THE CHARACTERIZATION OF VALYL TRANSFER RNA SPECIES FROM GREEN AND APLASTIDIC *EUGLENA GRACILIS* STRAIN Z

by

ALVIN A. STEIN

A dissertation submitted to the Graduate Faculty in Biology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1975

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

June 4, 1975
Date

Chairman of Examining Committee
Professor M. Selsky

June 6, 1975
Date

Executive Officer
Professor Louis G. Moriber

Prof. M. Belsky — Brooklyn College, Institution

Prof. C. Withner — Brooklyn College, Institution

Prof. C. Burdick — Brooklyn College, Institution

Prof. H. Lyman — SUNY, Stony Brook, Institution

Prof. G. Russell — Adelphi University, Institution

The City University of New York

ABSTRACT

Characterization of Valyl Transfer RNA Species from Green and Aplastidic Euglena gracilis Strain Z.

Utilizing optimal conditions for specific aminoacylation of valine to tRNA from green and aplastidic Euglena gracilis, and reversed phase chromatography (RPC-5), the isoaccepting species of valyl tRNA were characterized. Results indicated that there were four isoaccepting species for valine in wild type Euglena, one of which appeared to be chloroplast in origin since it was not found in aplastidic cells.

In aminoacylation reactions, synthetases derived from purified chloroplasts could only charge tRNA derived from chloroplast containing cells, inferring a specificity between chloroplast valyl tRNA and its cognate chloroplast synthetase. In addition, there were considerable quantitative differences noted among the non-chloroplast valyl tRNA species when light-grown, dark adapted wild type cells and a streptomycin-bleached mutant were compared by RPC-5 analysis.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF ILLUSTRATIONS

Page

INTRODUCTION

The development of the chloroplast is a complex, multifaceted process which, in the case of some organisms, involves massive and rapid structural and biochemical changes. For example, in the green flagellate alga Euglena gracilis, the conversion of the precursor body, the proplastid, to a fully developed chloroplast involves a 60-fold increase in volume requiring less than 72 hours. Concurrent with this increase in size are the formation and organization of numerous internal membranes and the synthesis of many diverse molecules.

Although a large body of knowledge exists concerning events leading to the formation of a fully developed chloroplast including structural and biochemical changes, very little is known about the mechanisms involved in initiating and regulating these processes. Among major questions requiring elucidation are: What is the precise role of, and what are the specific types of, light required for induction of chloroplast development? Where does the genetic information for the biosynthesis of a chloroplast originate? Where are the precursor components for the construction of the chloroplast synthesized? What are the regulatory mechanisms that integrate chloroplast development with other cellular functions?

The phenomenon of cytoplasmic inheritance was first reported by Correns in 1909 (as stated in Sager, 1972) and has come to mean to many that the cytoplasmic organelles may possess

a genetic system independent of the nucleus. Correns found that mutants of Mirabilis which contained abnormal chloroplasts showed segregation patterns which differed from the pattern of simple Mendelian segregation. The abnormal chloroplasts were transmitted by a mechanism that appeared to be dependent upon the transfer of the cytoplasm and which strongly suggested the presence of an extranuclear genetic system. Many other reports in the literature as well (i.e., Rhoades' study of iojop in maize) led to a revival of interest in the extent to which chloroplasts are autonomous.

It was critical to know if the chloroplast controlled all the aspects of its destiny, or was dependent upon extra-organellar influences for its survival.

In 1966 Schiff and Epstein proposed three general models describing possible degrees of chloroplast autonomy suggested by a variety of observations.

The first model proposed that the chloroplast was completely dependent upon the nuclear genome for every aspect of its construction and that the chloroplast was produced de novo in each generation.

At the other extreme an alternate model conceived of the chloroplast as a completely autonomous organelle which contained the necessary genetic information and synthesized all the molecules and structures necessary for its self-perpetuation. Finally, the more "moderate" model proposed that there was an

interaction between the nuclear and chloroplast genomes and accepted the possibility that the chloroplast must be capable of synthesizing some of the components for its own continuity and function. However, this hypothesis implied that the chloroplast depended to some extent upon the nuclear genome for certain aspects of its survival. A later modification of this model was offered by Schiff in 1971, in which he suggested that although it had its own unique genome, the chloroplast had become dependent upon the nucleus for one or more products required for its normal construction and functioning. Thus, a nuclear mutation could eliminate this product and lead to abnormal chloroplast development.

