UNIVERSITY OF MANITOBA

FUNCTIONAL STUDIES ON *RHODOSPIRILLUM RUBRUM* AND *RHODOPSEUDOMONAS PALUSTRIS* CELL-FREE PROTEIN SYNTHESIZING SYSTEMS

by

VERONICA SYLVIA FEENSTRA

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fullfillment of the Requirements
for the Degree of
Master of Science

Department of Microbiology
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ABSTRACT

Ribosome and S-100 fractions were extracted from Rs. rubrum, Rp. palustris, and E. coli cells and used in combination with each other in in vitro protein synthesis assays. All combinations were active but homologous systems (S-100 + ribosomes from the same source) were not always optimal, especially for P-Rs. rubrum. Whereas the phototrophic Rs. rubrum ribosomes were relatively unstable and had generally low protein-synthesizing activity, all other fractions were stable and highly active. It was possible to stimulate/inhibit in vitro protein synthesis by addition of a pigment-enriched PS-60 fraction. Generally, the lower the protein-synthesizing-activity the greater the degree of stimulation, with the exception of the homologous E. coli system which was significantly inhibited by all PS-60 fractions. A "pigment fraction" extracted from purified P-Rs. rubrum ribosomes had similar stimulatory/inhibitory effects on the in vitro protein-synthesizing systems. It is suspected that these stimulation/inhibition effects are exerted primarily on the S-100 fraction.

To my Parents, Sylvia and Anton Hurter,
and to my Husband, Maynard and our Son
Mark.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ATP	adenosine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpm	depreciations per minute
GTP	guanosine 5'-triphosphate
PEP	phosphoenol pyruvate
PMSF	phenylmethyl sulfonylfluoride
polyU	3'->5' polymer of uridilic acid
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
TCA	trichloroacetic acid
Tris	tri(hydroxymethyl)aminomethane

HISTORICAL AND INTRODUCTION

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The Phototrophic Bacteria

The phototrophic bacteria are widely distributed in nature. Although they can be found in almost any body of water as well as in soil, they tend to predominate in stagnant bodies of water containing hydrogen sulfide and an abundant amount of organic substances (Kondrat´eva, 1965). The presence of light and the microbial breakdown of organic matter define the ecological niches that allow these organisms to successfully compete with other microorganisms (Pfennig, 1978). Noted for their pigmented aggregate formation in rivers and lakes, throughout history, the presence of the purple sulfur bacteria has been recorded by historians, missionaries, physicians and scientists alike.

Taxonomically the phototrophic bacteria were initially divided into three families: Athiorhodaceae, the nonsulfur purple and brown bacteria, Thiorhodaceae, the purple sulfur bacteria, and Chlorobacteriaceae, the green sulfur bacteria (Kondrat´eva, 1965). This classification, however, was based primarily on differences in pigment composition and sulfur metabolism and has been updated in recent years on the basis of more sophisticated analysis on the physiology, metabolism, and genetics of these organisms. In the eighth edition of Bergey's manual of determinative bacteriology all phototrophic bacteria, with the exception of the blue-green bacteria, have been classified in the order Rhodospirillales, with the sub-orders Rhodospirillineae and Chlorobiineae (Pfennig and Trüper, 1974).

The suborder Chlorobiineae, or the green bacteria, is further subdivided into the family Chlorobiaceae, formerly Chlorobacteriaceae, and the recently added family Chloroflexaceae (Pierson and Castenholz, 1974). Whereas the members of the family Chlorobiaceae are mesophilic, nonmotile, strictly aerobic photoautotrophs, those of the family Chloroflexaceae are thermophilic, motile, photoheterotrophs and facultative chemoheterotrophs (Pierson and Castenholz, 1974; Madigan et al, 1974; Trüper, 1976).

There are also two families in the sub-order Rhodospirillineae, or the purple bacteria; Chromatiaceae, the purple sulfur bacteria, formerly Thiorhodaceae, and the Rhodospirillaceae, the purple and brown nonsulfur bacteria, formerly Athiorhodaceae. The Chromatiaceae are strict anaerobes and are characterized by their tolerance toward high sulfide concentration and light intensity, as well as their capacity to grow in the presence or absence of sulfide with simple organic substrates (Trüper and Pfennig, 1978). The Rhodospirillaceae, on the other hand, contain not only anaerobic phototrophs but also facultative chemoheterotrophs (Pfennig and Trüper, 1974). Belonging to this family are the genera Rhodopseudomonas and Rhodospirillum among which eight species can grow aerobically in the dark, i.e. Rp. palustris, Rp. capsulata, Rp. spheroides, Rp. gelatinosa, Rp. acidophila, Rp. sulfidophila, Rs. rubrum, and Rs. tenue (Uffen, 1978). When grown under aerobic conditions in the light they use carbon dioxide as the hydrogen acceptor for substrate oxidation and produce adenosine triphosphate (ATP) by photophosphorylation. When grown aerobically in the dark, the respiratory metabolism is used for the formation of ATP (Firsow and Drew, 1977), consequently a large proportion of organic substrate is decomposed with the liberation of carbon dioxide.

The ability of these organisms to grow in the dark, although readily demonstrable in the laboratory, is of little ecological importance because under natural conditions they do not thrive unless light is periodically available to them. As early as 1957, Cohen-Bazire et al had already demonstrated that Rs. rubrum and Rp. spheroides adjusted their cellular photosynthetic pigment contents according to the prevailing light intensity and oxygen tension. High oxygen tension both in the light and in the dark causes preferential synthesis of specific membrane proteins resulting in total blockage of pigment synthesis, inhibition of further production of the photosynthetic apparatus and induction of the oxidase system (Oelze and Drews, 1970). Shortly after shifting to anaerobic growth the cells lose their complex internal membrane system and synthesize an a-type cytochrome which probably functions as a terminal oxidase (Saunders and Jones, 1974). Adaptation to changes in oxygen partial pressure that is experienced when shifting from one physiological condition to another not only causes preferential synthesis of specific cellular components but also causes them to be synthesized at different rates (Firsow and Drew, 1977).

Phototrophically grown cells possess a well developed respiratory system and both the photosynthetic and respiratory enzyme assemblies are membrane associated and may have components in common (Saunders and Jones, 1974). Growth in the light involves the elaboration of many membrane-bound vesicles, known as the chromatophores, but light is not absolutely required for pigment synthesis since a culture of *Rp. spheroides* will turn red when grown in the dark under semi-anaerobic conditions (Cohen - Bazire *et al* 1957). High light intensities lead to a reduction in pigment synthesis and during adaptation to growth in low light conditions, conversely, there is a transitory increase in generation time and bacteriochlorophyll - a content of the cells (Uffen, 1973). Aerobically dark grown cells can readily adapt to photosynthetic growth with a lag period, the length of which is dependent on the light intensity and the initial pigment content.

Pigment synthesis in *Rs. rubrum* and *Rp. spheroides* can occur in the absence of cell division and vice versa, but, the synthesis of bacteriochlorophyll and carotenoids cannot proceed in the absence of protein synthesis. This has been demonstrated on growing cultures of *Rp. spheroides* where protein synthesis was inhibited by the addition of choromycetin or by sulfur starvation (Sistrom, 1962a).

Phototrophic bacteria are unique among the phototrophs with respect to the anaerobic nature of bacterial photosynthesis and for many years they have been used as model systems for investigating photosynthesis and related phenomena. Sufficient genetic information on these organisms however is severely lacking and only in the past decade has some progress been made into the genetic exchange systems of these organisms.

Extensive work has been done on mutant strains of the Rhodospirillaceae, particularily on Rp. spheroides, Rp. capsulata and Rs. rubrum. These studies have provided a great deal of information on such processes as electron transfer, nitrogen fixation, pigment biosynthesis, membrane development and differentiation. Typical mutants are quite readily obtained by ultraviolet irradiation or N-methyl-N-nitro-N-nitrosoguanidine mutagenesis with or without sub-subsequent penicillin screening. Spontaneous mutants have also been isolated but with less frequency. An excellent review on mutants of the Rhodospirillaceae is given by Saunders (1978). The following is a brief survey of some of these mutants and their usefulness in elucidating molecular mechanisms.

Many antibiotic mutants have been isolated, including those which are resistant to rifampin, streptomycin, nalidixic acid, and kanamycin. These types of mutants are helpful in elucidating mechanisms of energy conservation in the phototrophic bacteria. An example of a drug-resistant is the arsenate resistant mutant strain of Rp. capsulata which shows enhanced rates of photophosphorylation (Zilinsky et al, 1971).

The reactions involved in bacteriochlorophyll biosynthesis have been elucidated to a large extent with the use of "pigment mutants", i.e. mutants with blocks at specific stages of bacteriochlorophyll biosynthesis. Similarily mutants with specific defects in the respiratory or photosynthetic electron transfer system have also been isolated. Since the intracytoplasmic membrane of the purple nonsulfur bacteria accomodates both these systems, the resolution of the precise nature and arrangement of the components involved in either the respiratory or the photosynthetic electron transport system is complicated by their dual occurrence in the membrane. The use of mutants which have lesions that specifically affect either of these two systems can thus prove to be a very powerful tool in the analysis of these systems. An example of this is a mutant strain of Rs. rubrum (Picorel et al, 1977) which lacks bacteriochlorophyll P 800, a constituent of the photosynthetic reaction center, but can still grow photosynthetically. It has been proposed that two different kind of reaction centers coexist in the membranes of Rs. rubrum (Van Grondelle et al, 1976) but further work with these type of mutants is required before this problem can be resolved.

Studies on temperature-sensitive mutants of the *Rhodospirillaceae* are rather limited to date and the exact nature of the lesions affecting these strains is still unknown. It is hoped that with more extensive research it will be possible to elucidate interrelationships of photosynthesis and respiration in the purple nonsulfur bacteria.

Several other miscellaneous mutants have been isolated and are currently under investigation. Theoretically if appropriate classes of mutants can be coupled to efficient gene transfer systems, a thorough analysis of the photosynthetic processes should be possible in the *Rhodospirillaceae*.

A very important tool required in elucidating the genetics of the photosynthetic bacteria is the use of transducing phages. Although many attempts had been made in this direction through the years, the first virulent phage, specific for a member of the *Rhodospirillaceae*, was not isolated until 1968 (Freund-Mölbert *et al*, 1968). This phage, Rp-1, has a very restricted host range with only one out of seventeen *Rp. palustris* strains being able to propagate it. Since then several phages specific for *Rp. spheroides*, *Rp. palustris* and *Rp. capsulata* have been isolated. Their host range is also relatively narrow, (Mural and Friedman, 1974; Abeliovich and Kaplan, 1974), and to date no successful transduction has been reported involving any of them, with the exception of certain *Rp. spheroides* phages which are able to transfer some antibiotic resistance and nutritional markers, though with low frequencies (Saunders, 1978). The bacteriophage RS1 isolated for Rp. spheroides has been shown to have some sort of physiological specificity for the host. Rhodopseudomonas spheroides cells grown anaerobically show a partial resistance to RS1 as compared to aerobically grown cells (Abeliovich and Kaplan, 1974). It has been postulated that this might have some sort of ecological significance.

The only good system for genetic analysis in the phototrophic bacteria, known to date, is the gene transfer agent (GTA) discovered by Marrs for Rp. capsulata (Marrs, 1974). This transfer mechanism resembles the system of generalized bacteriophage transduction where samples of the donor DNA are transferred via deoxyribonuclease resistant particles and the transfer frequency is comparable to those for generalized transducing systems (Solioz et al, 1975). No viral activities have been found to be associated with the particles themselves although morphologically, the GTA particles themselves do resemble small bacterial viruses under the electron microscope (Saunders, 1978). The genetic transfer mediated by this GTA is limited to Rp. capsulata and does not extend to the other members of the Rhodospirillaceae. All regions of the bacterial genome thus far examined can be transferred and this system has therefore been used to map chromosomal regions about the size of an operon by Yen and Marrs (1976) who have successfully constructed a genetic map of a region containing markers for the production of bacteriochlorophyll and carotenoid in Rp. capsulata.

Since the GTA is limited to Rp. capsulata, other systems of genetic exchange must be searched for if one wants to extend the genetic analysis to other phototrophic bacteria. Such a system has recently been discovered for two species of Rhodopseudomonas. From Pseudomonas aeruginosa, the plasmid R68.45 has been transferred to Rp. spheroides and Rp. gelatinosa (Sistrom, 1977). This plasmid, when in P. aeruginosa, governs the properties of resistance to neomycin and enhancement of gene transfer (Kondorosi et al, 1977). Rhodopseudomonas spheroides strains carrying this plasmid are able to transfer not only the plasmid itself but also the chromosomal genes to other strains of Rp. spheroides, probably by conjugation (Sistrom, 1977). The presence of cryptic plasmids in phototrophic bacteria has been reported on several occasions (Gibson and Niederman, 1970; Saunders et al, 1976; Saunders, 1978), but transfer of chromosomal markers has only been demonstrated with the R plasmid R68.45, however, and it is hoped that this will eventually become a useful tool in the construction of genetic maps of Rp. spheroides and ultimately provide a means for manipulating genes within members of the Rhodospirillaceae. Since the Rhodospirillaceae provide attractive experimental systems for the integration of biochemical, biophysical, and genetic technologies, the recent trends in molecular biology make it more feasible to realize the full potential of these organisms with regards to establishing the mechanisms and functions of the photosynthetic apparatus.

As mentioned earlier, investigations on the purple sulfur bacteria have provided insight in the biosynthetic pathways of pigment formation as well as the relationship among light intensity, oxygen tension and pigment synthesis. Research in nucleic acid metabolism of these organisms has therefore been carried out with the hope that some information could be gained on the control mechanism of gene expression. Since differences in the rate of synthesis of pigments and enzymes exist between phototrophically and heterotrophically grown cells, differential gene expression must take place in these organisms under different physiological conditions.

As demonstrated by Lessie (1965b), Rp. spheroides cultures in which the synthesis of bacteriochlorophyll was repressed by increasing light intensity, showed an increase in the rate of total ribonucleic acid synthesis. Conversely, an increase in pigment synthesis was accompanied by an apparent cessation of net RNA synthesis. These results led to the assumption that different messenger RNA species are synthesized in organisms undergoing a transition from one growth phase to another. Attempts were therefore made to determine whether there were any changes in the mRNA populations by base composition analysis (Gray et al, 1964; Ferretti and Gray, 1967). It has been concluded that if there were any changes in the mRNA populations, they were undetectable by the methods employed. Later, other investigators have applied the more sensitive DNA-RNA hybridization competition techniques for the same problem, but again, no major differences could be observed between the phototrophic and the heterotrophic mRNA populations (Yamashita and Kamen, 1968; Witkin and Gibson, 1972b; Chow, 1976a). Because of the lack of qualitative differences in mRNA populations, it has been proposed that the level of regulation of protein synthesis in Rs. rubrum and Rp. spheroides is probably at the level of translation rather than of transcription.

Witkin and Gibson (1972a) had observed that the half-life of the unstable RNA species in anaerobically grown Rp. spheroides was much shorter than that of the aerobically grown cells (1.25 - 2.0 minutes versus 2.5 - 4.0 minutes), and transitions from aerobic to anaerobic conditions not only shortened the half-life of the unstable RNA but also caused a rapid drop in the proportion of stable RNA. Furthermore, an addition of puromycin or chloramphenicol to the cultures could affect the extent of degradation of the unstable RNA. It was therefore concluded that there was probably a difference in the ribosome populations between the phototrophic and heterotrophic organisms.

Similar studies had been carried out in Rs. rubrum cultures (Chow, 1976a). It has been shown that rifampicin caused an initial breakdown of about thirty percent of the pulse-labelled RNA in both phototrophic and heterotrophic Rs. rubrum. In the heterotrophic cells however, an additional degradation of RNA was observed at a later stage of rifampicin treatment and this secondary RNA degradation was probably caused by a breakdown of ribosomal RNA, especially the 23S species, as suggested by polyacrylamide electrophoresis results.

Two distinct ribosome populations, one for the phototrophic and one for heterotrophic cells, have also been proposed for Rp. palustris based on the observations that after the transition from anaerobic to aerobic growth conditions, the original Rp. palustris ribosomes are degraded followed by the reassembly of a new population of ribosomes (Mansour and Stachow, 1975).

If translational control were indeed taking place in these organisms by different ribosome populations, the question still remains whether this control were ultimately exerted at the level of transcription of the ribosomal RNA since evidence on the differences in rRNA had already been adduced (Lessie, 1965b; Marrs and Kaplan, 1970). Thus at this stage transcriptional control of gene expression in the phototrophic bacteria cannot be entirely ruled out.

Recently three distinct forms of DNA-dependent RNA and polyadenylic acid polymerases have been demonstrated in heterotrophic (Chow, 1977) and phototrophic Rs. rubrum (Chow, 1978). These enzymes are very similar in many unique properties, however several important differences do exist between the phototrophic and heterotrophic enzymes, such as the requirement for an additional purification step and a tighter DNA-enzyme association in the phototrophic enzymes as well as the apparent absence of the sigma subunit in the heterotrophic enzymes. Incidentally, similar studies on *Rp. spheroides* RNA polymerase have revealed neither structural nor functional differences between the phototrophic and heterotrophic enzymes (Jacobson, 1977).

