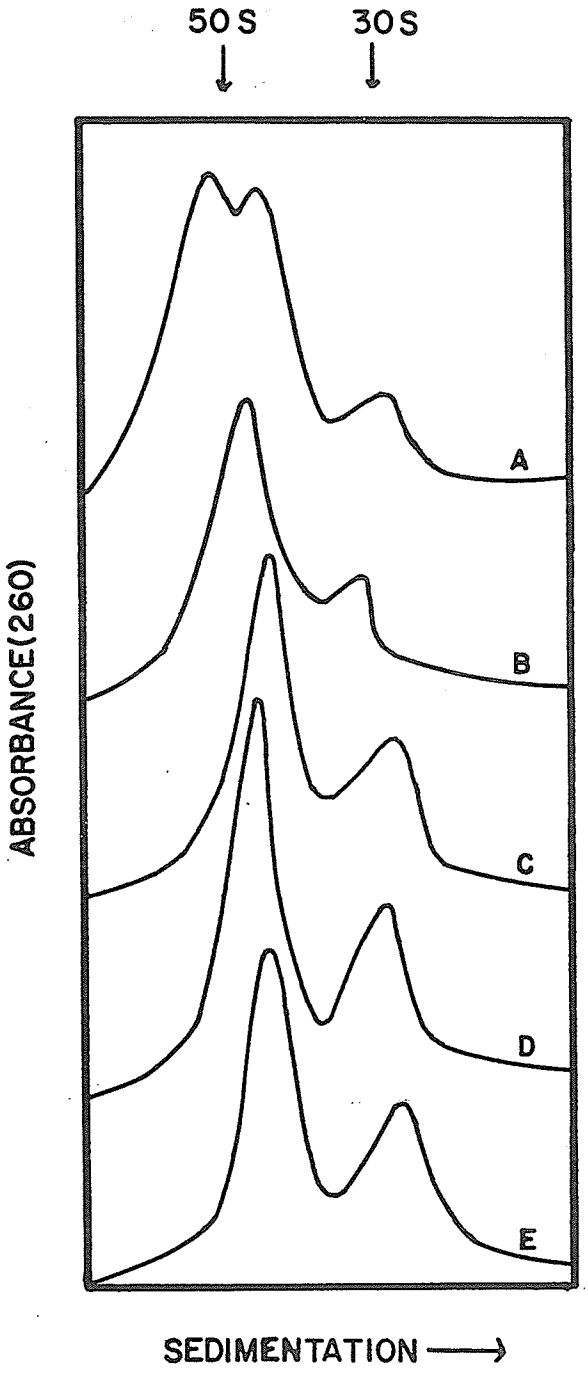
The response of ribosomes from heterotrophically - grown cells to different Mg^{++} concentrations is shown in Figure 4. The sedimentation profiles show that very little dissociation occurred at 0.01 M and 5.0 mM Mg^{++} (Figs. 4A and B) as judged by the amount of 30 S subunits. When Mg^{++} was lowered to 3.0 mM (Fig. 4C) substantial dissociation was evident. Lowering Mg^{++} levels down to 0.5 mM (Fig. 4E) did not induce further dissociation and the sedimentation patterns remained essentially the same as at 3.0 mM. The areas under the 30 and 50 S peaks were cut out and weighed to determine the relative amounts of each subunit. The 30 S: 50 S ratio was approximately ½ as would be expected if the mass of the 30 S subunit is ½ of the 50 S (Kurland, 1960) and if full dissociation of ribosomes had occurred.

At higher Mg^{++} levels, unusual profiles were obtained. Although the presence of 50 S and 30 S subunits, which are probably native subunits, was not unusual, the 2 peaks sedimenting near the 50 S region was not expected (Fig. 4A). Although this observation was not investigated further, Mg^{++} - induced aggregates of ribosomes in a conformation that resists sedimentation was suspected. Otherwise, the results are those expected of typical bacterial ribosomes (Tissières et al, 1959).

When ribosomes of autotrophically - grown T. novellus were

FIGURE 4. Sedimentation profile of ribosomes from heterotrophically - grown T. novellus. 2 A_{260} units were applied to 5 ml of 5-20% sucrose gradients in the buffers listed below then centrifuged at 50,000 r.p.m. for 105 min and analyzed as described in Methods. The arrows show the position of sedimentation of E. coli ribosomal subunits. Ribosomes were dialyzed and analyzed in:

A TK + 0.01 M Mg^{++}
B TK + 5 mM Mg^{++}
C TK + 3 mM Mg^{++}
D TK + 1 mM Mg^{++}
E TK + 0.5 mM Mg^{++}


50 S
30 S
A
B
C
D
E
ABSORBANCE(260)
SEDIMENTATION ⟶

analyzed in the same manner the results in Figure 5 were obtained. The profiles differed significantly from that obtained for heterotrophically - grown organisms. At 0.01 M Mg^{++} there was only a single 50 S peak with no evidence of 30 S native subunits. At lower Mg^{++} levels the amount of 30 S subunit increased, but even at 0.5 mM Mg^{++}, 30 S peak was only 1/10 of the 50 S peak. This indicates the ribosome was not fully dissociated under conditions which dissociated ribosomes of heterotrophically - grown cells. It should be noted that under the conditions of centrifugation used to obtain the profiles in Figures 4 and 5, 70 S ribosomes sediment to the bottom of the tube and can not be detected.

The ribosomes from autotrophically - grown T. novellus used to obtain Figure 5 were not treated with deoxycholate. To see whether the presence of membrane or similar material was responsible for preventing dissociation of subunits, ribosomes purified in the presence of deoxycholate were dialyzed and analyzed by sucrose gradient centrifugation in low Mg^{++} buffer. The results (Fig. 6) show that the profile obtained at 1.0 mM Mg^{++} was essentially the same as ribosomes purified without deoxycholate.

FIGURE 5. Sedimentation pattern of ribosomes from autotrophically grown T. novellus ribosomes in different Mg^{++} concentrations. The details of the experiment are the same as in the legend to Fig. 4.

A TK + 0.01 M Mg^{++}
B TK + 5 mM Mg^{++}
C TK + 3 mM Mg^{++}
D TK + 1 mM Mg^{++}
E TK + 0.5 mM Mg^{++}