Is the chloroplast autonomous? To demonstrate a complete or even partial autonomy it would be necessary to demonstrate that the chloroplast possessed:

a) Its own unique DNA;

b) A mechanism for the replication of this DNA, i.e., a chloroplast specific, DNA dependent DNA polymerase;

c) A mechanism for the transcription of this DNA, i.e., a chloroplast specific, DNA dependent RNA polymerase;

d) A protein synthesizing system including ribosomes, transfer RNAs and aminoacyl transfer RNA synthetases; also the ability to use the aforementioned to code unambiguously for, synthesize, and regulate at least part of its own biogenesis and functioning.

It is generally recognized that the chloroplast does indeed

possess all of the characteristics outlined in (a-d). The literature is voluminous in this area and has been thoroughly reviewed by Kirk and Tilney-Bassett (1967), Tewari (1971), Whitfield (1973), and Woodcock and Bogorod (1973). In addition, the bibliography lists many additional references which also support (a-d). Since the literature survey covered an extremely wide range of photosynthetic organisms (from algae to higher plants) it is assumed that the presence of all these chloroplast components is universal.

The identification of the site(s) containing genetic information for the synthesis of chloroplast specific or localized components is still debatable. The evidence for informational content of chloroplast DNA with respect to its encoding sequences of the other chloroplast nucleic acids has come primarily from hybridization studies. Studies of the coding sites or sites of synthesis of chloroplast proteins have been facilitated by the use of antibiotics of defined specificity. In addition, workers have used physiological shifts from growth in dark to growth in light, <u>in vivo</u> and <u>in vitro</u> incorporation studies and the utilization of mutants with biochemical or structural defects to obtain some of the findings that will be discussed in the following paragraphs.

There is an accumulating body of evidence which comes primarily from DNA-RNA hybridization studies that chloroplast DNA can code for chloroplast ribosomal RNA. Scott and Smillie (1967) found that chloroplast ribosomal RNA hybridized with

chloroplast DNA in Euglena to the extent of 1% at saturation. In the presence of excess unlabelled total rRNA from wild type cells, the extent of DNA-RNA hybridization was somewhat reduced. If chloroplast rRNA was added to the hybridization mixture the extent of hybridization was greatly lowered. Addition of unlabelled RNAs from dark grown Euglena, which contained no formed chloroplasts, did not reduce the formation of DNA-RNA hybrids, an indication that the binding of chloroplast rRNA to chloroplast DNA was specific.

Stutz and Rawson (1970) found that alkaline denaturation of Euglena chloroplast DNA resulted in the formation of two strands of different base composition and buoyant density. Hybridization studies with each strand indicated that the heavy strand bound nine times as much rRNA than did the lighter strand. Additional studies by these workers indicated that chloroplast DNA did not hybridize with 80S (cytoplasmic) rRNA.

Similar findings have been reported by Tewari and Wildman (1970) for tobacco where they found that the binding of chloroplast rRNA to chloroplast DNA varied from 1.4% - 1.7%, depending upon the method of isolation of the ribosomes. Chloroplast DNA was tested for homology with rRNA from cytoplasmic ribosomes and was found to hybridize to the extent of only 0.05%. Ingle et al (1971) reported for Swiss chard that chloroplast DNA hybridized with chloroplast rRNA five times more effieiently than it did with cytoplasmic rRNA.