In vitro translation and ribosomes

Ribosomes are the most complex components of the protein synthesizing machinery of the cell. Besides ribosomes the translation process requires mRNA, aminoacyl-tRNA's, GTP, Mg^{++}, K^{+}, NH_4^{+}, initiation factors (IF-1, IF-2, IF-3), elongation factors (EF-Tu, EF-Ts, EF-G), termination factors (RF-1, RF-2), and an energy generating system. This machinery translates mRNA into polypeptides in three stages: initiation, elongation, and termination.

The work of Zamecnik and his colleagues from 1950 onwards had made it possible to study protein synthesis in cell-free systems (Zamecnik, 1960). In the early systems, cell-free homogenates of rat liver were mixed with radioactive isotope labeled amino acid, ATP, and respiratory substrates. The fact that these mixtures were able to incorporate labeled amino acids into TCA insoluble material had clearly demonstrated that intact cells were not necessarily required for protein synthesis. Functionally active ribosomes were later partially purified by differential centrifugation from many other types of cells, such as reticulocytes and *E. coli*. Bacterial systems proved to have an advantage over animal systems since they were far more active in amino acid incorporation (Lengyel, 1969). Consequently the bacterial *in vitro* systems have been used as a major tool for studying the mechanisms of protein biosynthesis.

A relatively crude cell-free, protein synthesizing system can be prepared by disintegrating cells in aqueous buffers from which unbroken cells and cell debris are removed by low-speed centrifugation and small molecules removed by dialysis (Nirenberg and Matthei, 1961; Nirenberg, 1964). In such a system both endogenous and exogenous mRNA can be efficiently translated if appropriate amounts of ATP, GTP, an ATP generating system, proper ions, sulfhydryl compounds and (radioactively labeled) amino acids are included. When exogenous messenger is used the extracts should be preincubated to exhaust the endogenous messenger before addition of radioactive amino acids (Nirenberg and Matthei, 1961; Nirenberg, 1964).

A more refined system can be prepared by further fractionating the cell extract with high speed centrifugation. In general, an S-100 fraction is prepared to contain tRNA, the soluble factors G and T, aminoacyl tRNA synthetases together with many other less specified components (Nirenberg and Matthei, 1961; Lengyel, 1969). The S-100 fraction can subsequently be used as a starting material to purify any of these components (Modolell, 1972). Ribosomes are usually purified by the ammonium sulfate precipitation method of Kurland (Kurland, 1966). These purified components can then be used in combination to examine various aspects of protein biosynthesis. Using such systems, a detailed protein synthesis mechanism has been elucidated.

The first step in translation involves the formation of a 70S initiation complex which is formed with a 30S and a 50S ribosomal subunit, a mRNA and an f-Met-$tRNA_f^{met}$, in the presence of GTP and initiation factors. The mRNA binding process is a very complex one (Kurland, 1977). This is not unexpected since the mRNA has a variety of functions, such as providing the signals for initiation of protein synthesis for the codon-dependent binding of aminoacyl-tRNA, the coordinated movement of tRNA, and the termination of the translation process.

The functions of the three initiation factors are as follows: Initiation factor three (IF-3) binds to all free 30S subunits (Revel, 1972), and promotes the binding of the mRNA. Initiation factor two (IF-2) is involved in the binding of the f-Met-$tRNA_f^{met}$ to the 30S subunit, a process requiring the concomitant hydrolysis of GTP. It has been proposed that the major role of IF-1 is to promote the release of IF-2 from the initiation complex (Benne et al, 1973).

Elongation involves the addition of aminoacyl residues to the growing peptide chain in a cyclic fashion. An aminoacyl tRNA binds to the A-site of the ribosomes and then attaches itself to the mRNA-ribosome complex to form an aminoacyl-tRNA·EF-Tu·GTP complex. The next steps in this cycle are the hydrolysis of GTP to GDP and inorganic phosphate, and the subsequent release of EF-Tu·GDP complex from the ribosomes. The amino acid residues on the tRNA at the

P-site are then released from the tRNA molecule to form a bond with the α-amino group of the aminoacyl tRNA at the A site. This process is catalyzed by the peptidyl transferase which is a part of the 50 S ribosomal subunit. Removal of the deacylated tRNA from the P site follows, the peptidyl tRNA is shifted from the A site to the P site and simultaneously the mRNA slides with respect to the ribosome so that the next three nucleotides are exposed at the A site. The ribosome-bound GTP is cleaved and the cleavage products, together with EF-G, are released from the ribosome. Attachment of another aminoacyl-tRNA begins a new cycle.

Termination takes place when a terminator codon, (UAA, UAG, or UGA) on the mRNA is reached at the ribosomal A site. In procaryotes three release factors and GTP are required for the release of the polypeptide chain from the tRNA. The terminator condons promote the binding of the release factors to the ribosome (Lengyel, 1974).

Cell-free protein synthesis can be stimulated by addition of a number of compounds. For example an increase in polyphenylalanine synthesis has been observed in E. coli cell-free systems by the addition of polyamines which stimulate the binding of aminoacyl-tRNA to ribosomes (Igarashi et al, 1974). The fact that addition of spermidine or putrescine to a system with an optimal Mg^{++} concentration increases polypeptide synthesis indicates that these polyamines have a sparing effect on the Mg^{++} requirement. Further, many small molecular weight RNA species have been shown to have stimulatory activities in eucaryotic *in vitro* translation systems (Berns *et al*, 1975; Bester *et al*, 1975; Bogdanovsky *et al*, 1973; Kennedy *et al*, 1974). Similarly a small molecular weight RNA species associated with the ribosomes of heterotrophic *Rs. rubrum* has recently been shown to have an activating activity in the *Rs. rubrum* cell-free system (Chow, 1976 c). At the same time an inhibitor has also been observed in the above system. The chemical nature of this inhibitor, although still unclear, is believed to be an oligoribonucleotide or a very short sequence of double stranded RNA which has probably arisen from the activator molecule. There are many inhibitors for protein synthesis and their inhibitory mechanisms can be quite readily examined in cell-free systems. An example is that some antibiotics are anti-bacterial for gram-positive but not for gram-negative organisms. However, they have been shown to be inhibitory in cell-free systems prepared from both gram-positive as well as gram-negative organisms, as the difficulty in penetrating the outer layer of the gram-negative bacterial cell wall thus is removed (Pestka, 1977). Thus with the exception of one or two, virtually all inhibitors of protein synthesis *in vivo* are also inhibitors in cell-free systems.

The most thoroughly studied *in vitro* translation system is that of *E. coli*. Because most of the evidence concerning regulation at the level of translation has come from studies using cell-free systems, the majority of the regulatory studies has again been carried out in the

E. coli system. An example of this is given by O'Farrell and co-workers who have shown that the expression of the bacteriophage T4 gene 32 protein (P32) is regulated at the level of translation (Russel *et al*, 1976). This study on the functional half-life of the gene 32 messenger RNA has indicated that this mRNA is extremely stable. From these results, it has been concluded that the regulation of gene 32 expression is not at the transcriptional level, because this type of regulation cannot account for the rapidity with which P32 synthesis is repressed. Furthermore, derepression of P32 synthesis occurs in the presence of rifampicin which inhibits the initiation step of transcription.

The *E. coli* translational system has a very high degree of template specificity. Revel and co-workers (1972) showed that this specificity could be modified by changes in the physiological state of the cell. Such a modification in template specificity is also believed to be involved in the differential gene expression of sporulating *Bacillus subtilis* (Legault-Demare and Chambliss, 1974).

In vitro protein synthesizing systems have been described for several *Bacillus* species including *B. megaterium* (Deutscher *et al*, 1968), *B. thuringiensis* (Changchien and Aronson, 1970), and *B. subtilis* (Doi, 1971). Extensive work with *Bacillus* systems has indicated that translational control may play an important role in regulating the process of bacterial sporulation. It has been shown (Fortnagel and Bergmann, 1973) that a shift from exponential growth to sporulation in B. subtilis is associated with functionally different ribosomes. Similarily it has also been found that B. subtilis mutants resistant to protein synthesis inhibitors are deficient in sporulation capacity (Leighton, 1974). Similarily, Graham and Bott (1975), by looking at antibiotic resistant and conditional asporogenous mutants of B. subtilis have noted structural changes in ribosomal proteins of sporulating cells. Further, using functionally active 30 S and 50 S ribosomal subunits from B. subtilis, Guha and Szulmajster (1975, 1977) have shown that the 30 S ribosomal subunits are involved in translational control.

As for the facultatively phototrophic bacteria, a cell-free protein-synthesizing system has recently been developed for Rs. rubrum in order to examine the proposed translational control mechanisms in these cells (Chow, 1976 b). In this rather crude S-30 system only minor differences could be found between the phototrophic and the heterotrophic systems. For further analyses, Chow (1976 c) has used a more refined cell-free system containing purified Rs. rubrum ribosomes and S-100 fractions, with the well-characterized E. coli system as a reference. A number of differences have been discovered between the phototrophic and the heterotrophic fractions. In summary, the phototrophic ribosomes are highly active when combined with either the phototrophic or the heterotrophic S-100 fractions, whereas the heterotrophic ribosomes are active only when combined with the phototrophic S-100 fraction. Addition of a photosynthetic pigment-containing fraction (the PS-60 fraction) to the homologous heterotrophic system is, however, able to stimulate its activity. Other functional differences have also been noted between the phototrophic and the heterotrophic systems with regards to response to polyamines, $MgCl_2$ and biotin (Chow, 1976 b and 1976 c).

In the present study, an active homologous heterotrophic Rs. rubrum cell-free system has been developed by using a new variant strain of cells. The absorption spectra of the photosynthetic pigments in various fractions as well as the stimulatory activity of the PS-60 fractions have also been analyzed. This type of investigation has also been extended to Rhodopseudomonas palustris, another facultative phototroph. It is hoped that these studies will provide more information to the control mechanisms of protein synthesis in the phototrophic bacteria.

MATERIALS AND METHODS

Organisms

The organisms used throughout this study were two facultative phototrophs: *Rhodospirillum rubrum*, VF, a variant strain isolated in this laboratory, and *Rhodopseudomonas palustris*, ATCC 17002 obtained from Dr. S. Kaplan of the University of Illinois. In addition, *Escherichia coli*, obtained from our departmental stock cultures, was used as a reference strain because of its thoroughly studied properties.

Media

A) *Rhodospirillum rubrum* was grown in a liquid medium of the following composition, (Chow, 1976 a):

l-Glutamic acid	4.0 g
dl-Malic acid	3.5 g
Sodium citrate	0.8 g
$MgSO_4 \cdot 7H_2O$	0.2 g
KH_2PO_4	0.12 g
K_2HPO_4	0.18 g
$CaCl_2$	33 mg
Biotin	5 μg
Yeast extract	2.0 g
Distilled, deionized water up to	1000.0 ml

The pH was adjusted to 6.8 with a saturated solution of sodium hydroxide. Solid media were prepared by addition of 15.0 g of Difco Bacteriological agar to every liter of the above medium.

B) Rhodopseudomonas palustris was grown in a modified semi-synthetic liquid medium of Lascelles (Jacobson, 1977) containing:

l-Glutamic acid	1.47 g
dl-Malic acid	2.70 g
KH_2PO_4	0.50 g
K_2HPO_4	0.50 g
$(NH_4)_2HPO_4$	0.80 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$CaCl_2 \cdot 2H_2O$	53.00 mg
$MnSO_4 \cdot H_2O$	0.85 mg
$FeSO_4 \cdot 7H_2O$	2.78 mg
Na_3 Nitrilotriacetic acid	0.28 mg
Nicotinic acid	1.0 mg
Thiamine HCl	1.0 mg
Biotin	10.0 μg
Yeast extract	2.0 g
Distilled, deionized water up to	1000.0 ml

The pH was adjusted to 6.8 with a saturated solution of sodium hydroxide. Solid media were prepared by addition of 15.0 g of Difco Bacteriological agar to every liter of the above medium.

C) Escherichia coli was grown on a minimal salts-glucose medium of the following composition (Kurland, 1966):

KH_2PO_4	3.0 g
$Na_2HPO_4 \cdot H_2O$	6.8 g
NH_4Cl	2.0 g
Casamino acids	2.0 g
Glucose	5.0 g
$MgCl_2 \cdot 6H_2O$	0.2 g
Distilled, deionized water up to	1000.0 ml

The pH was adjusted to 7.8 with a saturated solution of sodium hydroxide. Solid media were prepared by addition of 15.0 g of Difco Bacteriological agar to every liter of the above medium.

Buffers

The buffer used for extraction and purification of various elements of the *in vitro* protein synthesizing system will hereafter be referred to as the standard buffer (S.B.). It has the following composition (Modolell, 1972):

10 mM Tris-HCl, pH 7.8

60 mM NH_4Cl

10 mM Mg-Acetate

6 mM β-Mercaptoethanol

To prevent protease activities, Rs. rubrum and Rp. palustris cells were washed in standard buffer containing 1.0 mM KCl and 3.54 mM phenylmethyl sulfonylfluoride (KCl-PMSF-S.B.) (Legault-Demare and Chambliss, 1974; Chow, 1976 b).

Growth Conditions

Rhodospirillum rubrum and Rp. palustris were routinely grown under phototrophic conditions as stab cultures by incubating in a Conviron Model E7 incubator, equipped with both fluorescent and incandescent lamps, for three days at 28^{o}C. They were subsequently stored at 4^{o}C and subcultured every week. Liquid cultures were prepared by transferring two to three needlefulls of stab cultures into 10 ml of liquid medium in a five inch screw-capped tube and grown phototrophically until mid-logarithmic phase of growth, as determined either by its characteristic color or by using a Klett-Summerson photoelectric colorimeter.

For large-scale preparations of phototrophic cultures, the above mentioned mid-logarithmic phase cultures were used as inoculum (1% v/v) by transferring them into Roux bottles filled with approximately 750 ml of medium. These were again incubated under phototrophic conditions in a Conviron incubator until mid-logarithmic phase of growth and subsequently transferred as a 7% inoculum (v/v) into 10 or 15 liter carboys which were once more incubated under phototrophic conditions. The cells were harvested by centrifugation in a Sharples centrifuge after 40 hours of incubation when they had reached mid-logarithmic phase of growth.

For large scale preparations of heterotrophically grown cells, 200 ml of sterile medium in a one-liter flask were inoculated from a stab culture and incubated at 28°C on a rotary shaker. The flasks were wrapped in aluminum foil to prevent exposure to light. In the early- to mid-logarithmic phase of growth (about 100-125 Klett units), the cultures were inoculated (2-3% v/v) into 10 or 15 liter glass carboys equipped with a gas inlet connecting to two spargers and a gas outlet. The carboys were also wrapped in aluminum foil to prevent exposure to light and vigorously aerated at 28°C. The cultures were harvested in early- to mid- logarithmic growth phase.

Large-scale preparations of *E. coli* cells were obtained by inoculating one-liter flasks containing 100 ml minimal salts-glucose medium the contents of which were subsequently transferred (1% v/v) into 10 or 15 liter carboys and incubated at 37°C with vigorous aeration. *Escherichia coli* cells were harvested in the early stationary phase of growth at approximately 200 Klett units.

Stock cultures of *Rs. rubrum* and *Rp. palustris* were prepared by centrifuging (20,000 x g) a ten ml culture, under asceptic conditions, and subsequently resuspending the pellet in one to two ml of sterile medium containing

40% (v/v) glycerol. These bacterial suspensions were stored in screw-cap vials at -76°C.

Harvesting of Cells

Cells were harvested by centrifugation in a Sharples steam-driven centrifuge at 37 lbs/in^2 and a flow rate of 200 to 250 ml per minute. The packed cells were washed twice with a KCl-PMSF-S.B., centrifuged in a Sorvall RC2-B centrifuge at 25,000 x g for 15 minutes, weighed and stored at -76°C. At all times during the harvesting procedure the cells were kept on ice.

Growth Curves

Growth curves of Rs. rubrum and Rp. palustris, grown either phototrophically or heterotrophically in 10 liter carboys, were constructed by the following methods:

a) Increases in optical density were measured in asceptically obtained 5 ml samples in a Klett-Summerson photoelectric colorimeter, equipped with a red filter, at intervals of three to six hours.

b) Dry weight of the cells was determined according to the method of Herbert et al (1971). At the appropriate time intervals a 5.0 ml sample was obtained asceptically, centrifuged at 25,000 x g for 15 minutes, washed twice with 5.0 ml distilled water, recentrifuged, and resuspended in 2.0 ml distilled water. The resuspended cells were than transferred to a pre-weighed drying plate which was then placed in a drying oven at 100-115^{o}C for 16 to 20 hours and reweighed on an analytical balance. All samples were analyzed in duplicate.

c) Determination of cellular protein content was carried out as follows: to a 5.0 ml bacterial culture sample 0.5 ml of 1.0 N NaOH was added and the mixture was vortexed briefly and heated to 100^{o}C for 15 minutes. The bacterial protein content was then determined by the Folin-phenol reagent according to the method of Lowry *et al* (1951). Crystalline lysozyme was used as a standard.

d) Colony forming units were determined by obtaining a sterile 1.0 ml sample from which sterile serial dilutions in the appropriate range (10^{2}-10^{7}) were made. One tenth ml of these diluted samples was asceptically transferred to a petri plate containing solid *Rs. rubrum* or *Rp. palustris* medium and incubated phototrophically or heterotrophically. For heterotrophically grown cells the petri plates were wrapped in aluminum foil and incubated upside down in a dark, 28^{o}C incubation room for one to two weeks, whereas phototrophic cells were incubated right-side up in a Gas-Pak jar and placed in a Conviron incubator with ilumination for one to two weeks.