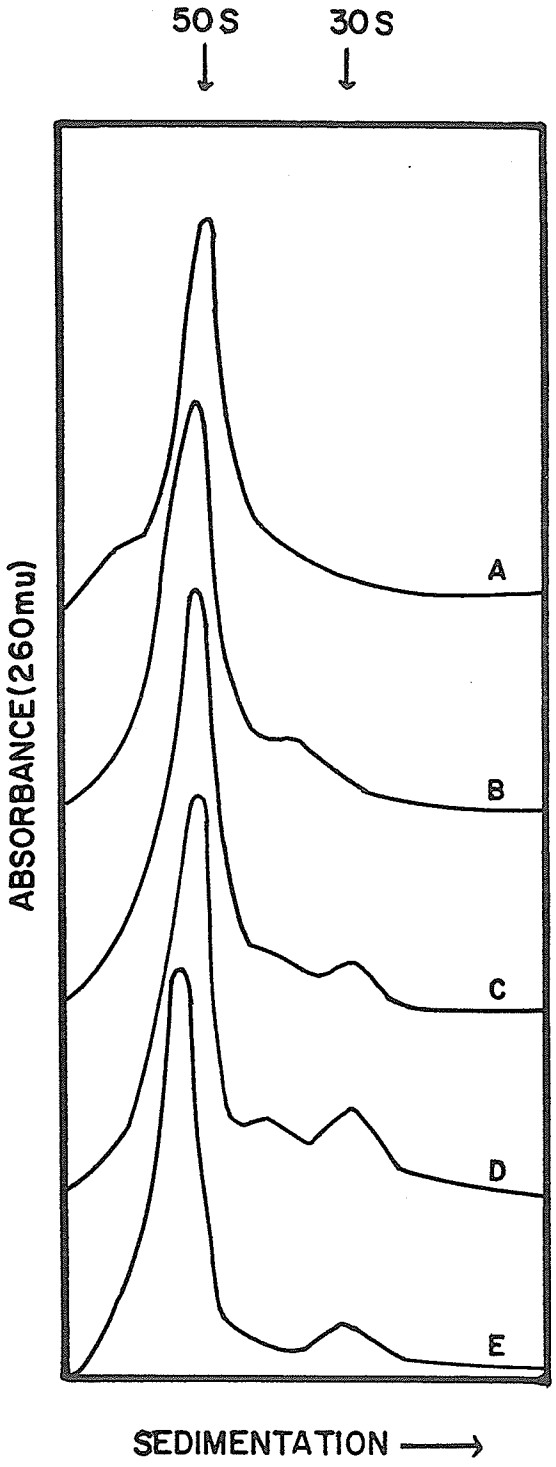
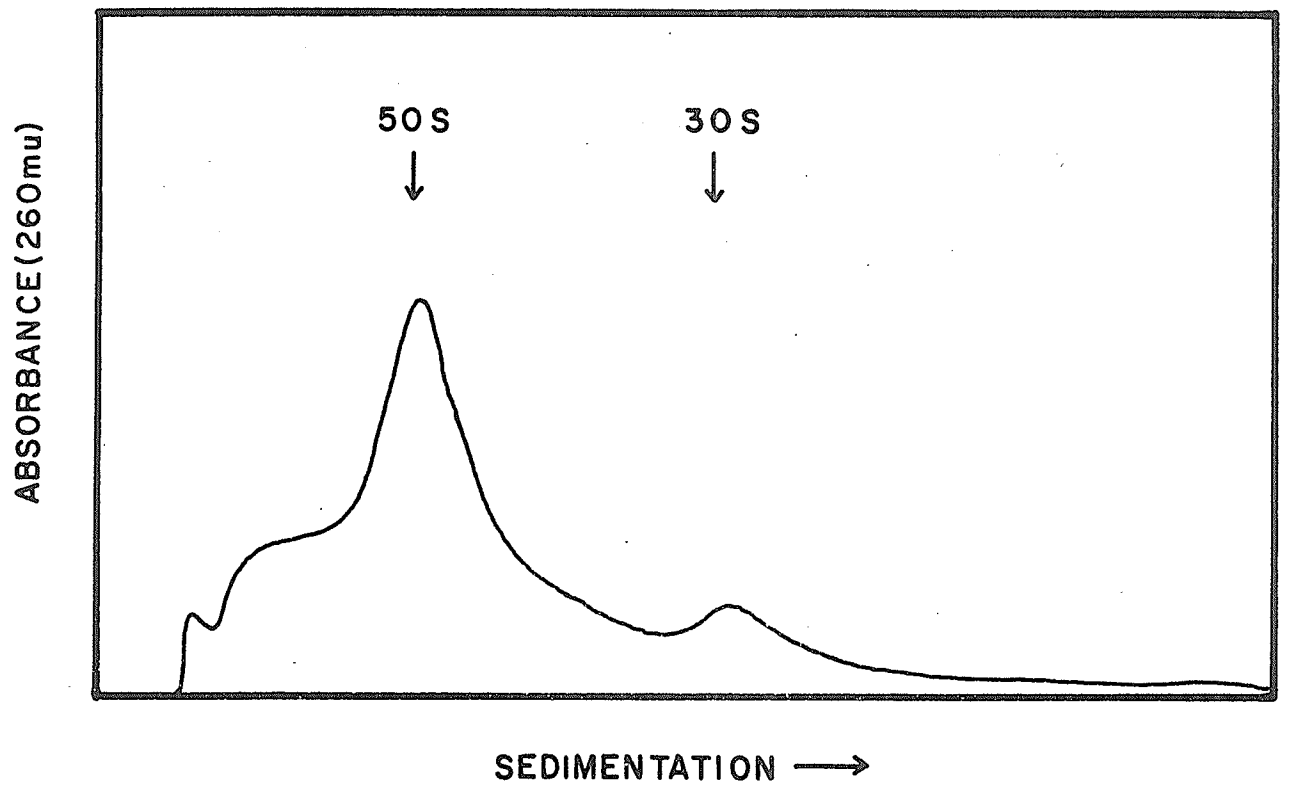

50S
30S
ABSORBANCE(260mu)
A
B
C
D
E
SEDIMENTATION ⟶

FIGURE 6. Sedimentation analysis of autotrophic T. novellus ribosomes prepared by DOC treatment. Sucrose density gradient centrifugation was carried out in TK + 1 mM Mg^{++} on 5-20% sucrose density gradient at 50,000 r.p.m. for 105 min in a SW 50.1 rotor. The arrows indicate 50 S and 30 S sedimentation positions of E. coli ribosomal subunits.


50S
30S
ABSORBANCE (260mu)
SEDIMENTATION

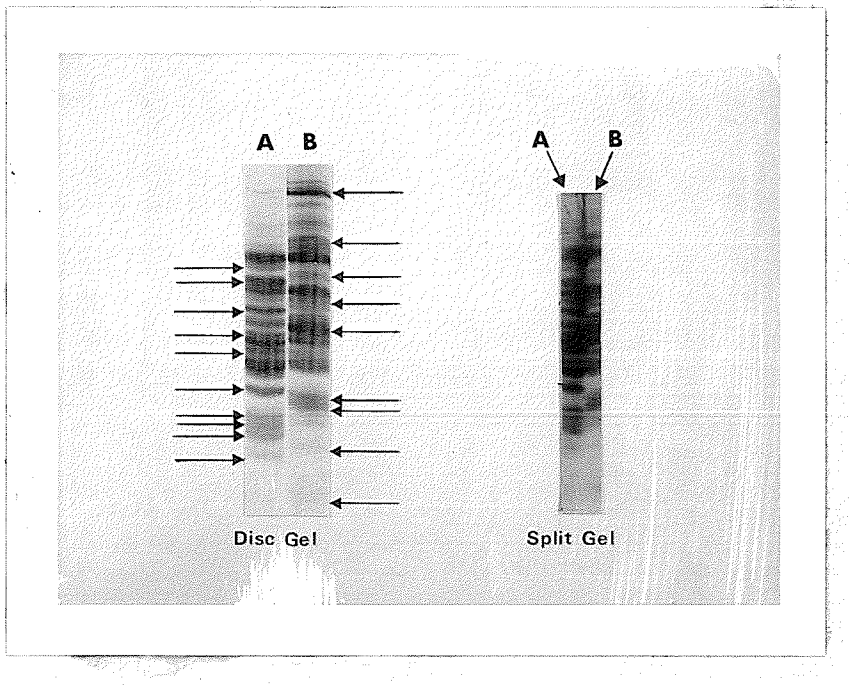
Electrophoretic Analysis of Ribosomal Proteins

To determine whether there was a structural basis for the difference in dissociation behaviour of ribosomes from glutamate - and thiosulfate - grown T. novellus, the protein components of the ribosomes were studied. Proteins were extracted in 67% acetic acid (Waller et al, 1961) in the presence of 0.1 M Mg acetate. The use of high Mg^{++} levels introduced by Hardy, Kurland, Voynow and Mora (1969) resulted in extraction of greater than 95% of E. coli ribosomal proteins.

Proteins extracted from autotrophically - and heterotrophically - grown ribosomes were solubilized in 8.0 M urea and electrophoresed in urea-polyacrylamide gels as described in "Methods" at pH 4.5. The results shown in Plate 1 (left) compare the electrophoretic patterns of ribosomal proteins of T. novellus grown under autotrophic (A) and heterotrophic (B) conditions. Surprisingly, a large number of differences in electrophoretic mobilities of protein bands were evident. Although 5 bands migrated to the same region in the gels for both types of ribosomes, 10 bands that appeared in gel A were absent in gel B and 9 bands that appeared in gel B were absent in A. The protein bands that appeared in only one type of ribosomes are indicated by arrows in Plate 1 (left). The fine bands that appeared at the top of the gels were not

PLATE 1

Electropherograms of ribosomal proteins extracted from autotrophically - and heterotrophically - grown T. novellus.



LEGEND. Proteins (100 μg) prepared from purified ribosomes were electrophoresed in urea-polyacrylamide gels either as disc gels (left) or split gels (right) as described in "Methods". The run was conducted at 10^{o} at 3 ma/gel tube for 3 h and stained with Coomassie Brilliant Blue. "A" and "B" represent ribosomal proteins from autotrophically - and heterotrophically - grown cells, respectively. Arrows pointing to protein bands in disc gels (left) indicate those that are found in only one of the gels, i.e., either A or B.

considered since they probably represent aggregates of proteins caused by oxidation or reduction (Kaltschmidt and Wittman, 1970).