In each of those two studies two different phenomena became obvious. First, although the hybridization of chloroplast rRNA to chloroplast DNA was considerably more efficient than that of the hybridization of cytoplasmic rRNA to chloroplast DNA, a small amount of the latter type of hybridization was always detectable. Second, there was a considerable amount of hybridization observed between nuclear DNA and chloroplast rRNA in both higher plants and algae. To explain the latter finding, Tewari and Wildman (1970) proposed the existence of two classes of chloroplast ribosomes, one of which was nuclear in origin - a model certainly in need of substantial proof. Ingle et al (1971) found in four different plants (wheat, Swiss chard, onion, and artichoke) that cytoplasmic rRNAs hybridized principally with a DNA of buoyant density 1.705g/ml and concluded that this represented nuclear DNA enriched in ribosomal RNA cistrons. Chloroplast and cytoplasmic rRNAs hybridized to about the same extent with this DNA. In a similar experiment with chloroplast DNA, they found that the chloroplast rRNA was much more effective in binding to it than was cytoplasmic rRNA. These authors, by comparing interspecies hybrids of cytoplasmic rRNA and nuclear DNA among the four previously mentioned plants, found a lack of species discrimination and concluded that the stability of these hybrids must be determined before any definite conclusions about nuclear origin of chloroplast rRNAs could be made. Stutz and Rawson (1970) found that in Euglena the heterologous hybrids are less stable than the homologous ones and concluded that this instability could be the result of pairing of similar but non-

identical sequences. Thomas and Tewari (1974) subsequently demonstrated that chloroplast rRNA from peas hybridized with chloroplast DNA from bean, lettuce, spinach, corn and oats to about the same degree. This indicated that the base sequences of these genes were very similar in all of these plants, and could be explained by the fact that, in general, ribosomal cistrons have remained more constant in evolution than has the majority of the DNA of the organism.

Stutz and Vandrey (1971) extracted chloroplast DNA from highly purified Euglena chloroplasts and obtained in addition to the usual band at 1.685 g/ml, a satellite band at 1.701 g/ml. Using both types of chloroplast DNA in hybridization experiments with purified chloroplast rRNA, they reported a six-fold increase in specific hybridization with the 1.701 g/ml fraction in contrast with that found with the 1.685 g/ml fraction. They concluded that there was a region of chloroplast DNA specifically enriched for chloroplast rRNA cistrons which was probably part of the 40μ circular DNA previously reported to be present in Euglena chloroplasts by Manning et al (1971).

Very little work has been reported on hybridization of chloroplast tRNAs to chloroplast DNA. Tewari and Wildman (1970) established that 0.4% - 0.7% of chloroplast DNA was involved in coding for chloroplast tRNA based on saturation-hybridization studies of total chloroplast tRNA from tobacco leaves with DNA from tobacco chloroplasts. Chloroplast rRNA did not compete for the tRNA sites on the chloroplast DNA, whereas chloroplast tRNAs

showed the expected competition. These hybridization values are equivalent to 4.4 - 7.9 x 10^5 daltons (M.W. of tobacco chloroplast DNA being 1.1 x 10^8 daltons). This amount of DNA would theoretically be sufficient to code for 20-30 tRNAs of molecular weight 25,000, assuming one tRNA/amino acid (exclusive of isoaccepting species).

Williams and Williams (1970) reported that chloroplast leucyl tRNA of Phaseolus hybridized to the extent of 0.025% of the chloroplast DNA. Assuming a molecular weight of 10^8 daltons for Phaseolus chloroplast DNA this represents a molecule of 25,000 dalton size, or the equivalent of one leucyl tRNA. Earlier, Schweiger (1967) had shown that enucleated Acetabularia could incorporate ^{14}C uracil into tRNA, which indicated that the cistrons in the chloroplast DNA might be transcribed.

The problem of determining the site of information for the synthesis of chloroplast proteins has been a formidable one. Most of the evidence reported appears to be of an indirect nature and deals primarily with the site of synthesis rather than the source of genetic information. Studies utilizing isolated chloroplasts have not provided much unambiguous information due to the extremely low rates of incorporation and the large number of proteins synthesized simultaneously. This often makes it difficult to identify specific proteins. By the use of "70S and 80S antibiotics" in conjunction with shifts from growth in the dark to growth in the light a considerable amount of information has

been obtained. The use of nuclear and chloroplast mutants should enable one to identify the codon source of such chloroplast specific proteins.