Extraction, purification and characterization of S-30, PS-60, S-100, and ribosome fractions

All the following extractions and purifications were carried out at 4°C. Cells washed with KCl-PMSF-S.B. were ground with two times (w/w) alumina and extracted with two volumes of the standard buffer containing three micrograms per ml deoxyribonuclease. The sample was subsequently centrifuged at 27,000 x g for 30 minutes (2x) to yield a supernatant known as the S-30 fraction.

The PS-60 and S-100 fractions were purified from the S-30 fraction according to the method of Chow (1976 c) with a minor modification. Centrifugation of the S-30 fraction at 60,000 x g for 50 minutes yielded a supernatant (S-60) and a pellet (PS-60). The PS-60 fraction which contains most of the photosynthetic pigments, was resuspended in two to three ml of the standard buffer. This sample was then dialyzed against two liters of the standard buffer overnight and stored in small aliquots at -76°C. Centrifugation of the S-60 fraction at 160,000 x g for two hours yielded another supernatant fraction. The top two thirdsof the final supernatant was carefully aspirated to avoid contamination with residual ribosomes and dialyzed in the same manner as the PS-60 fraction. This fraction constitutes the S-100 and it was also stored in small aliquots at -76°C.

Ribosomes were also purified from the S-30 fraction by the ammonium sulfate precipitation method of Kurland (1966). The purified ribosome samples were dialyzed against two liters of the standard buffer overnight, centrifuged at 27,000 x g for 10 minutes and the resulting supernatant stored in small aliquots at -76°C. The incubation step with puromycin (Chow, 1976 a) was omitted because no apparent effect on the protein synthesizing ability of the ribosomes could be observed with such a treatment.

Figure 1 is a schmematic diagram of the extraction procedures. Samples were thawed and re-frozen a maximum of three times before being discarded whereas any S-100 was not re-frozen after thawing.

All fractions, S-30, PS-60, S-100, and ribosomes were assayed for their protein content according to the method of Lowry et al (1951). Similarily an analysis of the pigment content was carried out for all of the above fractions by the following two methods:

a) To determine the relative concentrations of chlorophyll, yellow and red carotenoids in the various fractions these pigments were extracted according to the method of Cohen-Bazire et al (1957). A 0.1 ml sample was extracted with 4.9 ml of a mixture consisting of seven parts acetone and two parts methanol (v/v). The resulting mixture was vigorously vortexed for one to two minutes and subsequently centrifuged at 10,000 x g for 15 minutes. The resulting supernatant fraction was analyzed spectrophotometrically

KCl-PMSF-S.B. washed cells

ground with 2 x (w/w) alumina

↓

Extracted with 2 volumes of S.B.

containing 3 μg/ml DNase

↓

27,000 x g/30 min. (2 x)

↓

Supernatant Fluid (S-30 Fraction)

↓

60,000 x g/50 min. | Repeated Ammonium Sulfate Precipitation

Supernatant Fluid (S-60) | Pellet (PS-60 Fraction) | ↓

↓ | ↓ |

160,000 x g/2h | Resuspend in 2-3 ml S.B. | Ribosomes

↓ | | ↓

Supernatant Fluid | | Resuspend in S.B. at 300 A_{260} Units/ml

Top 2/3 (S-100 Fraction)

↓

Dialyze against 2 liters of S.B./overnight at $4^{\circ}C$; Store in small aliquots at $-76^{\circ}C$

Figure 1. Schematic summary of procedures used in the preparation of various fractions from Rs. rubrum and Rp. palustris (Chow 1976 b).

at the following wavelengths; 775 nm, 510 nm, and 456 nm, (A_{775}, A_{510}, and A_{456} respectively). The sample must be kept in the dark at all times during the extraction procedure and this was best achieved by wrapping the tube containing the sample in aluminum foil. Since bacterio-chlorophyll is relatively unstable the determination of pigment content was done as soon as possible after extraction. The equations for pigment determination in mg per 100 µl of sample were as follows:

$$\text{Chlorophyll} = (A_{775}) \times 2.19/20$$

$$\text{Yellow Carotenoids} = \frac{(\text{Corrected } A_{456} \times 0.397) - (\text{Corrected } A_{510} \times 0.355)}{20}$$

$$\text{Red Carotenoids} = \frac{(\text{Corrected } A_{510} \times 0.629) - (\text{Corrected } A_{456} \times 0.0728)}{20}$$

where: $\text{Corrected } A_{510} = A_{510} - (A_{775} \times 0.05)$

$\text{Corrected } A_{456} = A_{456} - (A_{775} \times 0.1)$

b) An analysis of the absorbance spectra of the various fractions was also carried out. The absorbance was measured both in the visible light region (340 nm - 1,000 nm) and in the ultra-violet light region (210 nm - 340 nm). The percent absorbance was calculated by using 510 nm and 280 nm respectively for the two light regions as 100%

absorbance. The resulting percent absorbance was plotted as a function of the wavelength.

Sucrose Gradients

It was found that the majority of pigments could be separated from the phototrophic Rs. rubrum ribosome fraction by centrifuging the sample through a 35 ml 5-30% linear sucrose gradient prepared in the standard buffer. Approximately 200 A_{260} units of ribosomes were carefully layered on top of the gradient and centrifuged in a Spinco SW 27 rotor at 50,000 x g for 16 hours at $4^{o}C$. One half ml fractions were collected from the bottom of the gradient and the A_{260} value of each fraction was measured to determine the position of the ribosome "peak". A pigmented pellet fraction remaining at the bottom of the gradient tube was subsequently resuspended in standard buffer of the original sample volume, dialyzed overnight against two liters of the standard buffer to remove any residual sucrose and stored in small aliquots at $-76^{o}C$.

The ribosome "peak" fractions were pooled from which ribosomes were recovered as a pellet by centrifuging at 160,000 x g for 20 hours in a Spinco Ti 60 rotor at $4^{o}C$. The resulting ribosome pellet was also resuspended in the original sample volume, dialyzed and stored in small aliquots at $-76^{o}C$.

Cell-free Protein Synthesis

In vitro protein synthesis was carried out according to the method of Chow (1976 b). The standard reaction mixture with a final volume 200 μl, contained the following:

Tris-HCl, pH	60 mM
NH_4Cl	66 mM
Mg-Acetate	7 mM
19 Amino acid mix (without phenylalanine)	0.05 mM of each amino acid
Tris-ATP, pH 7.8	1.5 mM
Tris-GTP, pH 7.8	0.045 mM
K-PEP	7.5 mM
Cysteine	0.05 mM
β-mercaptoethanol	6 mM
Dithiothreitol	2 mM
Pyruvate Kinase	5 μg
E. coli tRNA	50 μg
Poly U	100 μg
^3H-phenylalanine	2 μCi
Assay sample	0 - 85μl
H_2O, up to	200μl

The assay sample can consist of the following:

a)	S-30	30 μl
b)	S-100	50 μg Protein
	Ribosomes	6.6 A_{260} Units

c) S-100 — 50 μg Protein

Ribosomes — 6.6 A_{260} Units

PS-60 — as indicated in μg of protein

The reaction was carried out at 37^{o}C for 60 minutes. The radioactivity incorporated was determined by the hot trichloroacetic acid method (Modolell, 1971). An additional washing of the filters with 95% ethanol (6 ml), was included to suppress the quenching effect of the pigments present in the assay mixture.

Chemicals

The following chemicals were purchased from the Sigma Chemical Company: DNase I (from bovine pancreas), Tris-ATP, Tris-GTP, K-PEP, β-mercaptoethanol, dithiothreitol, pyruvate kinase, *E. coli* tRNA, polyuridylic acid (potassium salt), l-glutamic acid, dl-malic acid, egg-white lysozyme, and nicotinic acid.

The ^{3}H-phenylalanine (1 mCi/ml) was purchased from New England Nuclear Corporation.

Ultra pure ammonium sulfate and sucrose (density gradient grade) were obtained from Schwarz-Mann division of Becton, Brickinson and Company.

All other chemicals were reagent grade or better.

RESULTS

General Properties of Organisms

A variant strain of *Rhodospirillum rubrum* had been isolated in this laboratory by the conventional methods and was designated strain VF. Preliminary studies have shown that this strain was almost identical to the previously studied wild-type strain (Chow, 1976a,b,c) with respect to physiology and morphology but the ribosomes of the heterotrophic *Rs. rubrum* VF cells were much more stable when used for the *in vitro* protein-synthesis system (for detail, see later sections). When grown under phototrophic conditions the cells had the typical spiral-shaped morphology, and were about 0.8 to 1.0 μm in width and 10 μm in length as shown in Figure 2a, and, under heterotrophic conditions the cells generally tended to be shorter and somewhat pleomorphic (Figure 2b).

Rhodopseudomonas palustris, strain E1, was always pleomorphic, mostly ovoid to rod shaped with occasional slightly curved ones (Figures 2a and 2b). The size of individual young cells varied from 0.6 to 0.9 μm in width and 1.2 to 2.0 μm in length.

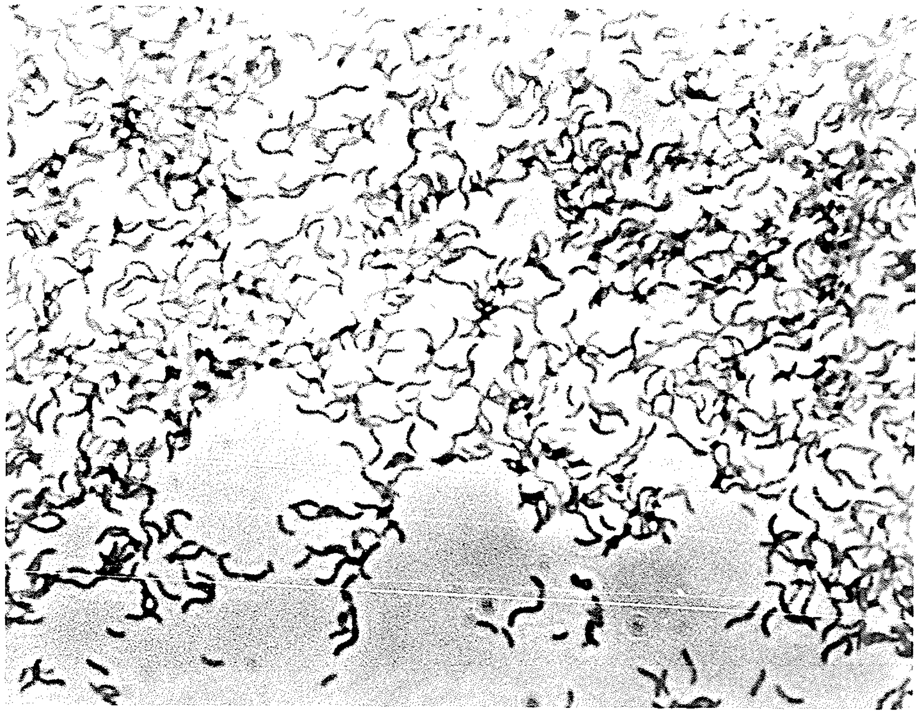
By mid-logarithmic phase of growth both phototrophic bacterial cultures were heavily pigmented; dark red for *Rs. rubrum* and brownish-red for *Rp. palustris*. Under heterotrophic conditions both *Rs. rubrum* and *Rp. palustris* cultures were light beige-pink by mid-logarithmic phase of growth. The cells or their fractions of the phototrophic

Figure 2. Phase contrast photomicrographs of *Rs. rubrum*, VF, cells. The cells were taken from a logarithmic growth phase culture, heat fixed, stained with crystal violet, and photographed under a Nikon phase contrast microscope (1600x).

(a) Phototrophic cells

(b) Heterotrophic cells

a)



b)

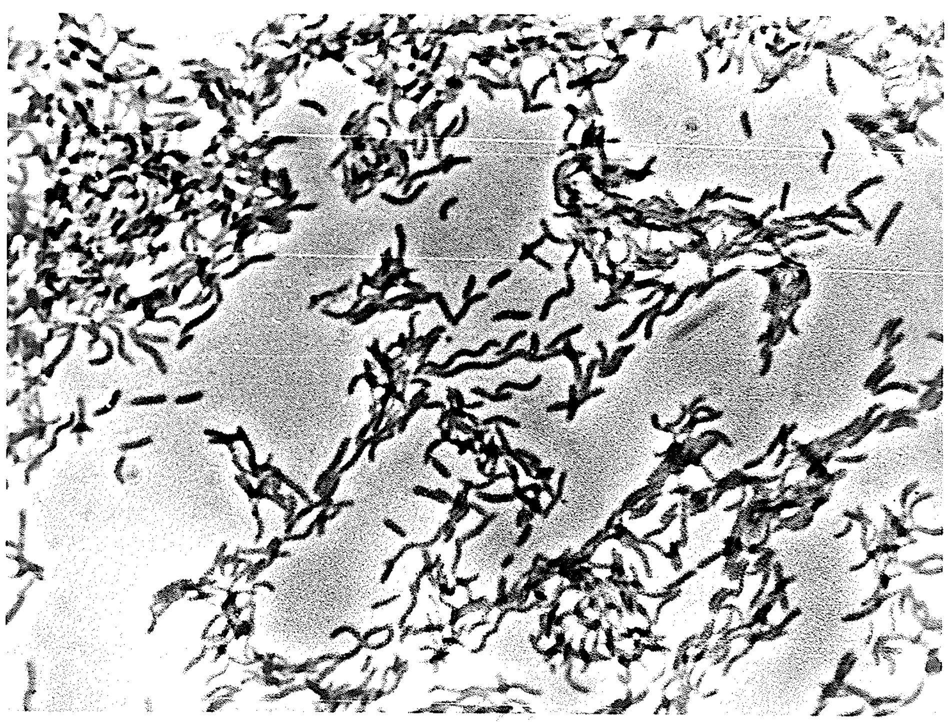
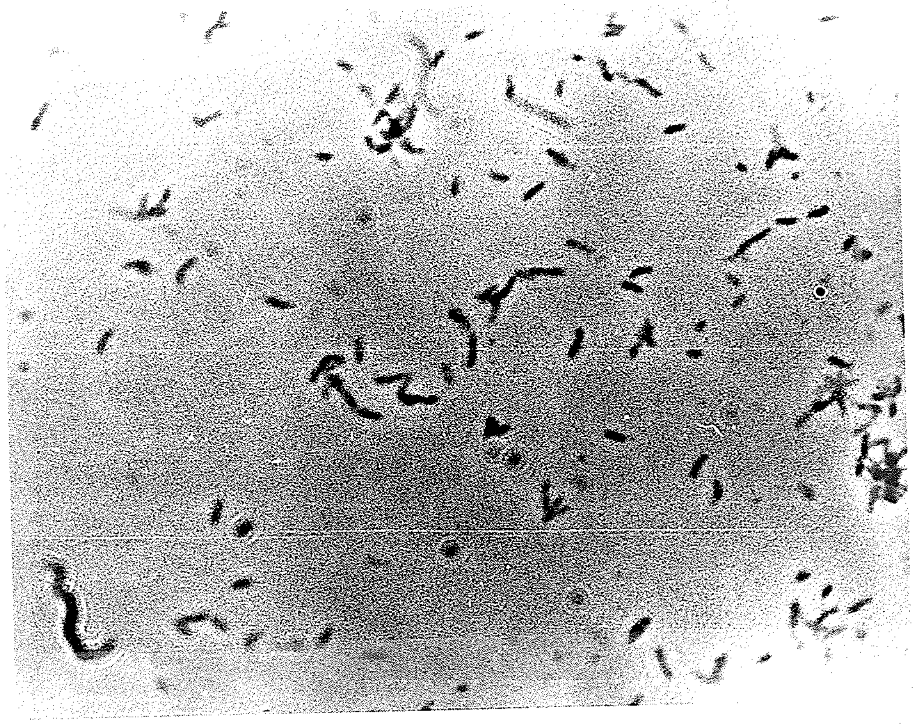


Figure 3. Phase contrast photomicrographs of _Rp. palustris_, El, cells. The cells were taken from a logarithmic growth phase culture, heat fixed, stained with crystal violet, and photographed under a Nikon phase contrast microscope (4000x).