Although these results were repeatable, differences in mobilities may arise from variances in electrophoretic conditions to which individual gels are exposed. To overcome these difficulties the "split gel" technique (Leboy et al, 1964), in which different protein samples are run in the sample gel, was employed. The results (Plate 1, right) showed that electrophoretic patterns were essentially the same as in the standard disc gels and that the differences observed were not artifacts of electrophoretic analysis.

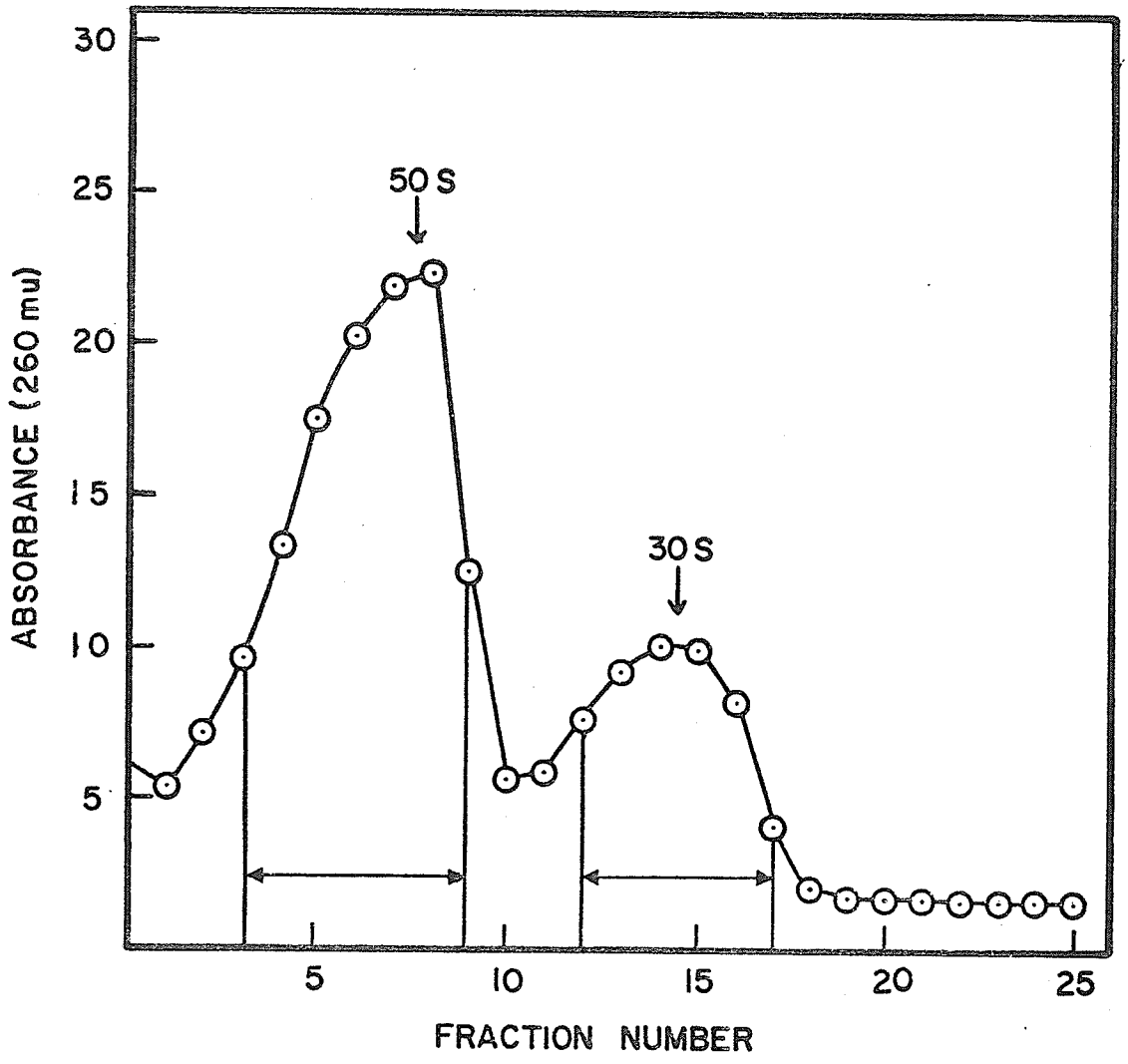
The analysis showed that there were major differences between ribosomal proteins derived from autotrophically - and heterotrophically - grown T. novellus. The results, however, did not indicate how many or which proteins are deleted or acquired as the organism alters its mode of metabolism. This is because the limitation of disc gel electrophoresis is that different proteins may have the same electrophoretic mobility and migrate as a single band. This is especially true for ribosomal proteins - for instance the 54 individual proteins of E. coli ribosomes resolve into more than 30 protein bands by disc gel electrophoresis (Kurland, 1971).

An attempt was made to determine whether the observed protein differences were confined to either or both of the ribosomal subunits. Unfortunately, despite many attempts, the isolation of large amounts of ribosomes from autotrophically - grown cells proved to be too difficult (see Discussion). However, it was possible to obtain relatively good electrophoretic protein patterns of ribosomal subunits from heterotrophically - grown T. novellus.

Ribosomal Subunits of Heterotrophically - Grown T. novellus

In order to obtain sufficient quantities of subunits for protein analysis, ribosomes were separated into subunits on a large scale. Approximately 900 A_{260} units of ribosomes (60 mg) in 3.0 ml were dialyzed for 6 h against TK buffer containing 1.0 mM Mg acetate. As established previously (see Fig. 4) this concentration of Mg^{++} resulted in good dissociation of ribosomes into subunits. One ml (300 A_{260} units) of dialyzed ribosomes was layered on 60 ml of a 5 to 20% sucrose gradient, made up in the same buffer as used for dialysis, and centrifuged for 12 h in a Spinco SW 25.2 rotor. Fractions were collected and suitably diluted aliquots were read at 260 mμ to determine the distribution of subunits in the gradient. The sedimentation profile is shown in Figure 7.

FIGURE 7. Separation of subunits of ribosomes from heterotrophically - grown T. novellus. Purified ribosomes were dialyzed for 6 h against TK buffer containing 1.0 mM Mg acetate. One ml samples (300 A_{260} units) were layered on 60 ml of a 5 to 20% sucrose gradient made up in the dialyzing buffer and centrifuged at 4^{o} for 12 h at 22,000 r.p.m. in a Spinco SW 25.2 rotor. After the run, the gradient tube was punctured and 1.5 ml fractions were collected. Aliquots of each fraction were diluted 1:10 with distilled H_2O and assayed spectrophotometrically at 260 mμ. The figure shows the absorbance profile for only 25 of the 40 fractions collected. The horizontal double-headed arrows show the fractions that were pooled.


30
25
20
15
10
5
ABSORBANCE (260 mu)
50 S
30 S
5
10
15
20
25
FRACTION NUMBER

Fractions containing 30 and 50 S subunits were individually pooled (Fig. 7) and precipitated with 0.7 vol ethanol (Staehelin, Maglott and Monroe, 1969). After allowing the mixture to stand overnight at -20^{o}, the precipitate was collected by low speed centrifugation (10,000 x g, 15 min) then dissolved in and dialyzed against TKM buffer. Subunits prepared in this manner were stored at a concentration of 200-300 A_{260} units/ml at -70^{o} until used.

To determine purity, subunits were diluted with TK buffer to a final Mg^{++} concentration of 1.0 mM, then 2.0 A_{260} units of each subunit were centrifuged on analytical sucrose gradients (Methods). The sedimentation profiles of the purified 30 and 50 S subunits are shown in Figures 8 and 9, respectively. By weighing the areas cut from tracings of each peak it was determined that the 30 S subunits were contaminated by approximately 10% with 50 S subunits and that the 50 S subunits were contaminated by less than 5% with 30 S subunits. Subunits prepared by this method were relatively pure with little cross-contamination.