Goodenough et al (1972) used antibiotics and nuclear mutants in Chlamydomonas to determine the sites of chloroplast component synthesis. They found that chloroplast ribosome production in wild type appeared to be insensitive to growth conditions and photosynthetic capacity of the chloroplast since cells grown phototrophically, mixotrophically or heterotrophically (in the dark) showed no change in chloroplast ribosome levels. This was also found to be true in a large number of mutant strains blocked in some phase of photosynthesis. One mutant, however, (ac-20) was exceptional in that it possessed low levels of chloroplast ribosomes when grown phototrophically and even lower levels when grown mixotrophically (up to a 90% reduction). Accompanying this reduction in chloroplast ribosomes were reductions in the levels of ribulose 1,5 diphosphate carboxylase, cytochrome 559, and Q (the flourescence quencher). In addition, plastid membrane organization and pyrenoid formation were affected. However, many other chloroplast specific proteins were unaffected. A summary of these findings is presented in Table B. These data were reinforced by experiments with light grown, wild type cells treated with chloramphenicol and spectinomycin (70S inhibitors) which gave results almost identical with those findings obtained with mixotrophically grown ac-20 cells. It was concluded that the components affected by low levels of chloroplast ribosomes

or 70S inhibitors were synthesized in chloroplasts. Cycloheximide (an 80S inhibitor) appeared to have an inhibitory effect on RuDPCase levels as did the 70S antibiotics, and it was suggested that the synthesis of this enzyme was more complex, perhaps, involving cytoplasmic as well as chloroplast ribosomes.

In Euglena, Smillie (1967) found that the synthesis of RuDPCase and NADP-glyceraldehyde 3-phosphate dehydrogenase were inhibited by chloramphenicol, and stimulated by cycloheximide. Smillie and Scott (1970) reported that there were two fructose 1,6 diphosphate aldolases in Euglena, one cytoplasmic and the other chloroplast in localization. The former was inhibited by cycloheximide and the latter by chloramphenicol. The effect of these two inhibitors on cytochrome 552 and ferridoxin NADP reductase was less conclusive, since although chloramphenicol inhibited both, cycloheximide also had an inhibitory effect (at concentrations higher than that required to inhibit cytoplasmic enzymes).

The most definitive evidence for the origin of the genetic information encoding for a chloroplast protein comes from the work of Wildman's laboratory. In a study by Kawashima and Wildman (1972), the authors present genetic evidence that the small subunit of Fraction I protein (ribulose 1,5 diphosphate carboxylase) of tobacco is inherited in a Mendelian fashion, since it is transmitted by both pollen and egg parent and appears to be coded for by nuclear DNA.

In a subsequent investigation Chan and Wildman (1972) studied the tryptic peptides from the large subunit of Fraction I protein obtained from a number of Nicotiana species, including one with an altered peptide. The altered peptide appeared in hybrids only when contributed by the female parent and it was concluded that the maternal mode of inheritance of this special peptide implied that the information for it resided in chloroplast DNA.

Kung et al (1972) showed that the inheritance of a specific Photosystem II chlorophyll-protein from Nicotiana was independent of the maternal parent and thus they concluded that this protein was coded for by nuclear DNA. Bourque and Wildman (1973) later reported that there were at least two electrophoretically distinguishable differences among the basic proteins of the 50S chloroplast ribosome subunit of two Nicotiana species. Neither polypeptide exhibited maternal inheritance in interspecific hybrids and they concluded that these proteins were also coded for by nuclear DNA.

Mets and Bogorod (1972) reported two erythromycin-resistant mutants of Chlamydomonas reinhardi, with altered proteins localized in the 52S ribosomal subunits of the 70S ribosome. One mutation was inherited in a Mendelian fashion (indicating that the coding information resided in the nuclear DNA) while the other mutant showed uniparental inheritance (indicating chloroplast DNA as the site of genetic information). Additional evidence

since then has indicated an interaction between the nuclear and chloroplast genetic systems in the biogenesis of chloroplast ribosomes.