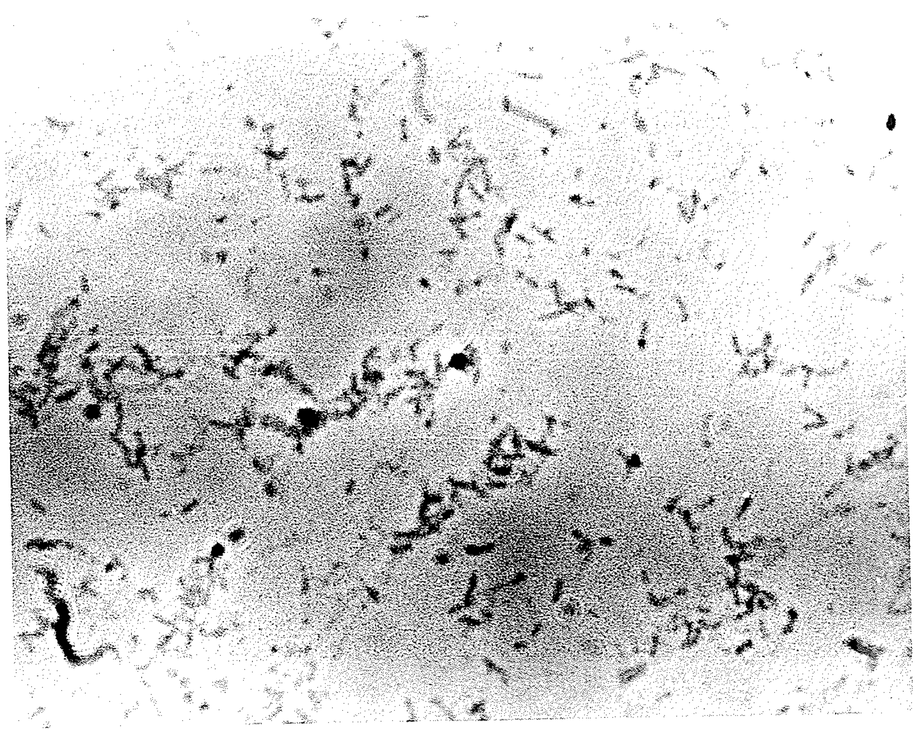
(a) Phototrophic cells

(b) Heterotrophic cells

a)



b)



and heterotrophic cultures will hereafter be referred to as P- and H-cells or fractions, respectively.

Upon harvesting a mid-logarithmic culture the average yields, given in wet weight, were as follows:

P-*Rs*. *rubrum* VF	1.0 g/l
H-*Rs*. *rubrum* VF	1.2 g/l
P-*Rp*. *palustris* El	1.3 g/l
H-*Rp*. *palustris* El	1.5 g/l
E. *coli*	2.5 g/l

Growth Curves

Figures 4 and 5 depict the growth curves of *Rs*. *rubrum* VF, and *Rp*. *palustris* El, respectively. Both organisms grew slightly faster under heterotrophic than under phototrophic conditions. The mean generation times of these cultures were as follows:

P-*Rs*. *rubrum* VF	6.6 hours
H-*Rs*. *rubrum* VF	6.3 hours
P-*Rp*. *palustris* El	7.0 hours
H-*Rp*. *palustris* El	6.7 hours

Preparation of S-30, PS-60, S-100, and Ribosome Fractions

As mentioned earlier (Materials and Methods), a number of fractions, such as S-30, PS-60, S-100 and ribosomes, were used for the study of *in vitro* protein synthesis. From 23 g (wet weight) of cells we were able to prepare two to three ml of PS-60 and 50 ml of S-100 fractions, and from 50 g (wet weight) of cells, we obtained approximately

Figure 4. Growth curves of Rs. rubrum VF, under (a) phototrophic and, (b) heterotrophic growth conditions.

(⊙——⊙) Optical density

(●——●) Colony Forming Units (CFU)

(◘——◘) Dry weight

(▲——▲) Protein content

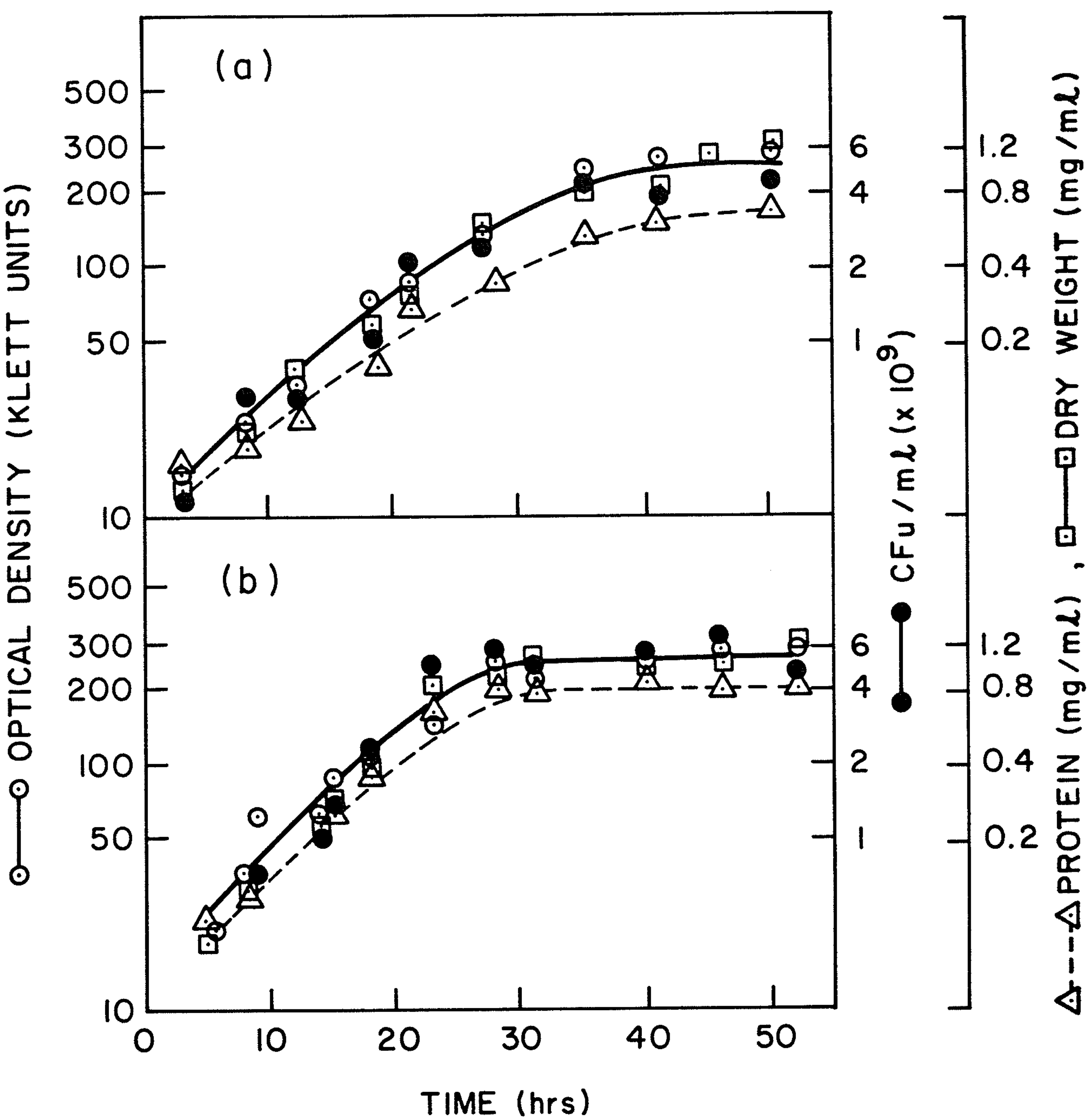

(a)
(b)
OPTICAL DENSITY (KLETT UNITS)
500
300
200
100
50
10
CFu / mℓ (x 10⁹)
6
4
2
1
1.2
0.8
0.4
0.2
PROTEIN (mg/mℓ) , DRY WEIGHT (mg/mℓ)
0
10
20
30
40
50
TIME (hrs)

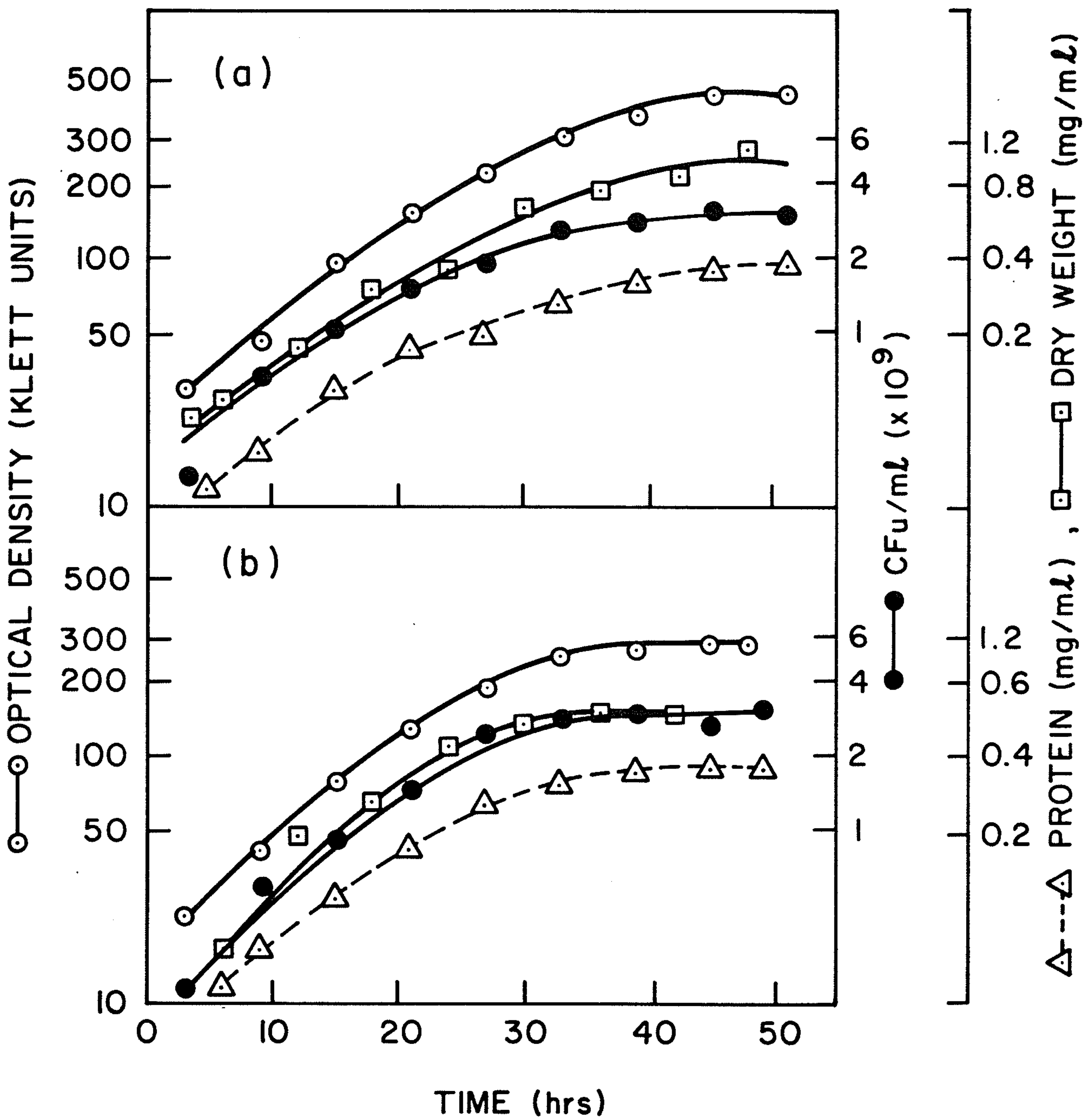
Figure 5. Growth curves of Rp. palustris, El, under (a) phototrophic and, (b) heterotrophic growth conditions.

(⊙———⊙) Optical density

(●———●) Colony Forming Units (CFU)

(▫———▫) Dry weight

(▲———▲) Protein content


(a)
(b)
OPTICAL DENSITY (KLETT UNITS)
500
300
200
100
50
10
CFu/mℓ (x 10^9)
6
4
2
1
DRY WEIGHT (mg/mℓ) , PROTEIN (mg/mℓ)
1.2
0.8
0.6
0.4
0.2
0
10
20
30
40
50
TIME (hrs)

2,000 A_{260} units of purified ribosomes.

General Properties of S-30, PS-60, and S-100 Fractions

Since the cells used in this study were heavily pigmented, some of their fractions were also heavily pigmented, especially for the PS-60 fraction which contained an enriched pigment content (Table 1). Under our growth conditions the non-sulfur purple bacteria contained two to three major types of pigments; yellow and red carotenoids and bacteriochlorophylls, and the physiological conditions had greatly influenced the composition of the pigments.

The absorption spectra of the various fractions were measured in the visible light region and the results are shown in Figures 6 to 9. According to the results of Cohen-Bazire *et al* (1957), the (yellow and red) carotenoids have absorption maxima at 450 nm, 477 nm, and 510 nm, whereas bacteriochlorophylls absorb strongly at 590 nm, 805 nm, and 875 nm. Figure 6 shows the absorption spectra of *Rs. rubrum* S-30 and PS-60 fractions. The S-30 fraction was essentially equivalent to the total cell extract and would therefore be considered as such in this instance. In the phototrophic *Rs. rubrum* PS-60 sample (Figure 6a) absorption maxima were found at the following wavelengths: 375 nm, 510 nm, 590 nm, 875 nm, and 920 nm. Corresponding peaks were also present in the S-30 fraction (Figure 6a) with the noted exception of the latter two absorption peaks at 875 nm and 920 nm, indicating that the PS-60 fraction was enriched in bacteriochlorophylls. In the H-*Rs. rubrum*

Table 1. Color and pigment content of S-30, PS-60 and S-100 fractions.

Cells	Fractions	Color	Pigment content in µg per mg of protein*		
			Bacterio-Chlorophyll	Yellow Carotenoids	Red Carotenoids
P-*Rs. rubrum*	S-30	deep red	8.09	0.04	0.31
	PS-60	deep red	6.18	0.06	1.18
	S-100	pink	0.27	0	0.08
H-*Rs. rubrum*	S-30	beige pink	4.95	0	0.21
	PS-60	beige pink	3.56	0.06	0.31
	S-100	yellow	0.09	0	0.01
P-*Rp. palustris*	S-30	deep red	1.75	0	0.08
	PS-60	deep red	2.36	0	0.12
	S-100	yellow	0.06	0	0.01
H-*Rp. palustris*	S-30	beige pink	1.03	0.01	0.08
	PS-60	beige pink	1.02	0	0.09
	S-100	yellow	0.01	0	0.01

*The pigment content was determined by the methods of Cohen-Bazire *et al* (1957) and the protein content was determined by the methods of Lowry *et al* (1951). These results represent an average of three determinations.

Figure 6. Absorption spectra of Rs. rubrum S-30 and PS-60 fractions.

Phototrophic (a) and heterotrophic (b) Rs. rubrum S-30 and PS-60 fractions were diluted to about the same optical density at 510 nm and their absorption spectra were measured at the indicated wavelengths. The absorbance at various wavelengths was standardized by using the absorption value at 510 nm as 100%. The absolute absorbance values at 510 nm, before dilution, for P-S-30, P-PS-60, H-S-30, and H-PS-60 were 4.84, 14.98, 1.64, and 4.94 respectively.