Proteins were extracted from 30 and 50 S subunits with 67% acetic acid and analyzed by disc gel electrophoresis at pH 4.5 as described in Methods. The protein bands that appeared after staining are shown in Plate 2. The patterns obtained for 30 and 50 S proteins are distinct from each other except for a few bands

FIGURE 8. Sedimentation profile of purified 30 S ribosomal subunits from heterotrophically - grown T. novellus. Subunits were diluted with TK buffer to give a final Mg^{++} concentration of 1.0 mM. Then 2 A_{260} units were layered on a 5 ml 5 to 20% sucrose gradient made in TK buffer with 1.0 mM Mg^{++} and centrifuged at 4^{o} for 105 min at 50,000 r.p.m. in a Spinco SW 50.1 rotor. The gradient was analyzed with a flow-through cell attached to a Gilford Model 2000 spectrophotometer at 260 mμ.

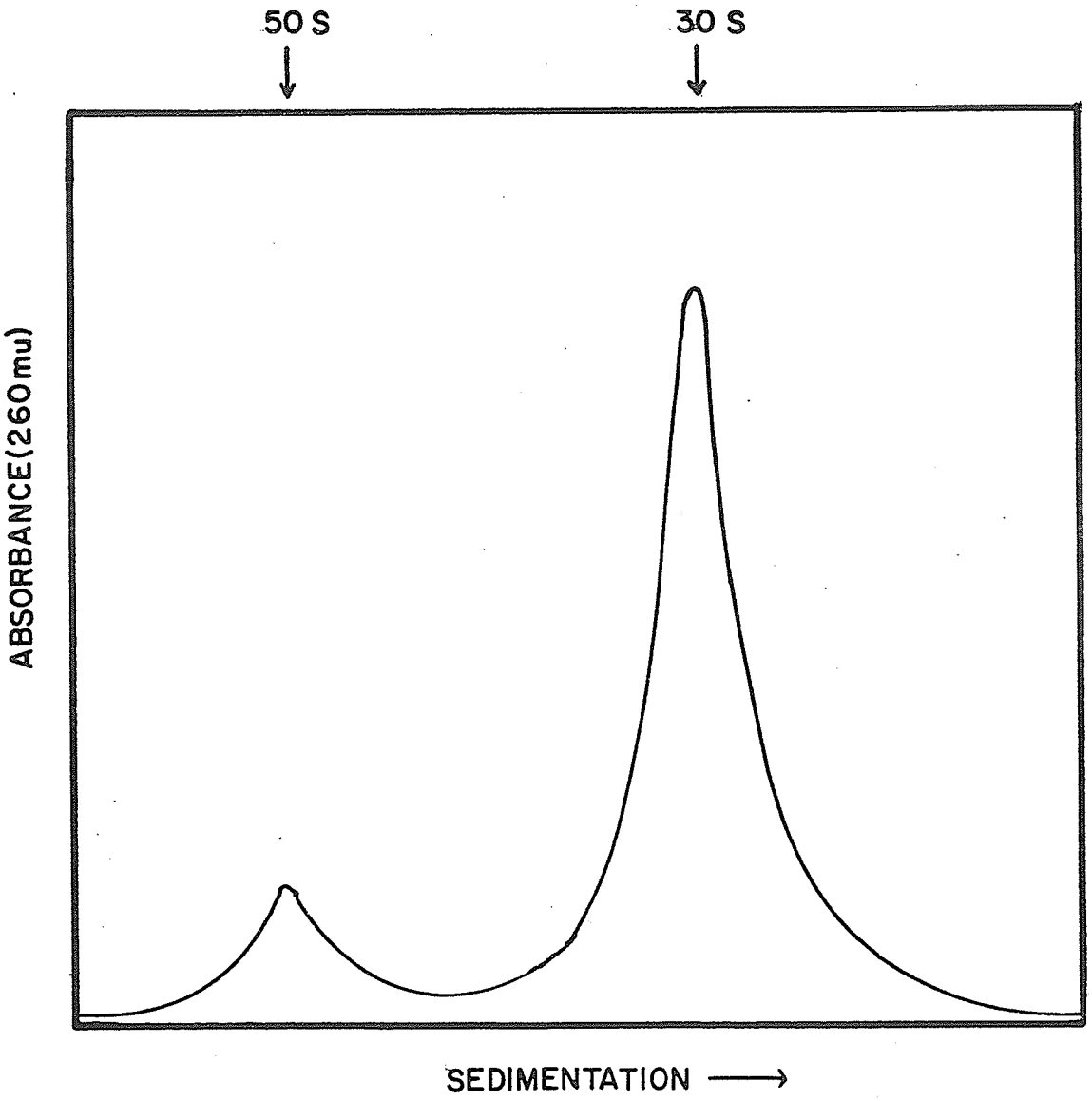
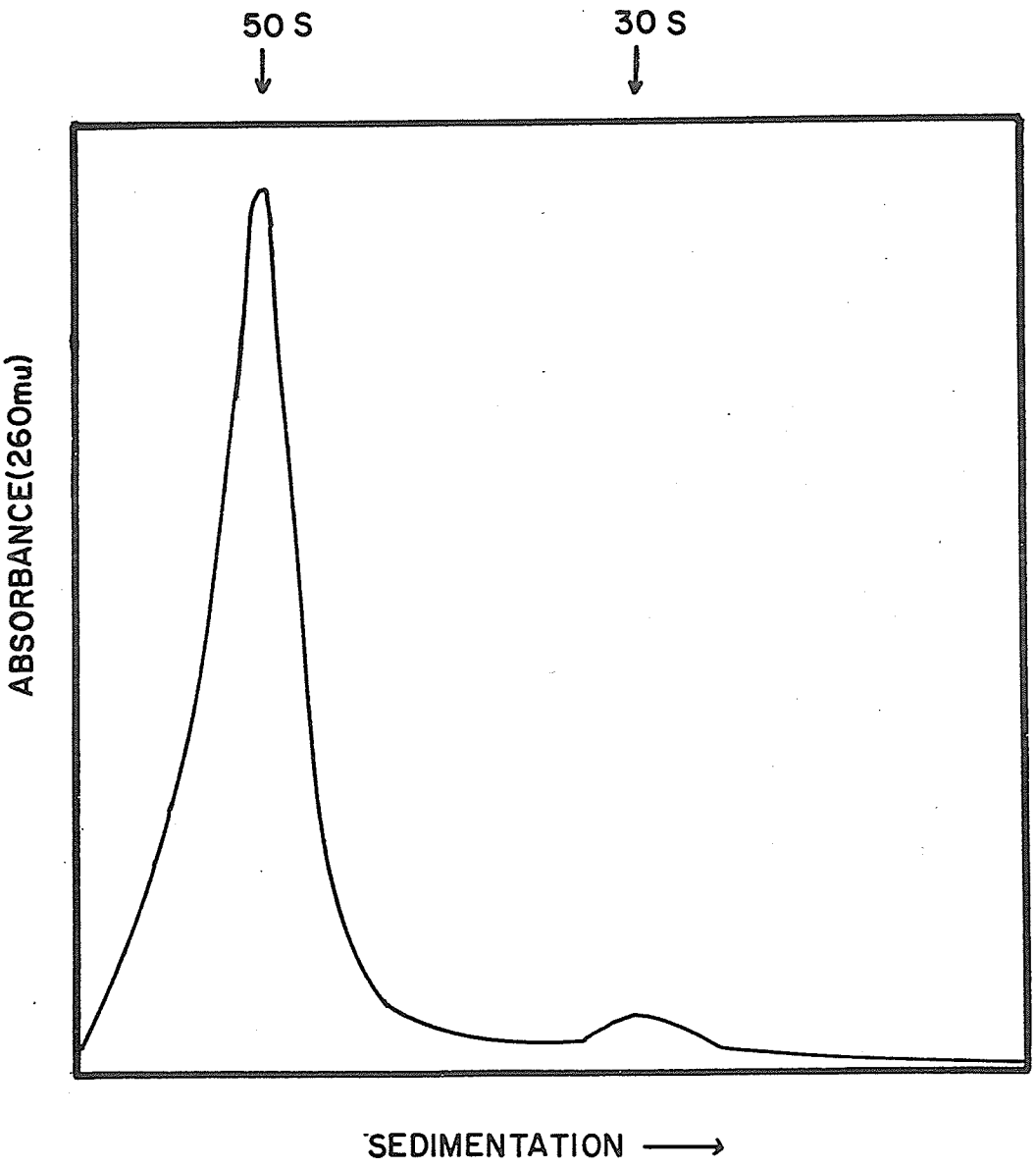