It would appear, then, that the two extreme models describing chloroplast autonomy, namely complete dependence on the nuclear genome or complete self-reliance on the chloroplast genome can be rejected. Instead it seems that chloroplasts rely on the nuclear genome and its protein synthesizing machinery as well as their own. Recently, evidence has appeared which suggests a bi-directional mutualistic existence between the nuclear and chloroplast genomes. For example, Blamire et al (1974) described experiments in which chloroplast protein synthesis was implicated in the regulation of nuclear DNA replication in Chlamydomonas reinhardi. Utilizing a group of 70S-specific antibiotics they found that each of these drugs inhibited nuclear DNA replication at concentrations which had no effect on chloroplast DNA replication.

Hoober and Stegman (1973) studied the synthesis and regulation of a major chloroplast membrane polypeptide which was formed on 80S ribosomes in the cytoplasm of Chlamydomonas reinhardi Mutant y-1. These authors proposed that a regulatory protein synthesized in the chloroplast inhibits the synthesis of a mRNA (site of its transcription possibly in the chloroplast), which codes for this chloroplast membrane polypeptide. This regulatory protein was believed to be synthesized on chlorpplast ribosomes since its production was inhibited by chloramphenicol.

The activity of this regulatory protein was affected by the conversion of protochlorophyllide to chlorophyll, and these authors hypothesized that protochlorophyllide may act as a corepressor.

Thus it is apparent that chloroplast autonomy and development are closely intermeshed with the activities of other cellular components and that these interactions are multidirectional. That much more unequivocal information is required before any theory concerning chloroplast biogenesis and autonomy could be proven is indeed an understatement.

The green flagellate Euglena gracilis has been chosen as experimental material here because it offers many advantages for the study of chloroplast development. It can be grown rapidly in axenic culture, producing relatively large quantities of biological material. Its chloroplasts, by growth of the organism in the dark for several generations, can reform precursor bodies - the proplastids - which contain very little internal structure, no detectable chlorophyll, and no photosynthetic capacity. Re-exposure of these dark grown cells to the light results in the reformation of fully developed and functional chloroplasts. This entire developmental process can occur in the absence of growth by maintaining cells on a "resting" medium developed by Stern et al (1964), which makes it possible to study the developmental processes under conditions in which variables introduced by cell division are eliminated. In addition, Euglena can be permanently bleached by growth on streptomycin (Provasoli et al, 1948), exposure to ultraviolet radiation (Lyman et al, 1959),

or growth above 32° C (Pringsheim and Pringsheim, 1952). Each of these treatments can be carried out without effect on cell viability. Aplastidic cells could thus be utilized to possibly ascertain the physical location and sites of genetic information of numerous chloroplast specific components. A detailed description of the developmental events leading to the formation of a mature chloroplast in Euglena gracilis has been described by Schiff (1970) and thus only the major features pertinent to this study will be discussed here.

Dark grown cells of Euglena contain proplastids about 1-2μ in diameter, bound by a membrane two layers in thickness. (Klein et al, 1972). Ribosomes (68S), smaller than those in the cytoplasm, are present in the proplastid. The proplastid contains prolamellar bodies which are composed of tubular elements and as many as three per proplastid have been observed. Several thylakoid membranes are attached to the prolamellar body and cytoplasmic intrusions into the proplastid are extensive. Mitochondria are often observed in close association with the proplastids. Upon exposure to light, plastid membrane formation becomes evident in 30 minutes, and thylakoids form and increase in number until a maximum is reached. After four hours of development in the light, the thylakoids fuse into stacks called lamellae, and lamellar formation is linear from about 14 hours to 72 hours (Ben Shaul et al, 1964). At about 24 hours there is a tremendous expansion in the length of the proplastid and by 24-36 hours the plastid is elongate. The pyrenoid (site of

paramylon synthesis) is formed during 14 to 24 hours of development, and by 72 hours the development of a mature chloroplast is complete (Ben Shaul et al, 1964).