(———) PS-60

(-----) S-30

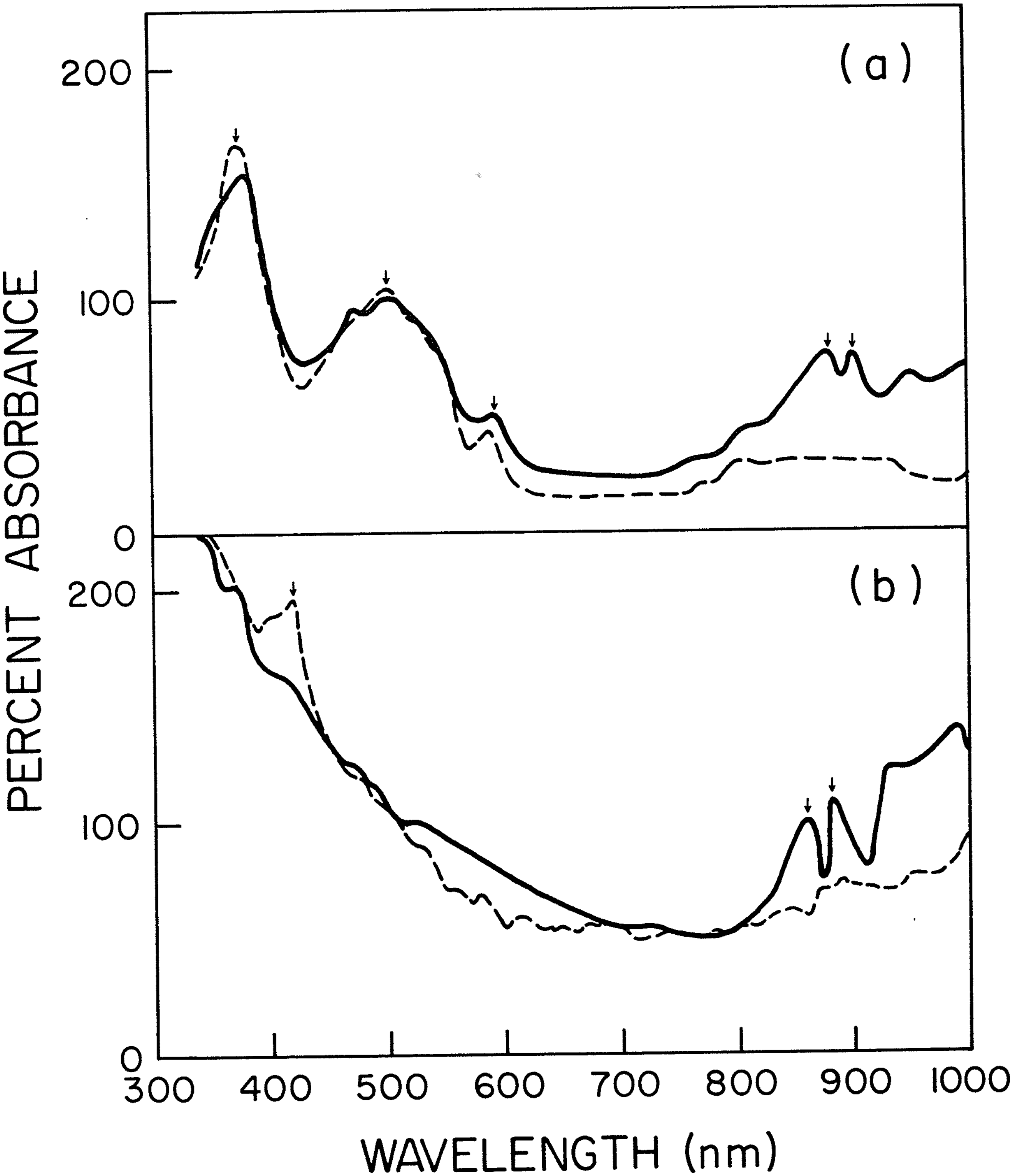

(a)
(b)
200
100
0
200
100
0
PERCENT ABSORBANCE
300
400
500
600
700
800
900
1000
WAVELENGTH (nm)

Figure 7. Absorption spectra of _Rp._ _palustris_ S-30 and PS-60 fraction.

Phototrophic (a) and heterotrophic (b) _Rp._ _palustris_ S-30 and PS-60 fractions were diluted to about the same optical density at 510 nm and their absorption spectra were measured at the indicated wavelengths. The absorbance at various wavelengths was standardized by using the absorption value at 510 nm as 100%. The absolute absorbance values at 510 nm, before dilution, for P-S-30, P-PS-60, H-S-30 and H-PS-60 were 1.78, 3.51, 0.78, and 1.31 respectively.

(——) PS-60

(----) S-30

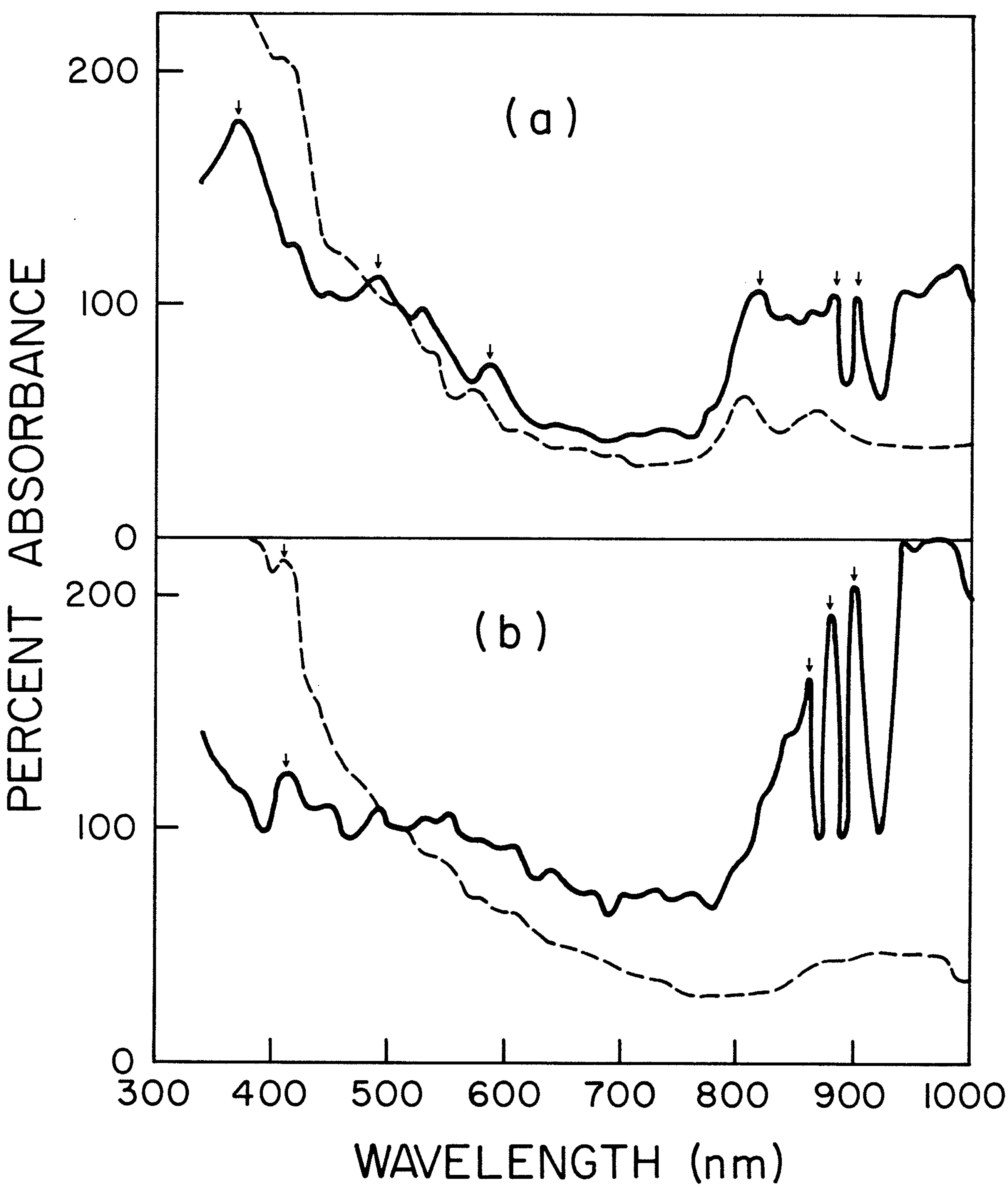

(a)
(b)
PERCENT ABSORBANCE
WAVELENGTH (nm)
0
100
200
300
400
500
600
700
800
900
1000

Figure 8. Absorption spectra of Rs. rubrum S-100 and ribosome fractions.

Phototrophic (a) and heterotrophic (b) Rs. rubrum S-100 and ribosome fractions were diluted to about the same optical density at 510 nm and their absorption spectra were measured at the indicated wavelengths. The abosrbance at various wavelengths was standardized by using the absorption value at 510 nm as 100%. The absolute absorbance values at 510 nm, before dilution, for P-S-100, P-ribosomes, H-S-100, H-ribosomes were 0.76, 2.15, 0.93, and 1.15, respectively.

(———) Ribosomes

(----) S-100

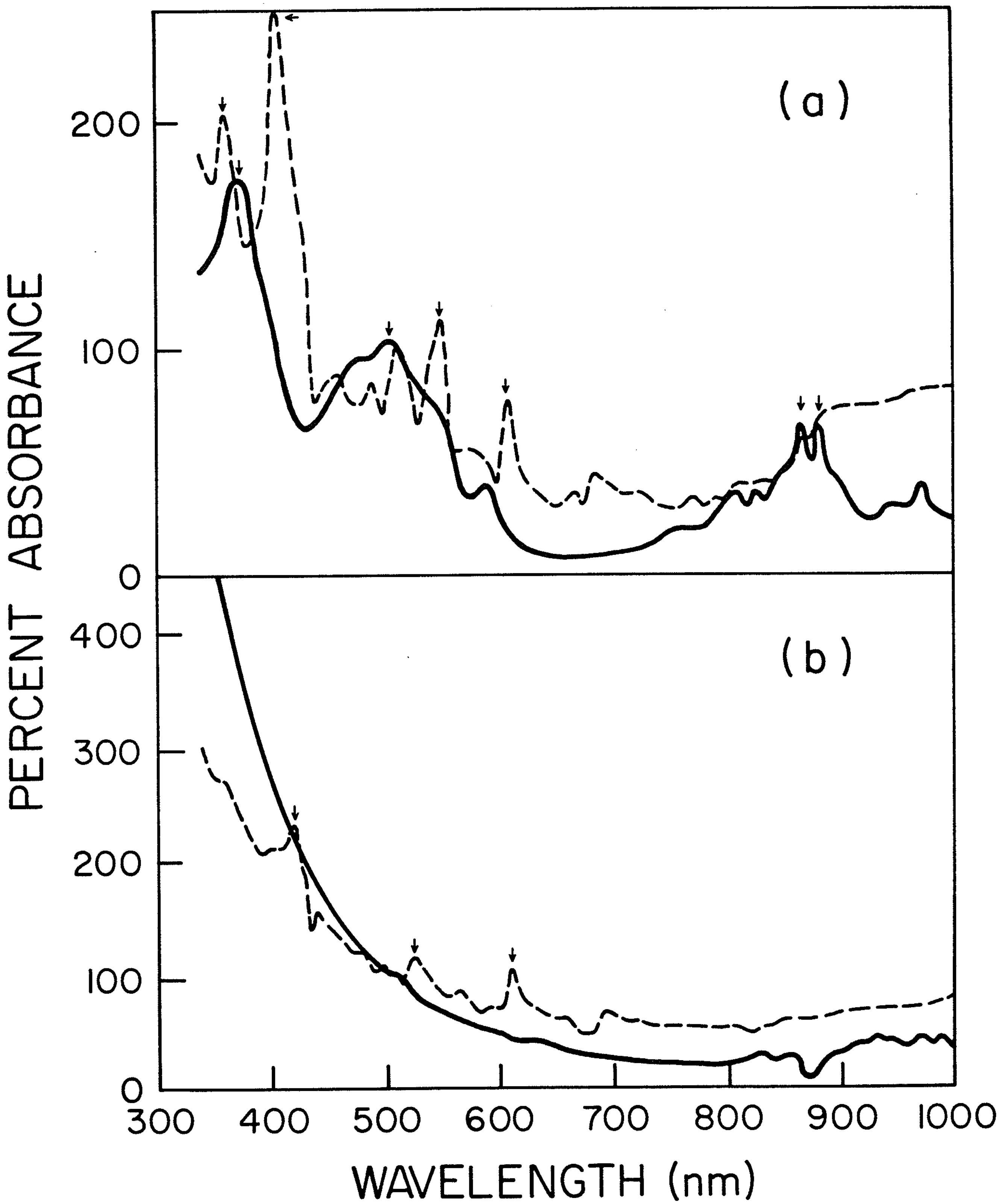
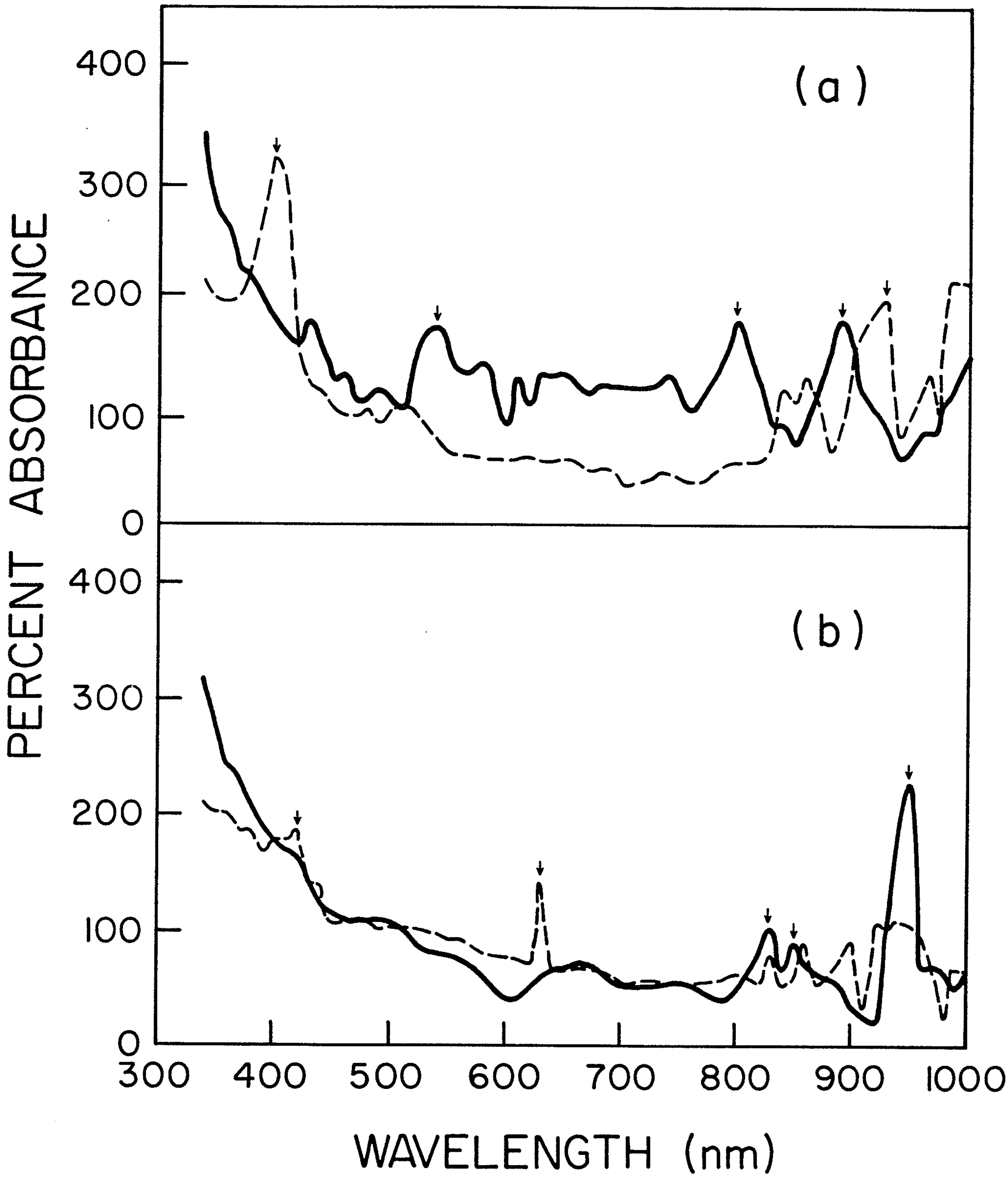

(a)
(b)
PERCENT ABSORBANCE
0
100
200
300
400
WAVELENGTH (nm)
300
400
500
600
700
800
900
1000

Figure 9. Absorption spectra of _Rp_. _palustris_ S-100 and ribosome fractions.

Phototrophic (a) and heterotrophic (b) _Rp_. _palustris_ S-100 and ribosome fractions were diluted to about the same optical density at 510 nm and their absorption spectra were measured at the indicated wavelengths. The absorbance at various wavelengths was standardized by using the absorption value at 510 nm as 100%. The absolute absorbance values at 510 nm, before dilution for P-S-100, P-ribosomes, H-S-100, and H-ribosomes were 1.45, 0.35, 3.35, and 0.28, respectively.

(——) Ribosomes

(----) S-100


400
300
200
100
0
(a)
400
300
200
100
0
(b)
300
400
500
600
700
800
900
1000
PERCENT ABSORBANCE
WAVELENGTH (nm)

samples (Figure 6b) the S-30 fraction showed an absorption maximum at 425 nm which was not found in the PS-60 fraction. This absorption peak corresponded to a carotenoid peak. Similar to the phototrophic samples, the heterotrophic PS-60 fraction was also significantly enriched in bacteriochlorophylls, absorbing at 850 nm and 875 nm.

In the Rp. palustris samples (Figure 7), both the P- and H-fractions absorbed strongly in the carotenoid region (300 to 510 nm) whereas the (P- and H-) PS-60 fractions showed very strong absorption in the region from 800 to 1,000 nm, indicating that the Rp. palustris PS-60 fractions were also enriched in bacteriochlorophylls.

Absorbance spectra of Rs. rubrum and Rp. palustris S-100 and ribosome fractions were also measured and shown in Figures 8 and 9. The noteworthy peaks in the P-Rs. rubrum (Figure 8a) ribosomes were found at 360 nm, 510 nm, 865 nm, and 875 nm, whereas the S-100 fraction absorbed strongly at 340 nm, 410 nm, 515 nm, 610 nm, 675 nm, and 900 to 1000 nm. The H-Rs. rubrum ribosome and S-100 fractions contained a relatively low amount of pigments as reflected by their absorption spectra (Figure 8b).

Phototrophic Rp. palustris S-100 and ribosome fractions (Figure 9a) showed many absorption peaks which were present at 395 nm, 825 nm, 850 nm, 925 nm, and 975 nm for the S-100 fraction, and at 535 nm, 800 nm, and 875 nm, as well as a broad absorption band from 560 nm to 740 nm for the ribosome fraction. The H-Rp. palustris fractions

(Figure 9b) also had relatively little pigments, however absorption peaks at 625 nm for the S-100 fraction and at 825 nm, 850 nm, and 950 nm for the ribosomes were observed.