50 S
30 S
ABSORBANCE(260mu)
SEDIMENTATION ⟶

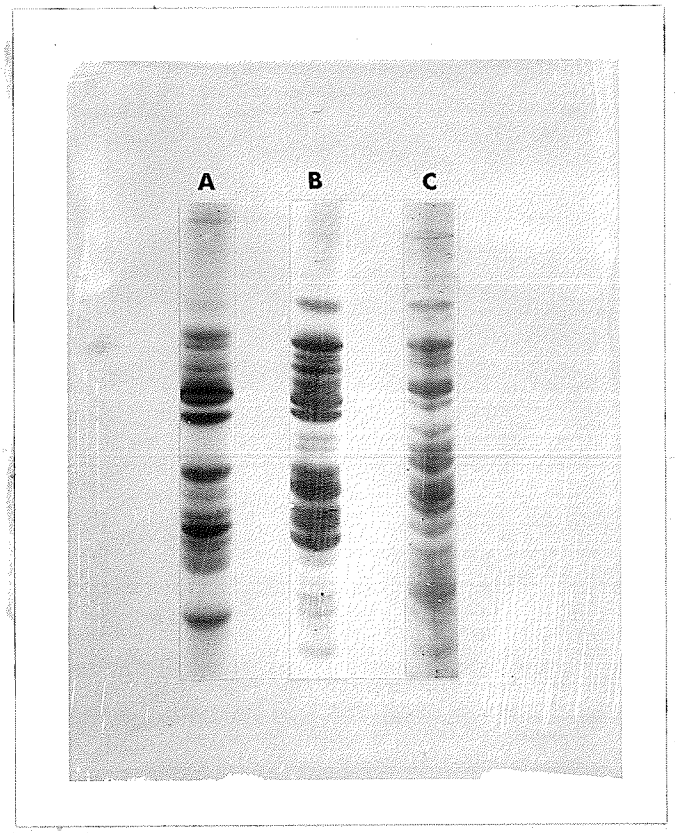
FIGURE 9. Sedimentation profile of purified 50 S ribosomal subunits of heterotrophically - grown T. novellus. Details are the same as that in legend to Figure 8 except 50 S subunits were analysed.


50 S
30 S
ABSORBANCE(260mμ)
SEDIMENTATION ⟶

LEGEND. Proteins were extracted with 67% acetic acid and 50 µg samples were electrophoresed into urea-polyacrylamide gels at pH 4.5 as described in "Methods". Running time was 4 h at 10^{o} at a constant current of 3 ma/gel tube. The gels were stained with Coomassie Brilliant Blue and destained with 10% TCA (Methods). The proteins were extracted from 30 S subunits (A), 50 S subunits (B) and intact ribosomes (C).

PLATE 2

Electrophoretic patterns of ribosomal proteins of heterotrophically - grown T. novellus.



that appear to migrate at the same rate. As stated earlier, migration of protein bands at the same rate during disc electrophoresis does not indicate the proteins are identical. The 30 S protein pattern shows 14 protein bands and the 50 S, 20 bands. This compares favourably with the 16 bands and 21 bands found with the E. coli 30 and 50 S subunits, respectively (Kurland, 1971) representing 21 and 34 proteins, respectively (Kaltschmidt and Wittman, 1970).

Since the same total protein load was placed on all gels (50 µg), the protein pattern of proteins from intact ribosomes (Plate 2C) appears fainter than those of subunit proteins (Plates 2A and B). This is to be expected since the proteins would be diluted among more bands. However, it is possible to see that the pattern of the proteins from intact ribosomes is a composite of the patterns of that from subunit proteins.

Although the data, by itself, does not distinguish the protein differences between subunits of ribosomes prepared from autotrophically - and heterotrophically - grown organisms, it does help in characterizing ribosomes of the latter.

In vitro Polypeptide Synthesis

The activity of T. novellus ribosomes in polypeptide synthesis was tested in a system that incorporated ^{14}C-phenylalanine into TCA - insoluble precipitates using polyuridylic acid as messenger RNA. The system was originally developed by Nirenberg (1961) for E. coli. To establish some parameters for the T. novellus system, protein - synthesizing components of heterotrophically - grown cells were used. This procedure was followed to conserve, as much as possible, ribosomes and cells of autotrophically grown T. novellus.

S-30 fraction, (containing ribosomes and supernatant factors), S-100 fraction, (containing supernatant factors only), crude ribosomes and purified ribosomes were prepared as described in Methods. The incorporation assay was carried out using these components as described in Table 1 and Methods. These results show that T. novellus ribosomes are capable of forming polypeptides in vitro in a poly U - directed system. (Table 5)

The optimal concentration of S-100 required for polypeptide synthesis was determined using the standard assay (Methods) with variable amounts of S-100 and 9.2 A_{260} units of ribosomes. The results shown in Figure 10 indicate that incorporation of ^{14}C-phenylalanine into TCA - insoluble precipitates required little

TABLE 5

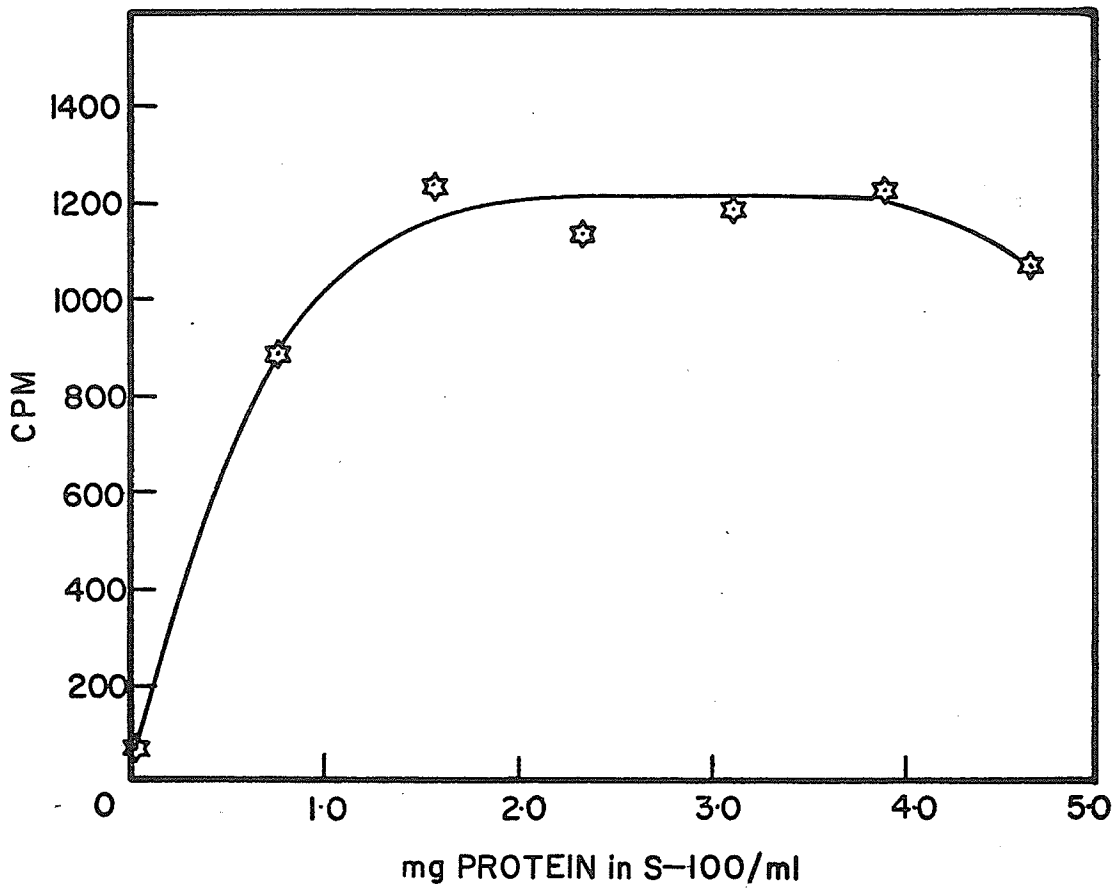
Incorporation of ^{14}C-phenylalanine into polypeptides by *T. novellus* (heterotrophically-grown) cell-free system.