In summary, all the PS-60 fractions showed enhanced absorption peaks in the long wavelength region (800 to 1000 nm, Figures 6 and 7) suggesting a higher concentration of bacteriochlorophylls, and, generally speaking, the S-100 and ribosome fractions of the P-cells showed several absorption peaks throughout the visible light region whereas the corresponding H-samples had far fewer and less pronounced peaks (Figures 8 and 9).

The absorption spectra of these fractions in the ultraviolet light region had also been measured (Materials and Methods), however no significant absorption peaks were found and thus the results were not included here.

In vitro Protein Synthesis Assays

(A) S-30 system

The S-30 is a relatively crude protein synthesizing system which contains all of the factors required for protein synthesis as well as many other components with undetermined functions (Modolell, 1971). In general, an active S-30 protein-synthesizing system indicates the presence of biologically functional ribosomes and S-100 fraction which can then be purified from this fraction. The protein-synthesizing activities of the *Rs. rubrum* and *Rp. palustris* S-30 system however, were not at all reproducible. They incorporated various amounts of ^{3}H-phenylalanine ranging from 20,000 to 200,000 dpm for any given S-30 fraction and for this reason the S-30 system had not been routinely used in this study except for the initial activity measurement.

(B) S-100 plus ribosome system

To eliminate the possibility of background counts, the S-100 and ribosome fractions were separately tested for their activity in an *in vitro* assay mixture. The results (Table 2) demonstrated that all the ^{3}H-phenylalanine incorporated was less than the background counts (2,909 dpm, incorporated in the absence of both the S-100 fraction and ribosomes in a poly U directed assay system).

Figure 10 depicts the effect of varying the ribosome/S-100 ratio on the protein-synthesizing activity of the homologous P-*Rs. rubrum* system. As the ratio increased, the amount of ^{3}H-phenylalanine incorporated also increased, but started to level off when the ratio had reached 2/1 and higher. A 2/1 ratio of ribosomes/S-100 was therefore chosen for all the following *in vitro* protein synthesis assays.

A time kinetics study of the homologous *Rs. rubrum*, *Rp. palustris*, and *E. coli* *in vitro* protein-synthesis systems was carried out and the results are shown in Figure 11. After 10 to 15 min the incorporation of ^{3}H-phenylalanine had reached 80 to 90% of its maximal value. The optimal incorporation was attained after 20 min incubation period in all cases,

Table 2. The activity of various fractions in *in vitro* protein synthesis assay systems*

S-100	Ribosomes	dpm incorporated
-	-	2,909
P-*Rs. rubrum*	-	1,472
H-*Rs. rubrum*	-	1 948
P-*Rp. palustris*	-	2,813
H-*Rp. palustris*	-	2,019
E. coli	-	2,081
-	P-*Rs. rubrum*	819
-	H-*Rs. rubrum*	1,110
-	P-*Rp. palustris*	2,334
-	H-*Rp. palustris*	1,009
-	*E. coli*	606

*The reaction mixture (Chow, 1976c) contained 50 µg S-100 proteins or 6.6 A_{260} units of ribosomes where indicated. This mixture was incubated at 37°C for 60 min and the amount of radioactive phenylalanine incorporated was determined by the hot trichloroacetic acid precipitation procedure as described in "Materials and Methods".

Figure 10. ^{3}H-phenylalanine incorporated by phototrophic Rs. rubrum systems with varying ribosome/S-100 ratios.

The reaction mixtures containing a constant amount of S-100 protein (50 μg) were incubated at 37°C for 60 min with the indicated ribosome/S-100 (protein/protein) ratios. The amount of ^{3}H-phenylalanine incorporated was determined by the hot trichloroacetic acid method as described in "Materials and Methods".

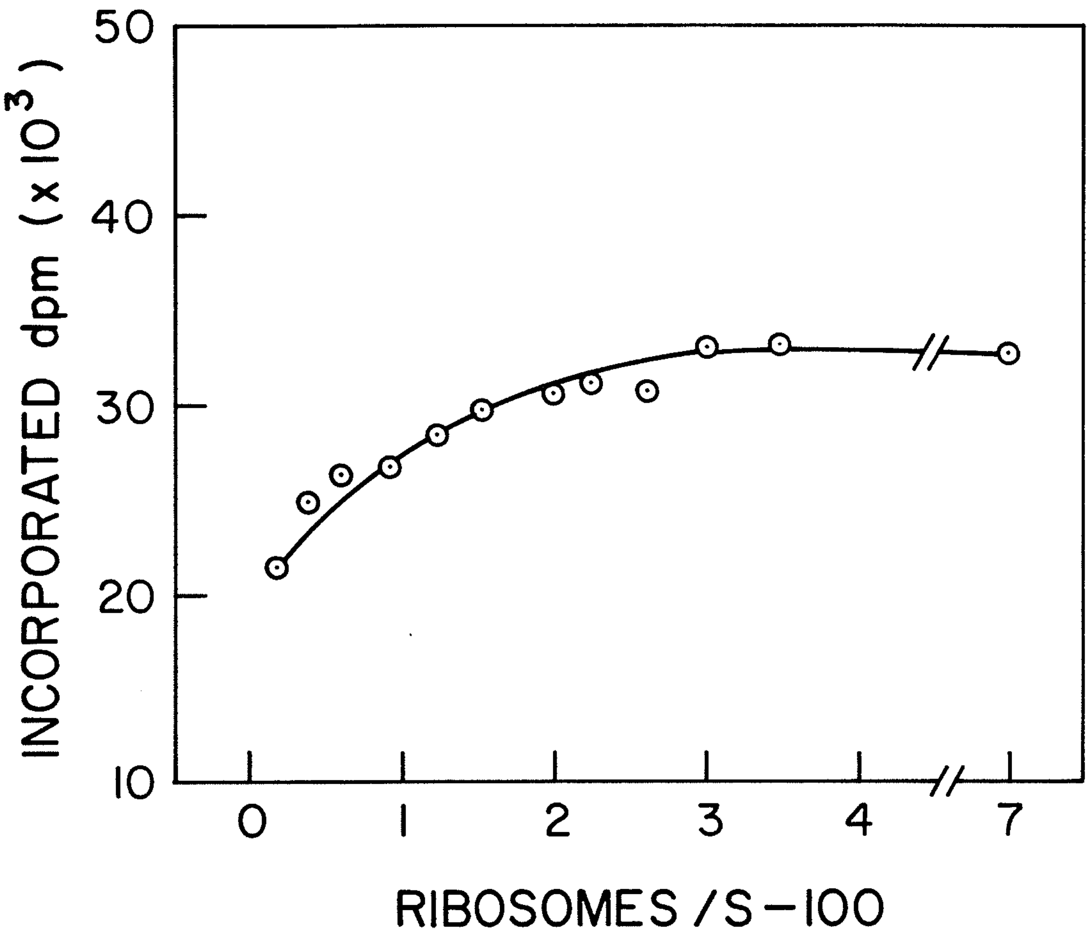

50
40
30
20
10
INCORPORATED dpm (x 10^3)
0
1
2
3
4
7
RIBOSOMES / S-100

Figure 11. Kinetics of ^{3}H-phehylalanine in a poly U directed *in vitro* protein synthesis system.

The complete reaction mixture containing 6.6 A_{260} units of ribosomes and 50 μg of S-100 protein from the same organism was incubated at 37°C for the indicated times. The amount of incorporated ^{3}H-phenylalanine was determined by the hot trichloroacetic acid method as described in "Materials and Methods". Note change in scale in (e).

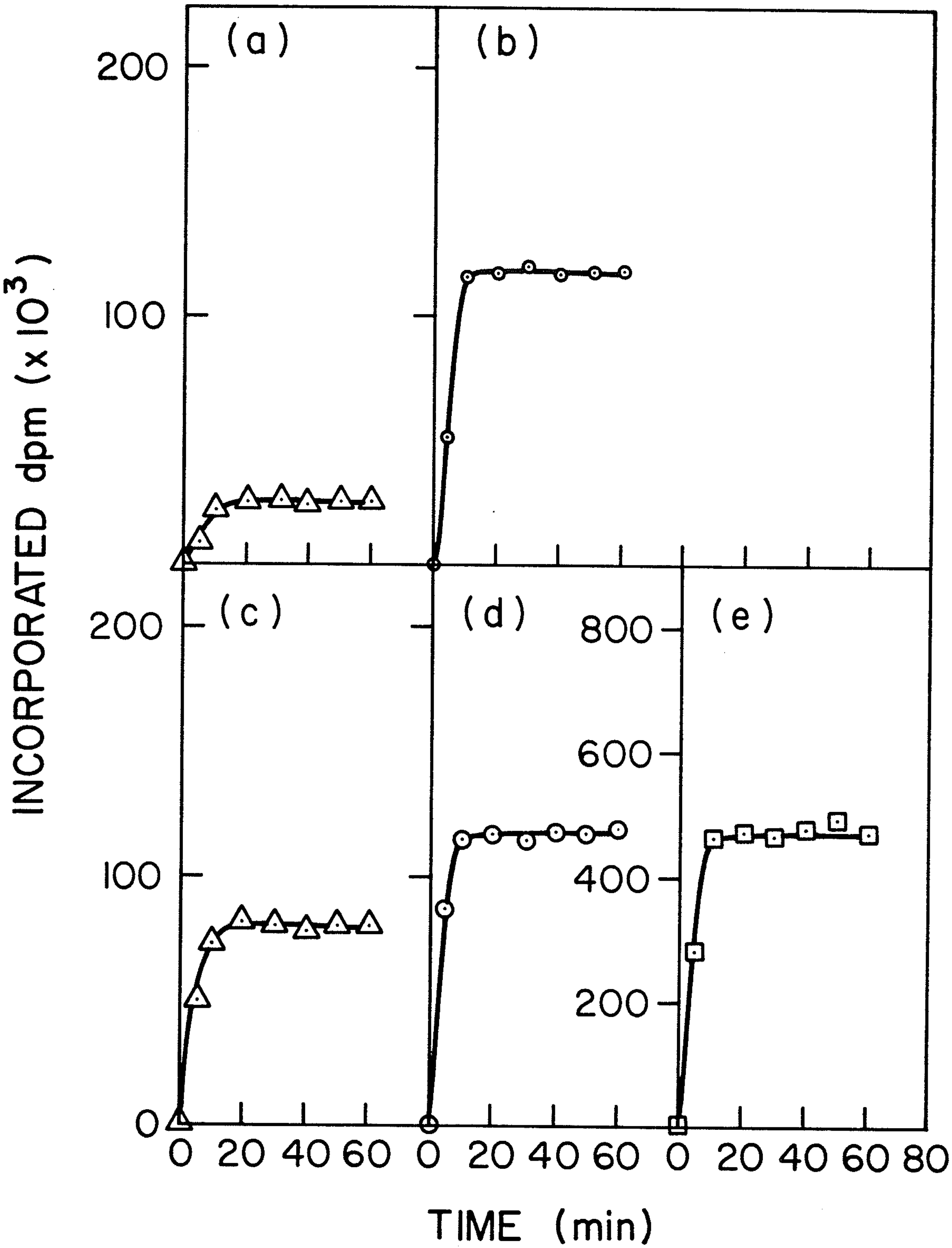
(a) P-*Rs. rubrum*

(b) H-*Rs. rubrum*

(c) P-*Rp. palustris*

(d) H-*Rp. palustris*

(e) *E. coli*


(a)
(b)
(c)
(d)
(e)
200
100
0
800
600
400
200
0 20 40 60 0 20 40 60 0 20 40 60 80
INCORPORATED dpm ($\times 10^3$)
TIME (min)

and a constant level was maintained for up to 60 min. Consequently a 60 min incubation period was used for all subsequent *in vitro* assays.

The various S-100 and ribosome fractions obtained from (P- and H-) *Rs. rubrum*, (P- and H-) *Rp. palustris*, and *E. coli* cells could be mixed in a number of combinations to test their *in vitro* protein synthesizing activity. Table 3 shows the average amount of ^{3}H-phenylalanine incorporated for the various S-100 and ribosome combinations. With the exception of the P-*Rs. rubrum* ribosomes, which showed a variability of up to 40,000 dpm from one batch of purified ribosomes to another, the results were highly reproducible. For the P-*Rs. rubrum* ribosomes only those ribosome samples that showed high activity were used. It is interesting to note that the homologous systems did not always show the highest activity. All S-100 fractions and all ribosomes were active but some combinations were better than others. For instance *E. coli* and H-*Rp. palustris* S-100 provided a high activity when combined with any of the ribosomes tested whereas the H-*Rs. rubrum* S-100 fraction showed a low activity when combined with (P- and H-) *Rp. palustris* ribosomes. As for the ribosomes, H-*Rs. rubrum* and *E. coli* samples showed a relatively high activity with virtually all of the S-100 fractions, but the P-*Rs. rubrum* ribosomes had high activities only when combined with H-*Rs. rubrum* or *E. coli* S-100 fractions.

When comparing the activities of the various homologous systems, the P-*Rs. rubrum* system exhibited the lowest

Table 3. A comparison of *in vitro* protein synthesis activities by various S-100 and ribosome combinations*

Ribosomes	S-100				
	P-*Rs. rubrum*	H-*Rs. rubrum*	P-*Rp. palustris*	H-*Rp. palustris*	*E. coli*
P-*Rs. rubrum*	<u>30,638</u>	96,036	68,461	49,068	82,301
H-*Rs. rubrum*	108,502	<u>140,251</u>	158,807	120,485	155,735
P-*Rp. palustris*	153,593	46,215	<u>92,878</u>	114,787	142,016
H-*Rp. palustris*	100,693	23,818	144,691	<u>136,835</u>	143,785
E. coli	148,261	145,872	58,184	131,503	<u>589,314</u>

*The results represent an average of three determinations. A ribosome/S-100 ratio of 2/1 was used and the reaction mixture was incubated at 37°C for 60 min. The amount of ^3H-phenylalanine incorporated was determined by the hot trichloroacetic acid method as described in "Materials and Methods". Activities in homologous systems are underlined.

activity and the order of activity is as follows: P-Rs. rubrum < P-Rp. palustris < H-Rp. palustris $\leq$ H-Rs. rubrum < E. coli. The higher activity observed in the homologous H-Rs. rubrum system was of particular interest, because in a previous report by Chow (1976c) who used fractions from wild-type Rs. rubrum cells, it was shown that the homologous H-Rs. rubrum system had very low activity. However, this low activity could be stimulated to many folds higher by addition of the PS-60 fraction of the phototrophic Rs. rubrum. Further, this P-Rs. rubrum PS-60 fraction inhibited the activity of other Rs. rubrum systems in which photosynthetic pigments were present, such as P-S-100 + P-ribosomes and H-S-100 + P-ribosomes (Chow, 1976c). Because of these interesting findings, the effects of the PS-60 fractions of both phototrophic and heterotrophic Rs. rubrum and Rp. palustris were tested on various protein-synthesizing systems.

Effects of PS-60 Fractions on Protein-Synthesizing Activity

Before the effects of the (P- and H-) Rs. rubrum and Rp. palustris PS-60 fractions on various protein-synthesizing systems were analyzed, the possible residual protein-synthesizing activities in these fractions were first tested. As shown in Tables 4 and 5, these fractions exhibited a relatively low activity when tested alone or when tested in combination with increasing amounts of ribosomes or S-100 fractions (compare results in Tables 4 and 5 with results in Table 3).

Table 4. Incorporation of ^{3}H-phenylalanine by PS-60 fractions and by PS-60 fractions + ribosomes*

Ribosome Source	Ribosome Concentration A_{260} units	PS-60 fractions	dpm incorporated
-	-	P-*Rs*. *rubrum*	716
-	-	H-*Rs*. *rubrum*	1,972
-	-	P-*Rp*. *palustris*	2,096
-	-	H-*Rp*. *palustris*	2,184
P-*Rs*. *rubrum*	7 14 21	P-*Rs*. *rubrum*	12,377 14,198 11,801
H-*Rs*. *rubrum*	7 14 21	H-*Rs*. *rubrum*	17,715 18,134 17,001
P-*Rp*. *palustris*	7 14 21	P-*Rp*. *palustris*	16,173 15,149 12,773
H-*Rp*. *palustris*	7 14 21	H-*Rp*. *palustris*	10,113 13,178 12,256
E. *coli*	7 14 21	P-*Rs*. *rubrum*	12,373 11,101 12,178
E. *coli*	7 14 21	P-*Rp*. *palustris*	9,314 12,701 12,567

*The reaction conditions were the same as described in Table 3, except that S-100 fractions were omitted in all reaction mixtures. Four hundred µg of PS-60 proteins or 400 µg of PS-60 protein + increasing amounts of ribosomes were included in the reaction mixtures, as indicated.