T. novellus components*	Assay Mixture**	c.p.m. in TCA precipitated
S-30	-	1,113
S-30	no poly U	136
S-100	-	68
S-100	no poly U	67
S-100 + ribosomes	-	637
S-100 + ribosomes	no poly U	241

**Assay mixture - The components of the assay mixture are outlined in Table 1 (Methods). Modifications to this mixture are noted in this column. Details of the incorporation assay are given in Methods.

* The volume of the reaction mixture was 0.5 ml and, when used, S-30 was at a concentration of 1.5 mg protein/ml; S-100 was at 1.5 mg protein/ml; and ribosomes at 9.2 A_{260} units/ml.

FIGURE 10. Effect of S-100 fraction on ^{14}C-phenylalanine incorporation by a cell-free T. novellus (heterotrophically - grown) system. The assay system, described in Methods, contained 9.2 A_{260} units of ribosomes in each tube.

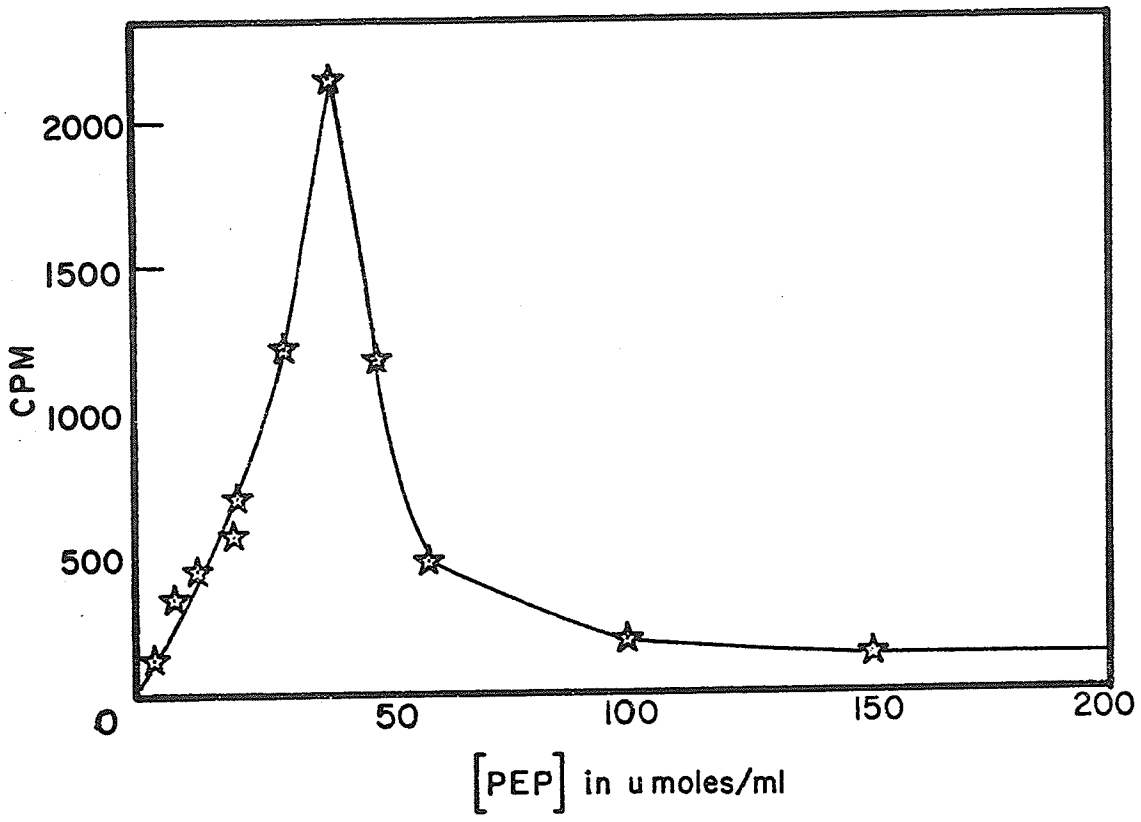

1400
1200
1000
800
600
400
200
0
CPM
1·0
2·0
3·0
4·0
5·0
mg PROTEIN in S—100/ml

S-100. At 1.0 mg S-100 protein/ml in the assay system incorporation was almost at a maximum. Increasing S-100 5-fold in the system did not alter incorporation activity. Since concentrations of phosphoenolpyruvate are critical in incorporation assays, the effect of varying concentrations of this reagent in standard incorporation assays with constant amounts of S-100 (1.55 mg protein/ml) and ribosomes (9.2 A_{260} units/ml) was tested. The results (Fig. 11) show that incorporation is strongly influenced by PEP, rising to a sharp maximum at PEP concentration of 40 μmoles/ml then being strongly inhibited at higher levels.

The reason for the behaviour of the assay system to PEP is not understood. Since PEP served as a energy source in the assay it seemed peculiar that an excess should result in such marked inhibition. Perhaps the higher levels altered the ionic conditions due to the use of the trisodium salt of PEP.

Using identical conditions the incorporation of ^{14}C-phenylalanine into TCA insoluble precipitate by in vitro systems of both autotrophically - and heterotrophically - grown T. novellus were compared. In these assays 4.6 A_{260} units of ribosomes from either autotrophically or heterotrophically - grown T. novellus and 0.75 mg protein from the corresponding S-100 fraction were incubated in poly U - directed incorporation assay mixtures for

FIGURE 11. Effect of [PEP] on ^{14}C-phenylalanine incorporation by heterotrophic T. novellus in vitro polypeptide synthesizing system. The amounts of S-100 (1.552 mg protein / ml) and ribosome (9.2 A_{260} mμ) were kept constant in all reactions. The CPM represents TCA - precipitable polyphenylalanine. The reaction mixture is described under Materials and Methods.


2000
1500
1000
500
0
CPM
50
100
150
200
[PEP] in u moles/ml

30 min at 28°. Under these conditions incorporation by components of autotrophically - grown cells was 1/5 that of heterotrophically - grown organisms. Counts incorporated by the former were about 140 c.p.m. and by the latter about 650 c.p.m.

Although these findings were not extended, they showed that there is a vast difference between the *in vitro* protein - synthesizing capabilities of the 2 types of cells.

DNA Analysis

The results, on the whole, point to gross differences between *T. novellus* cultured under different conditions. To see whether differences could be detected at the genetic level, an analysis of the GC content of DNA from both types of cells was carried out. DNA was extracted from autotrophically - and heterotrophically - grown cells and analyzed by buoyant - density centrifugation in CsCl (Methods).

The results, presented in Figures 12 and 13, show that DNA of heterotrophically - and autotrophically - grown cells sediment where the refractive indexes of CsCl are 1.4004 and 1.4016, respectively. From this data, the GC content of the DNA was calculated (Methods). As seen in Table 6, the DNA of autotrophically - grown *T. novellus* has a GC content of 65.4% compared to 52.1% for heterotrophically - grown cells. Although the analysis

FIGURE 12. The cesium chloride density gradient centrifugation of the DNA of heterotrophic T. novellus. One A_{260} unit of DNA was suspended in 5 ml of Cs Cl solution (ρ = 1.857g/cc) and centrifuged at 40,000 r.p.m. in a Spinco SW 50.1 rotor at 25^{o} for 44 h. The circles represent the absorbance of each fraction at 260 mμ and crosses represent refractive index. Details are given in Methods.

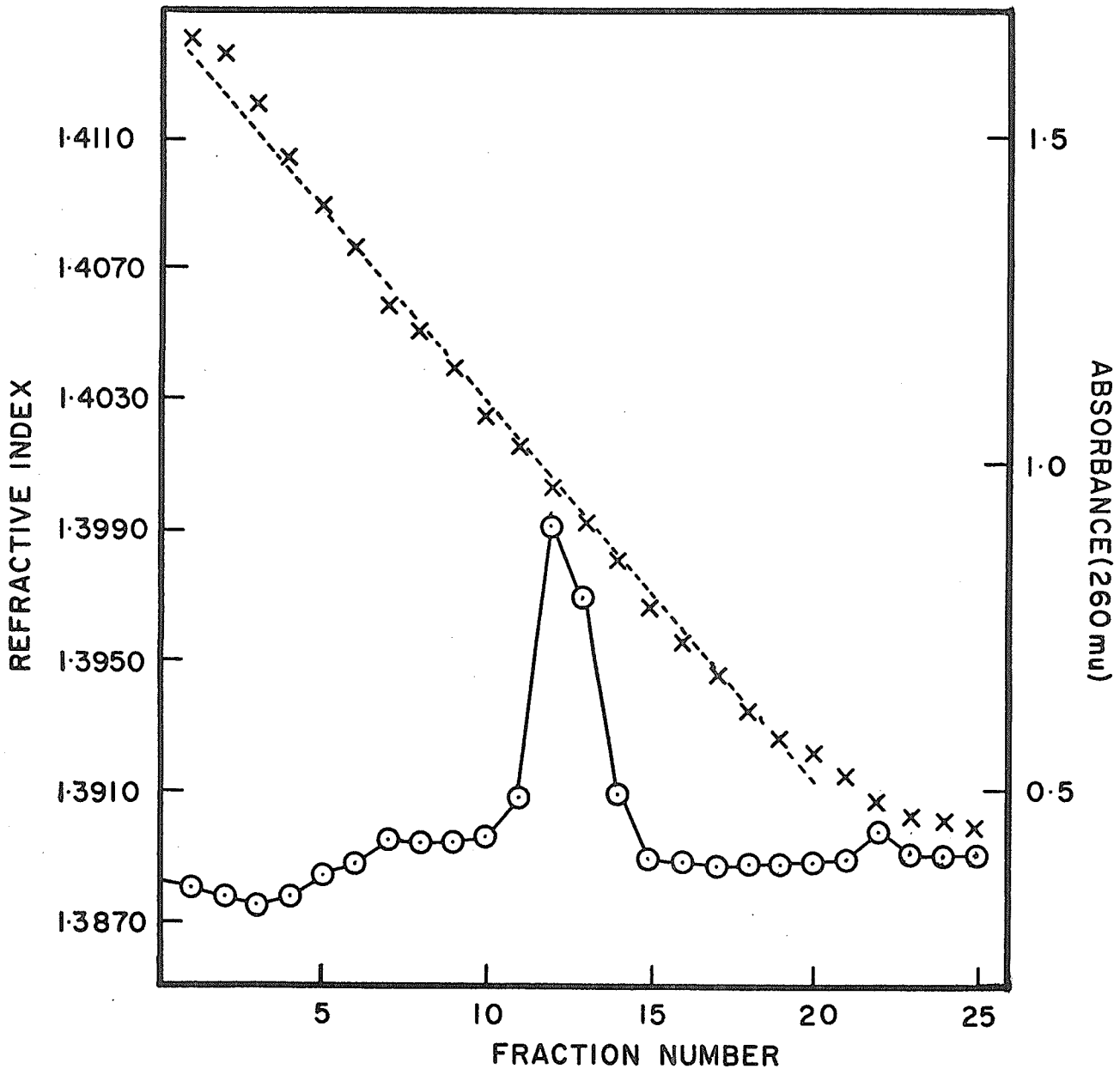

1·4110
1·4070
1·4030
1·3990
1·3950
1·3910
1·3870
REFRACTIVE INDEX
1·5
1·0
0·5
ABSORBANCE(260 mμ)
5
10
15
20
25
FRACTION NUMBER

FIGURE 13. Cesium chloride density gradient centrifugation of the DNA of autotrophic T. novellus. One A_{260} unit of DNA was suspended in 5 ml of Cs Cl solution (ρ = 1.857 g/cc) and centrifuged at 40,000 r.p.m. in a Spinco SW 50.1 rotor at 25° for 44 h. The circles represent absorbance at 260 mμ and crosses represent refractive index.

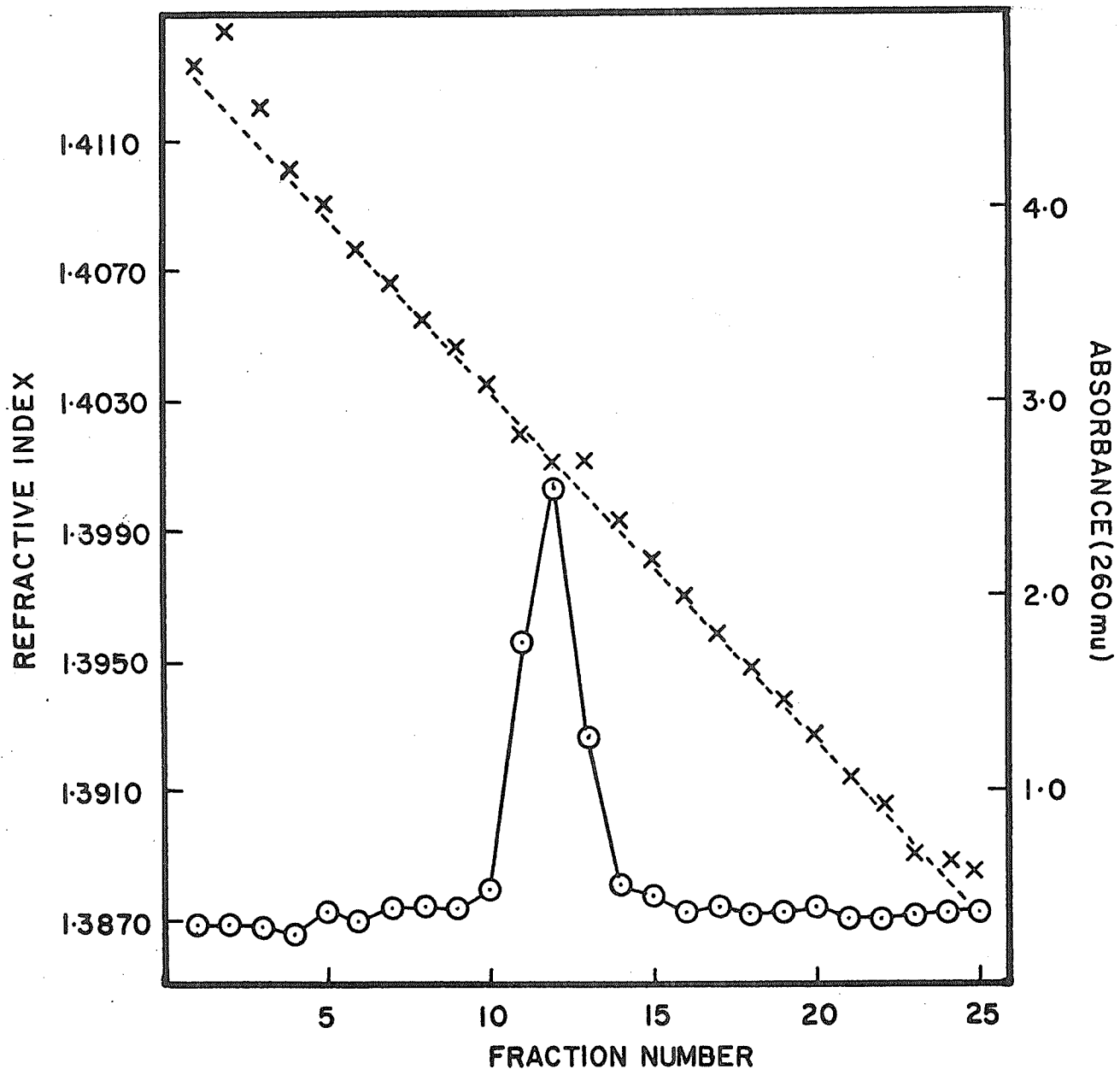

1·4110
1·4070
1·4030
1·3990
1·3950
1·3910
1·3870
REFRACTIVE INDEX
4·0
3·0
2·0
1·0
ABSORBANCE(260mu)
5
10
15
20
25
FRACTION NUMBER

TABLE 6

GC content of *T. novellus* DNA

Growth Condition	Refractive index*	Density g/cc*	% GC*
heterotrophic	1.4004	1.7118	52.1
autotrophic	1.4016	1.72412	65.4

* Details of DNA analysis are given in Methods.

of DNA from autotrophically - grown cells agree well with published data (Jackson, Moriarty, and Nicholas, 1968), the discrepancy of 13.3% in GC content between the 2 types of DNA is difficult to explain.