Table 5. Incorporation of ^{3}H-phenylalanine by PS-60 and S-100 fractions*

S-100	PS-60			
	P-*Rs. rubrum*	H-*Rs. rubrum*	P-*Rp. palustris*	H-*Rp. palustris*
P-*Rs. rubrum*	660	3,096	1,472	1,948
H-*Rs. rubrum*	4,846	4,118	5,140	5,368
P-*Rp. palustris*	2,840	1,836	2,604	3,626
H-*Rp. palustris*	2,886	2,286	1,582	3,970
E. coli	3,488	1,094	4,142	3,538

*The reaction conditions were the same as described in Table 3. except that in the reaction mixture ribosomes were omitted and 400 μg proteins of the various PS-60 fractions were included.

When PS-60 fractions were added to complete assay mixtures, i.e., mixtures containing both S-100 and ribosomes, a strong stimulating effect had been observed in almost all protein-synthesizing systems which contained at least one fraction (either the S-100 fraction or the ribosomes) of the *Rs. rubrum* or *Rp. palustris* origin, but an inhibitory effect existed on the homologous *E. coli* systems (Tables 6, 7, 8, and 9). Within limits, no obvious differences could be found among the P-*Rs. rubrum* (Table 6), the H-*Rs. rubrum* (Table 7), the P-*Rs. palustris* (Table 8) and the H-*Rp. palustris* (Table 9) PS-60 fractions, with respect to their stimulatory and inhibitory effects. However, the degree of these effects was apparently dependent on the nature of the protein-synthesizing system, and, as a general rule, the lower the original activity, the greater the stimulatory effect. This point could be illustrated by comparing the orders of the protein-synthesizing activity of the homologous systems in the absence of the PS-60 fractions (P-*Rs. rubrum* < P-*Rp. palustris* < H-*Rp. palustris* ≤ H-*Rs. rubrum* < *E. coli*, Table 3) and in the presence of the PS-60 fractions (*E. coli* < H-*Rs. rubrum* ≤ H-*Rp. palustris* < P-*Rp. palustris* < P-*Rs. rubrum*, Table 10).

Since the P-*Rs. rubrum* PS-60 fractions showed relatively higher stimulatory and inhibitory effects (Table 6), it had been chosen for two additional studies, i.e., concentration and ribosome/S-100 ratio effects.

Table 6. The effect of P-Rs. rubrum PS-60 fraction on the *in vitro* protein synthesizing activity*

Ribosomes	S-100				
	P-Rs. rubrum	H-Rs. rubrum	P-Rp. palustris	H-Rp. palustris	E. coli
P—Rs. rubrum	166,769 (544.3)	134,026 (139.6)	66,186 (96.7)	114,657 (233,7)	183,847 (274.4)
H-Rs. rubrum	224,727 (207.1)	242,666 (173.0)	196,365 (123.6)	186,198 (149.6)	251,600 (161.6)
P-Rp. palustris	197,701 (128.7)	134,094 (290.2)	334,000 (359.4)	147,878 (128.7)	149,814 (105.5)
H-Rp. palustris	208,315 (206.9)	129,440 (546.3)	308,802 (213.4)	133,207 (97.4)	167,285 (116.4)
E. coli	219,492 (148.1)	137,292 (94.1)	141,386 (242.8)	107,504 (81.7)	154,807 (27.2)

*The conditions employed were the same as those described in Table 3, except that P-Rs. rubrum PS-60 fraction was added at a final concentration of 400 μg protein per assay mixture. Figures shown in parentheses are percent incorporation using the values shown in Table 3 as 100%.

Table 7. The effect of H-Rs. rubrum PS-60 fraction on the in vitro protein synthesizing activity*

Ribosomes	S-100 P-Rs. rubrum	H-Rs. rubrum	P-Rp. palustris	H-Rp. palustris	E. coli
P-Rs. rubrum	149,885 (489.2)	162,649 (156.8)	183,831 (268.5)	147,339 (300.5)	152,686 (185.5)
H-Rs. rubrum	227,167 (209.4)	219,139 (156.3)	219,908 (138.5)	200,473 (166.4)	148,024 (95.0)
P-Rp. palustris	203,162 (132.3)	172,708 (373.6)	137,050 (147.5)	182,275 (158.7)	196,464 (138.4)
H-Rp. palustris	249,209 (247.5)	105,762 (443.9)	244,807 (169.2)	150,576 (110.1)	163,760 (113.9)
E. coli	273,708 (184.6)	142,807 (97.9)	107,581 (184.7)	140,619 (106.9)	189,221 (33.2)

*The conditions were the same as those described in Table 3, except that H-Rs. rubrum PS-60 fraction was added at a final concentration of 400 µg protein per assay mixture. Figures shown in parentheses are percent incorporation using the values shown in Table 3 as 100%.

Table 8. The effect of P-Rp. palustris PS-60 fraction on the in vitro protein synthesizing activity*

Ribosomes	S-100 P-Rs. rubrum	H-Rs. rubrum	P-Rp. palustris	H-Rp. palustris	E. coli
P-Rs. rubrum	138,127 (450.8)	96,995 (101.0)	131,148 (191.6)	73,676 (150.2)	96,431 (117.2)
H-Rs. rubrum	338,873 (312.3)	173,598 (123.8)	191,464 (120.6)	279,442 (231.9)	192,920 (123.3)
P-Rp. palustris	306,442 (199.5)	181,214 (392.0)	184,320 (198.3)	246,596 (214.6)	271,016 (190.9)
H-Rp. palustris	282,406 (280.4)	203,640 (854.7)	279,751 (193.3)	225,887 (165.1)	280,764 (195.3)
E. coli	290,264 (196.6)	170,948 (117.2)	260,421 (447.2)	125,927 (95.7)	267,998 (46.0)

*The conditions were the same as those described in Table 3, except that P-Rp. palustris PS-60 fraction was added at a final concentration of 400 μg protein per assay mixture. Figures shown in parentheses are percent incorporation using the values shown in Table 3 as 100%.

Table 9. The effect of H-Rp. palustris PS-60 fraction on the in vitro protein synthesizing activity*

Ribosomes	S-100				
	P. Rs. rubrum	H-Rs. rubrum	P-Rp. palustris	H-Rp. palustris	E. coli
P-Rs. rubrum	97,256 (317.4)	173,598 (106.5)	115,720 (169.0)	64,868 (132.2)	85,179 (103.5)
H-Rs. rubrum	227,384 (209.6)	165,205 (117.8)	210,584 (132.6)	224,191 (186.1)	165,012 (106.0)
P-Rp. palustris	245,344 (159.7)	174,713 (377.9)	233,663 (251.4)	180,054 (156.7)	167,767 (118.2)
H-Rp. palustris	240,948 (239.3)	171,648 (720.5)	180,598 (124.8)	203,061 (148.1)	301,793 (209.9)
E. coli	292,602 (197.4)	148,089 (101.5)	137,424 (236.0)	233,355 (177.4)	212,106 (37.2)

*The conditions were the same as those described in Table 3, except that H-Rp. palustris PS-60 fraction was added at a final concentration of 400 μg protein per assay mixture. Figures shown in parentheses are percent incorporation using the values shown in Table 3 as 100%.

Table 10. Summary of the effects of PS-60 fractions on various in vitro protein synthesis systems*

Ribosome	S-100: P-Rs. rubrum	H-Rs. rubrum	P-Rp. palustris	H-Rp. palustris	E. coli
P-Rs. rubrum	450.4 (317.4-544.3)	126.0 (101.0-156.8)	181.5 (96.7-268.5)	204.2 (132.2-300.5)	170.2 (103.5-274.4)
H-Rs. rubrum	234.6 (207.1-312.3)	144.0 (117.8-173.0)	128.8 (120.6-138.5)	183.5 (149.6-231.9)	121.5 (195.0-161.6)
P-Rp. palustris	155.05 (128.7-199.5)	358.4 (290.2-392.0)	239.2 (147.5-359.4)	164.7 (128.7-214.6)	138.3 (105.5-190.0)
H-Rp. palustris	243.5 (206.9-280.4)	640.6 (443.9-854.7)	175.2 (124.8-213.4)	130.2 (97.4-165.1)	158.9 (113.9-209.9)
E. coli	181.7 (148.1-197.4)	102.7 (94.1-117.2)	277.7 (184.7-447.2)	115.4 (81.7-177.4)	35.9 (27.2-46.0)

*These figures are the averages of the figures given in Tables 6 to 9. In parentheses are given the ranges of stimulation or inhibition in the presence of various PS-60 fractions. Homologous systems are underlined.

As presented in Figure 12, the P-*Rs*. *rubrum* PS-60 fraction started to stimulate the homologous P-*Rs*. *rubrum* protein-synthesizing activity at a very low concentration (20 to 100 μg), and this stimulatory effect continued to increase, though at a lower rate, as the concentration of the PS-60 fraction increased. This stimulating effect was not as marked in other phototrophic bacterial systems; a concentration higher than 100 μg/assay was required for the H-*Rs*. *rubrum* (Figure 12a) and the P- and H-*Rp*. *palustris* (Figure 12b) systems. As for the *E*. *coli* system (Figure 13), the P-*Rs*. *rubrum* PS-60 fraction, as expected, inhibited its activity at an almost linear rate.

As shown earlier (Figure 10), a ratio of 2/1 for the P-*Rs*. *rubrum* ribosome/S-100 ratio was required for an optimal protein-synthesizing activity and higher ribosome/S-100 ratios did not increase the activity to any greater extent. In the presence of the P-*Rs*. *rubrum* PS-60 fraction, however, a higher ribosome/S-100 ratio (about 3/1, Figure 14) was found necessary for maximal activities. These results suggested to us that the PS-60 fraction was probably providing a factor(s) present mainly in the S-100 fractions but not in the ribosomes.

Photosynthetic Pigments in Ribosomes

Since the PS-60 fractions were able to stimulate protein-synthesis and since they were enriched in photosynthetic pigments, it was suspected that the pigment

Figure 12. Effect of P-_Rs_. _rubrum_ PS-60 fraction on _in vitro_ protein synthesizing activity of _Rs_. _rubrum_ and _Rp_. _palustris_ systems. The reaction conditions were the same as described in Table 3, except for the inclusion of the indicated amount of P-_Rs_. _rubrum_ PS-60 fraction.

(a) _Rs_. _rubrum_ systems

(b) _Rp_. _palustris_ systems

⊙——⊙ homologous phototrophic systems (i.e., PS-100 + P-ribosomes)

▲——▲ homologous heterotrophic systems (i.e., H-S-100 + H-ribosomes)

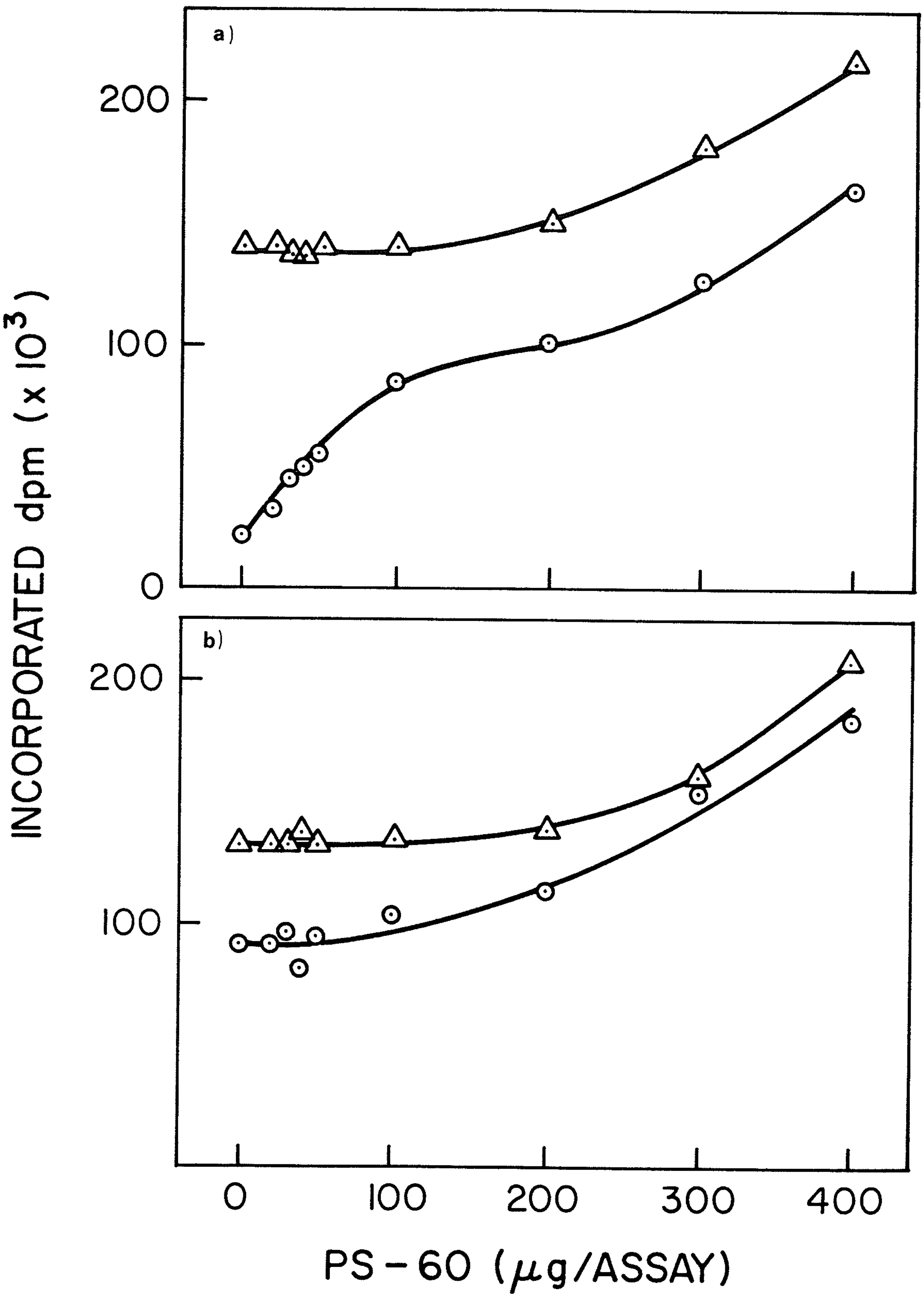
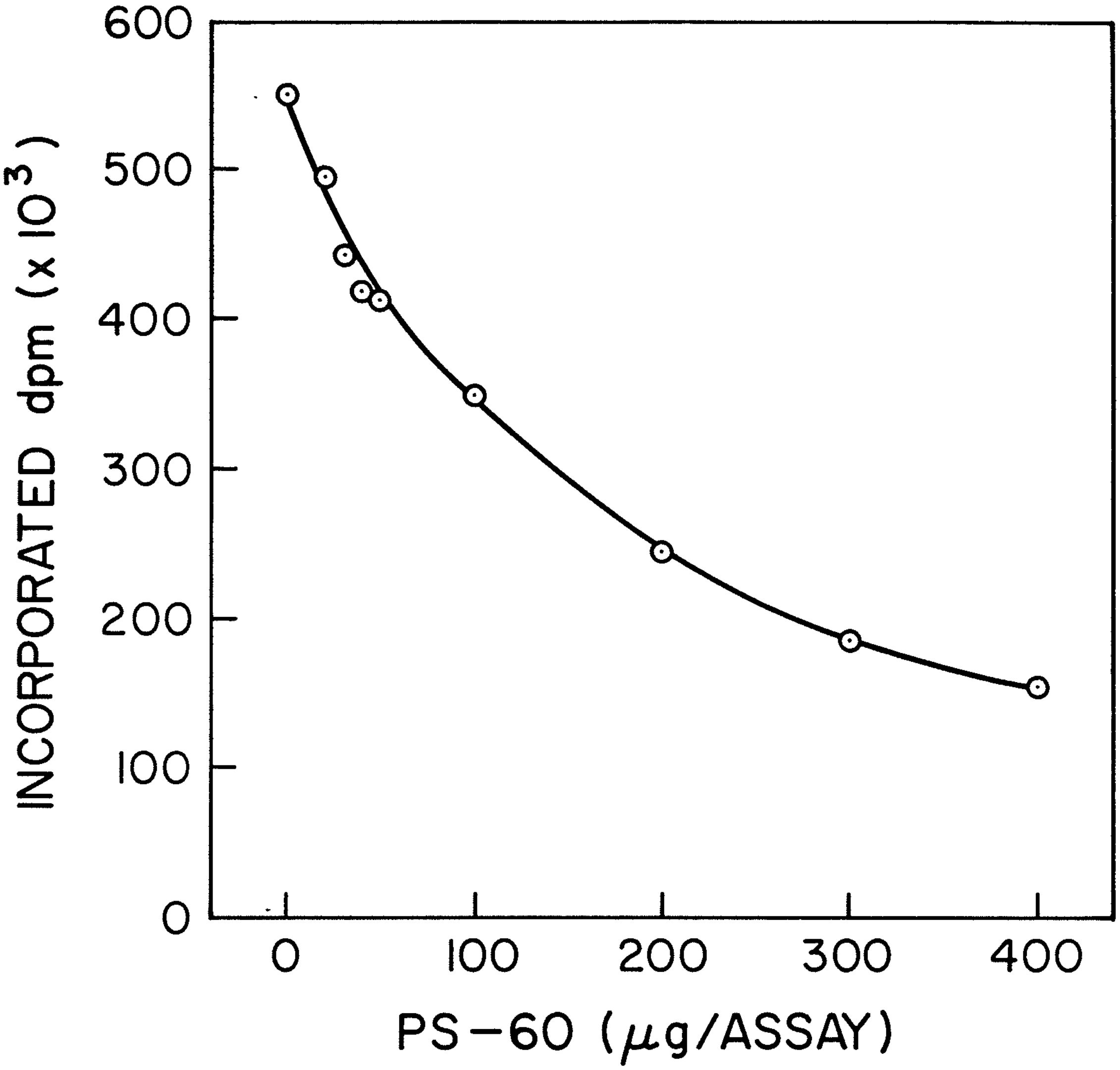

a)
b)
200
100
0
200
100
INCORPORATED dpm (x 10^3)
0
100
200
300
400
PS - 60 (μg/ASSAY)

Figure 13. Effect of P-Rs. rubrum PS-60 fraction on in vitro protein synthesizing activity of E. coli system. The reaction conditions were the same as described in Table 3, except for the inclusion of the indicated amount of P-Rs. rubrum PS-60 fraction.