To see whether the analytical procedure was at fault, GC content of DNA from autotrophically grown cells was also analyzed spectrophotometrically by the 3 term analysis for native DNA described by Felsenfeld (1971). The absorbance measurements are shown in Table 2. The manipulations to obtain u_1, u_2, u_3 and S_3, S_4, S_5 from A_{260} readings are given in Methods. The AT concentration calculated as described in Methods was 32.4%, (or 65.4% GC). This corresponds well with the figure obtained by CsCl density centrifugation and indicates that the technique used for DNA analysis are valid. It seemed doubtful; therefore, that the difference in GC content between the 2 types of DNA could be attributed to faulty analytical procedure.

V. DISCUSSION

T. novellus is unique in the genus *Thiobacillus* in its ability to grow as a facultative autotroph. This characteristic is not uncommon among microorganisms having been recognized in *Hydrogenomonas* species, some photosynthetic bacteria, and in *Desulfovibrio desulfuricans* (Woods and Lascelles, 1954). Although previous studies with *T. novellus* (see Historical) showed enzymes for CO_2 fixation and thiosulfate oxidation must be induced for adaptive growth as an autotroph, little has been done to determine whether broader physiological changes occur in adapting organisms.

Growth studies reported here and elsewhere (see Historical and Results) showed that *T. novellus* cells growing autotrophically adapted readily to heterotrophic growth. However, adaptation of cells from heterotrophic to autotrophic growth required a long adaptation period. In this study approximately 3-4 weeks were required for the gradual adaptation of cells to thiosulfate. It was highly unlikely that this period of adaptation represents the time required only for synthesis of enzymes for thiosulfate oxidation and CO_2 fixation since derepression or induction of enzyme synthesis is a relatively fast process.

That profound changes take place in the cell during adaptation was indicated by the differences between organisms

grown under the 2 conditions. For instance, cells grown heterotrophically could be suspended to a smooth homogeneous suspension in buffers, whereas autotrophically grown cells formed aggregates and clumps, indicating changes in surface structures. Possibly these alterations allow the cell to tolerate acidic conditions during growth on thiosulfate. It is known that cultural conditions influence the fatty acid composition of T. novellus (Levin, 1972) suggesting that membrane changes may also take place during adaptation.

In the reverse direction, i.e., adaptation from autotrophy to heterotrophy, there is only a short lag before cells begin to grow (Fig. 3). It is therefore likely that the organism has or can readily elaborate components required for heterotrophic growth (LeJohn et al; 1967). The time period required for adaptation to autotrophy (3-4 weeks), on the other hand, suggested changes in addition to regulation of enzyme synthesis were involved. It was with this point in mind that differences were sought for in protein synthesizing components of T. novellus grown under 2 cultural conditions.

The results showed marked differences in ribosome content of cells grown autotrophically and heterotrophically. Approximately 112.5 A_{260} units (7.3 mg) of purified ribosomes were extracted from

1 g heterotrophically-grown T. novellus whereas only 1.6 mg could be extracted from 1 g of autotrophically-grown cells. This represents a 4.6 fold difference in ribosome content. If this difference is real, it may account for the slow growth of T. novellus in thiosulfate. As stated in Results, it was difficult to break cells grown autotrophically which may account for low ribosome yields. However, since the yields remained low despite using various disruption and extraction procedures, it is quite likely that the difference in ribosome content is real, and may reflect differences in protein-synthesizing capacities of cells growing under the 2 cultural conditions.

An analysis of ribosomes from T. novellus grown as heterotrophs and autotrophs showed the protein contents were 42.2 and 46.6% respectively. This agrees fairly well with 37% reported for E. coli (Tissières et al, 1959) and other bacteria, e.g., Halobacterium, 40%, (Bayley and Kushner, 1964). The difference of 4.6% between ribosomes of autotrophically - and heterotrophically-grown cells suggested that not only does ribosome content of the 2 types of cell differ, but also the ribosome structure itself. Although the slight difference may be attributed to contaminating proteins, further analyses showed the ribosomes vary significantly.

It is well known that lowered Mg^{++} concentrations dissociate

ribosomes into 2 unequal subunits (Chao, 1957; Tissières et al., 1959). The response of ribosomes of autotrophically - and heterotrophically - grown T. novellus to Mg^{++} differed considerably as shown in Figures 4 and 5. Whereas, ribosomes from T. novellus grown as a heterotroph dissociated readily at 3.0 mM Mg^{++}, ribosomes of autotrophically - grown cells failed to show dissociation at even 0.5 mM Mg^{++}. The reasons for the results presented in Figure 5 are difficult to interpret. It may mean that cells grown autotrophically have very few 30 S subunits or that lowered Mg^{++} does not fully dissociate ribosomes but results in an altered conformation of the ribosome which sediments around 50 S.

Although, the molecular mechanism is not understood, the results indicated that T. novellus grown under 2 conditions elaborate ribosomes which differ in their dissociation patterns. Since ribosomes from deoxycholate - treated extracts of autotrophically - grown cells produced identical results, it does not appear that binding of these ribosomes to a lipid fraction such as membranes was responsible.

Major differences were also detected when the ribosomal proteins of T. novellus grown either as an autotroph or heterotroph were analyzed by disc gel electrophoresis. The results shown in Plate 1 show that only 5 protein bands were common to

ribosomes derived from both types of cells, and that 9 to 10 protein bands were specific only for one type of ribosome. This indicates that major modification of ribosome structure must occur when cells adapt to different cultural conditions.

The fact that the protein complement of ribosomes can change in response to growth conditions has been reported in E. coli systems (Deusser and Wittman, 1972) so that these results were not surprising. However, the replacement of at least 9-10 proteins represents a very basic change in ribosome structure. Electrophoresis in 2-dimensions will be required to identify the proteins that are replaced, nevertheless, analysis by one dimensional electrophoresis clearly showed that changes do occur. It is unlikely that changes of such magnitude could result from binding of cytoplasmic proteins since such artifacts would be removed during the purification process. Further support for this idea comes from the data obtained from in vitro polypeptide assays. Ribosomes from autotrophically - grown T. novellus functions 1/5 as well as equivalent amounts of ribosomes of heterotrophically - grown cells with supernatant factors of the latter (Results). This indicates that the ribosomes from the 2 types of cells are functionally as well as structurally different.

Taken together, the results, indicate that adaptation of T. novellus to different cultural conditions involves changes in

cell organelles as well as requirement for specific enzymes. Similar results have been reported for E. coli that grow in minimal and enriched media (Deusser et al, 1972). Although this may explain, in part, the time required for adaptation to autotrophy, it does not explain why adaptation to heterotrophy proceeds rapidly. Perhaps autotrophically growing cells can function with few changes as heterotrophs whereas reciprocal adaptation requires major changes. Time course analyses of adapting organisms would be required for such studies and have not been carried.

An alternative explanation may be that the process of adaptation is actually a screening process that enriches an autotrophic organism in a population of heterotrophs. During autotrophic growth, heterotrophic cells may be maintained by cross feeding processes and vice versa. Supporting this contention is the DNA difference between the 2 types of organism. It seems unlikely that autotrophically - and heterotrophically - grown cells should have different GC contents if they were the same organism and suggests the cells growing under the 2 conditions are, indeed, different organisms.

Johnson and Chow (1973) have also shown GC contents of T. novellus grown autotrophically and heterotrophically were

similar to that reported here. Furthermore they isolated a bacteriophage that could infect both types of cells indicating that although the GC content was different, the cells were identical according to phage type. More work is required to resolve this crucial question.

The work presented here outlines in broad details that adaptive processes may require more than simple regulation of enzyme synthesis, and involves changes in protein synthesizing components. The major handicap in the pursuit of these studies was the inability to obtain large quantities of cells under autotrophic conditions for more critical studies of ribosomal proteins and the involvement of ribosomes in protein synthesis. Nevertheless, the work suggests that there may be a functional basis for heterogeneity in ribosomes (see Historical) and that protein complement of ribosomes change with cultural conditions possibly to accomodate different messenger RNA'S that are produced.

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