600
500
400
300
200
100
0
INCORPORATED dpm (x 10^3)
0
100
200
300
400
PS-60 (μg/ASSAY)

content in our protein-synthesizing components (i.e., S-100 and ribosomes) was playing an important role in determining the final (protein-synthesizing) activity. In this study, we had analyzed the pigment content in various ribosome samples, especially in the P-Rs. rubrum ribosomes which might contain different amounts of pigments in different preparations. The pigment content of other ribosomes samples (of the H-Rs. rubrum, the P- and H-Rp. palustris) was, however, generally low and without much variation (Table 11).

It has also been found that the majority of the pigments could be removed from the P-Rs. rubrum ribosomes by centrifugation in a sucrose gradient. The profile of such a gradient is shown in Figure 15. Fractions 10 through 17 which showed a relatively higher A_{260} value were pooled and subsequently used as "centrifuged ribosomes" (Tables 11 and 12). At the bottom of the gradient, we found a heavily pigmented pellet which contained a large amount of photosynthetic pigments, especially bacteriochlorophylls (Table 11). This pellet was resuspended in the standard buffer (designated as the "pigment fraction", Table 11) and was tested for its activity in various protein-synthesizing systems (Table 12).

Similar to the PS-60 fractions (Tables 4 and 5), the "pigment fraction" did not have much protein-synthesizing activity when combined with either ribosomes or the S-100 fractions (lines 1-4, Table 12), and showed a stimulatory effect when added into systems containing (uncentrifuged) P-Rs.

Figure 14. Effect of P-*Rs. rubrum* PS-60 fraction on the ribosome/S-100 ratio.
The reaction was carried out under the same conditions as described in Figure 10, except that 400 μg of P-*Rs. rubrum* PS-60 proteins were included in the mixtures. For comparison the amount of ^{3}H-phenylalanine incorporated by phototrophic *Rs. rubrum* system in the absence of PS-60 fraction (Figure 10) is also included.

▲——▲ PS-60 added

⊙——⊙ PS-60 not added

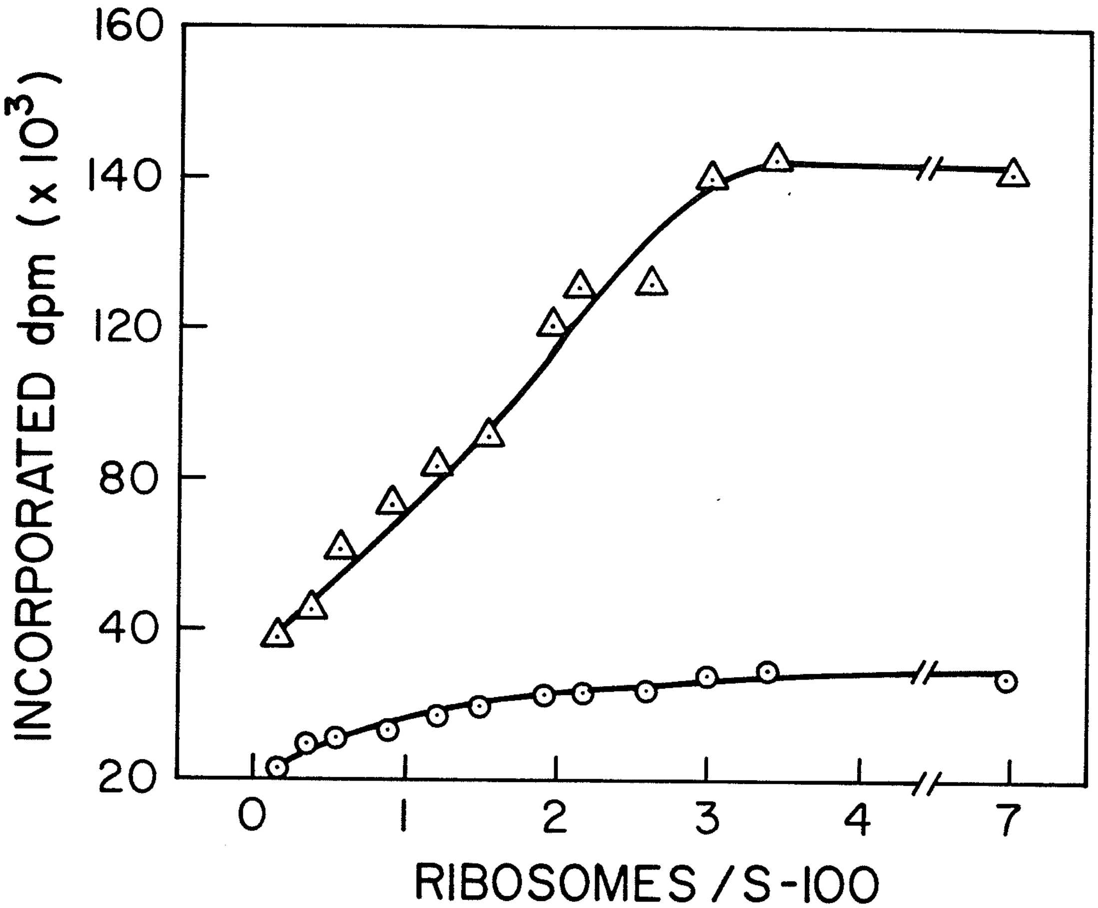

160
140
120
80
40
20
INCORPORATED dpm (x 10^3)
0
1
2
3
4
7
RIBOSOMES / S-100

Table 11. Pigment content in ribosomes and associated fractions*

Cells	Fractions	Pigment content in μg/mg of protein		
		Bacterio-Chlorophylls	Yellow Carotenoids	Red Carotenoids
P-Rs. rubrum	Ribosomes[(a)]	0.01 - 2.56	0	0.001 - 0.110
	"Centrifuged ribosomes"[(b)]	0.007	0	0
	"Pigment fraction"[(c)]	2.01	0.101	0.201
H-Rs. rubrum	Ribosomes[(a)]	0.010	0	0
P-Rp. palustris	Ribosomes[(a)]	0.014	0	0
H-Rp. palustris	Ribosomes[(a)]	0	0	0

*These results represent an average of 4 preparations. For P-Rs. rubrum ribosomes, see text and Figure 15.

(a) Ribosomes without further sucrose gradient centrifugation.

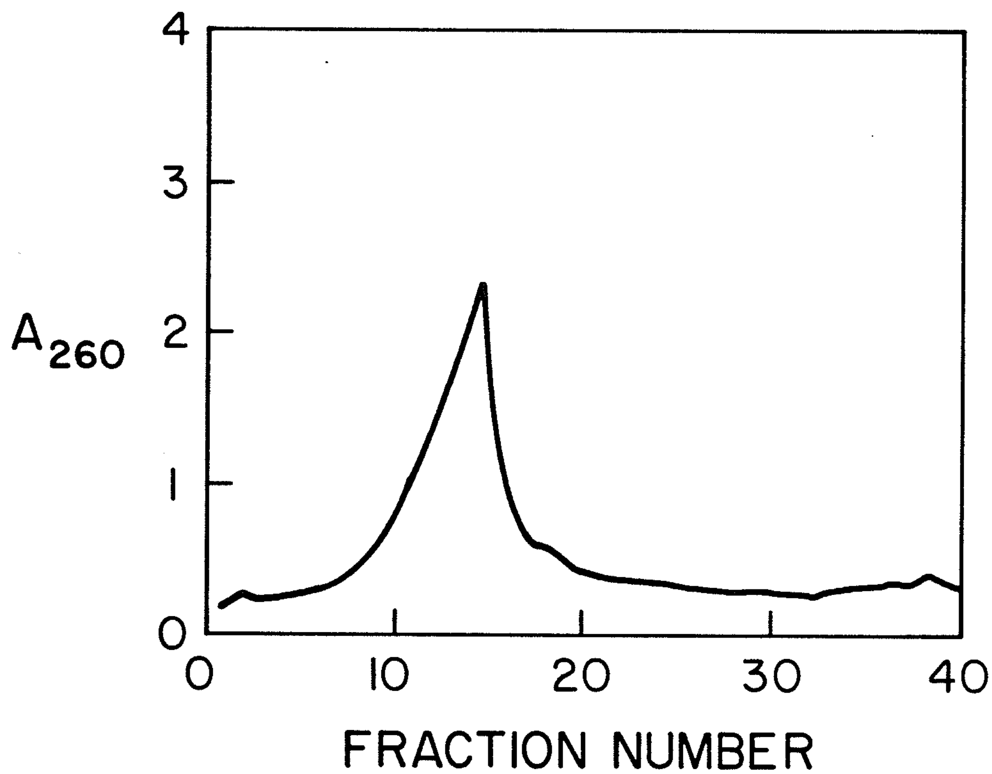
(b) Ribosomes after sucrose gradient centrifugation (see Figure 15).

(c) "Pigmented fraction" was recovered from the bottom of the sucrose gradient after centrifugation (see Figure 15).

In this experiment (also for Figure 15 and Table 12), the pigment content of the P-Rs. rubrum ribosomes before centrifugation was 2.04, 0, and 0.09 μg/mg protein, respectively, for bacteriochlorophylls, yellow and red carotenoids.

Figure 15. Sucrose gradient profile of P-Rs. rubrum ribosomes.

Approximately 200 A_{260} units of purified P-Rs. rubrum ribosomes were applied to a linear 5-30% (w/v) sucrose gradient in standard buffer and centrifuged in a Spinco SW27 rotor at 50,000 x g for 16 hours at 4°C. The fractions were collected as described in "Materials and Methods".



A260
4
3
2
1
0
0
10
20
30
40
FRACTION NUMBER

Table 12. Effect of the "pigment fraction" on *in vitro* assay systems.

Experiment	S-100 (50 µg of protein)	Ribosomes (7 A_{260} units)	"Pigment fraction" (50 µg protein per assay)	dpm of ^{3}H-phenylalanine incorporated
1	-	P-*Rs. rubrum*[a]	+[c]	0
2	P-*Rs. rubrum*	-	+	1,201
3	H-*Rs. rubrum*	-	+	878
4	*E. coli*	-	+	1,180
5	P-*Rs. rubrum*	P-*Rs. rubrum*[a]	-	37,765
6	H-*Rs. rubrum*	P-*Rs. rubrum*	-	88,219
7	*E. coli*	P-*Rs. rubrum*	-	107,121
8	P-*Rs. rubrum*	P-*Rs. rubrum*	+	48,408
9	H-*Rs. rubrum*	P-*Rs. rubrum*	+	154,172
10	*E. coli*	P-*Rs. rubrum*	+	144,249
11	P-*Rs. rubrum*	*E. coli*	-	98,840
12	H-*Rs. rubrum*	*E. coli*	-	156,649
13	P-*Rs. rubrum*	*E. coli*	+	263,664
14	H-*Rs. rubrum*	*E. coli*	+	526,106
15	*E. coli*	*E. coli*	-	525,398
16	*E. coli*	*E. coli*	+	158,464
17	P-*Rs. rubrum*	P-*Rs. rubrum*(s)[b]	-	892
18	H-*Rs. rubrum*	P-*Rs. rubrum*(s)	-	17,301
19	*E. coli*	P-*Rs. rubrum*(s)	-	24,972
20	P-*Rs. rubrum*	P-*Rs. rubrum*(s)	+	1,249
21	H-*Rs. rubrum*	P-*Rs. rubrum*(s)	+	86,911
22	*E. coli*	P-*Rs. rubrum*(s)	+	42,640

(a) P-*Rs. rubrum* - phototrophic *Rs. rubrum* ribosomes before sucrose gradient centrifugation.

(b) P-*Rs. rubrum*(s) - phototrophic *Rs. rubrum* ribosomes after sucrose gradient centrifugation, *i.e.*, "centrifuged ribosomes".

(c) +/- - in the presence or absence of the "pigment fraction", respectively.

rubrum ribosomes (lines 5-10, Table 12) or (P- or H-) *Rs. rubrum* S-100 fractions (lines 11 - 14, Table 12). Its inhibitory effect on homologous *E. coli* systems was rather strong (lines 15 and 16, Table 12). The activity of the "centrifuged ribosomes" was much lower than that of their uncentrifuged counterparts (compare lines 17 - 22 with lines 5 - 10, Table 12), however, addition of the "pigment fraction" was able to stimulate the activity (lines 17 - 22, Table 12).

DISCUSSION

With respect to morphology (Figure 2), growth characteristics (Figure 4), and the absorption spectra of the S-30, PS-60 (Figure 6) and S-100 (Figure 8), the newly isolated Rs. rubrum VF strain cells were very similar, if not identical, to the previously studied wild-type Rs. rubrum cells (Chow, 1976a,b,c, and unpublished data). However, a major difference existed between these two types of cells in their cell-free, protein-synthesizing activity. A detailed comparison is shown in the following table in which the figures are extracted from Table 3 of this thesis (for strain VF) and a previous report (Table 1 of Chow, 1976c, WT stands for the wild-type).

	S-100			
Ribosomes	P(VF)	H(VF)	P(WT)	H(WT)
P(VF)	30,638	96,036	-	-
H(VF)	108,502	140,251	-	-
P(WT)	-	-	92,366	105,524
H(WT)	-	-	169,862	9,516

The very low activity of the wild-type homologous heterotrophic system was believed to be caused by the instability of the wild-type (S-100 and ribosome) fractions which contained very little photosynthetic pigments (Chow, 1976c). The VF heterotrophic fractions, though not containing much more pigments than the wild-type fractions, were rather stable as reflected by their high activities. On the contrary, the VF phototrophic fractions showed a lowered activity than their wild-type couterparts. It has also been demonstrated that the VF phototrophic ribosomes contained a variable amount of pigments (Table 11) whereas the wild type ribosomes were always heavily pigmented (Chow, 1976c). The reason(s) for the variation in pigment content and in protein-synthesizing activity in these two types of cells is still unclear and whether there is a direct relation between these two parameters remains to be seen.

Once we had established the optimal conditions for cell-free, protein synthesis assays in terms of ribosome/S-100 ratio and time kinetics (Figures 10 and 11), more intensive studies were carried out by using not only the *Rs. rubrum* fractions but also those of another facultatively phototrophic bacterium, *Rp. palustris* as well as the intensively studied *E. coli* cells. With various combinations of S-100 and ribosome fractions, we had made several interesting observations (Table 3) one of which is that the homologous systems did not always display the maximal activity, especially for the P-*Rs. rubrum* system, and, to a lesser degree, the P-*Rp. palustris* system. Because of the generally high activities of the various combinations, these systems may, in the future, be employed as a useful tool for analyzing important aspects of protein synthesis in the phototrophic bacteria.

Another point of interest lies in the effects of the PS-60 fractions on the cell-free, protein-synthesizing activity. Regardless of their source, all PS-60 fractions showed an enhanced absorption in the long wavelength region

(800-1000 nm, Figures 6 and 7), indicating an enrichment of specific groups of bacteriochlorophylls (Clayton, 1966). Moreover, as summarized in Table 10, the stimulatory effect of these fractions was far stronger on the homologous phototrophic systems than on the homologous heterotrophic systems, and they even rendered an inhibition on the pigment-free E. coli system. At present it is still unclear whether these (stimulatory and inhibitory) effects are caused by the pigments *per se* or by the proteins (or other unidentified components) associated with these pigments. However, the change in the optimal ribosome/S-100 ratio from 2/1 in the absence of the PS-60 fraction (Figure 10) to 3/1 after the addition of the PS-60 fraction (Figure 14) has led us to suspect that the PS-60 fractions were supplying certain component(s) present, but not in adequate amounts, in the bacterial S-100 fractions. This component(s), if it exists, must also be associated with (at least) the phototrophic Rs. rubrum ribosomes, since "pigments" isolated from them had a similar stimulatory and inhibitory effect (Table 12).

Somewhat related to our findings, Ludden and Burris (1976) have recently demonstrated that Rs. rubrum chromatophores are able to stimulate the (Rs. rubrum) nitrogenase activity *in vitro*. Attempts to isolate and purify the stimulatory component(s) from the PS-60 and ribosome fractions are currently being made in this laboratory.

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