One of the first physiological changes detected upon light exposure was the conversion of protochlorophyll(ide) to chlorophyll(ide) which was thought to be the inductive signal to the cell for the onset of chloroplast development (Schiff et al, 1961). In addition, there was a large increase in oxygen uptake, most of which was irreversible, with a small reversible increase attributed to photorespiration. The increase in respiration (perhaps mitochondrial) was thought to be able to provide the energy and intermediates needed for chloroplast development. As chloroplast development proceeded, the photorespiratory component of oxygen uptake decreased until at four hours there was a net increase in oxygen evolution. It was therefore suggested that the beginning of photosynthesis occurred at approximately four hours after light exposure (Schiff et al, 1963). These findings were in good agreement with studies of CO_2 fixation (Stern et al, 1964) and with the time of formation of the first photosynthetic lamellae. Chlorophyll synthesis and photosynthetic ability increased very slowly from 0 to 12 hours, and both rose sharply from about 14 hours until completion of development at 72 hours, an observation which correlated well with the increase in lamellar formation reported during this period (Stern et al, 1964). A number of proteins, apparently chloroplast localized, appeared or increased in amount during this period (Lewis et al, 1965).

Various photosynthetic enzymes and related compounds such as TPN triose phosphate dehydrogenase (Hudok and Fuller, 1965), RuDPC (Fuller and Gibbs, 1959), TPN-transhydrogenase (Lazzarine and San Pietro, 1963) and cytochrome 552 (Perine et al, 1964) also appeared or increased in amount during this period.

The findings by Holowinsky and Schiff (1969) that a brief period (about 90 minutes) of low level illumination followed by a dark period would eliminate the usual lag period in chlorophyll formation (potentiation) when cells are returned to light aided in the study of the early stages of chloroplast development. Since the optimal length of the dark period for potentiation was equal, in effect, to 12 hours of continuous light, it was assumed that the short exposure to light allowed those events which would ordinarily take place in the light to also take place in its absence. This allowed a separation of the early inductive phase from the later one since the latter could be studied in the dark. The action spectrum for induction during pre-illumination indicated that the protochlorophyllide to chlorophyllide conversion actually triggered the process.

Two separate findings have indicated that the developing chloroplast does not obtain energy and precursors from its own photosynthesis. Stern et al (1964) showed that considerable chloroplast development took place prior to the development of full photosynthetic competence and that the optimal light intensity for development occurred at 150 foot-candles, a level far below the 2,000 foot-candles required for saturation intensity

for photosynthesis. Schiff et al (1968), using DCMU, a specific inhibitor of electron flow from PSII, found that it completely blocked CO_2 fixation but only diminished chlorophyll synthesis by 20%. Plastids that developed in the presence of DCMU reached normal size and had chloroplast structures, pigments, and the capacity for photosynthesis even when DCMU blocked photoreduction and noncyclic phosphorylation. This suggested that the reducing power and ATP required for these processes must be supplied to the developing plastid by the rest of the cell. These results were in good agreement with the known fact that many organisms such as Chlamydomonas, Chlorella, and some higher plants do not require light for chlorophyll synthesis or chloroplast development (Bishop, 1966).

Zeldin and Schiff (1967) have shown that light induction of chloroplast development resulted in incorporation of labelled RNA precursors into both chloroplast and non-chloroplast RNAs and they suggested that RNA synthesis in both chloroplast and the cytosol was activated by light. These results were consistent again with the idea that chloroplasts could obtain at least some of their building blocks from the rest of the cell. These results also suggested the possibility that the signals that were involved in chloroplast development must affect, in addition, sites outside the chloroplast.

Thus in Euglena the greening situation is reminiscent of adaptive enzyme formation. Presented with light as a substrate,

dark grown cells of Euglena produce the necessary machinery to use this light, and this induction and subsequent chloroplast development involve the coordination of many diverse activities. The question to be posed is, "How are these events regulated?".

The conversion of the proplastid to a fully mature chloroplast involves drastic changes in cellular metabolism. There appears to be little doubt that these developmental events require the synthesis of many new molecules such as RNA and protein. Concurrent with these syntheses the cessation of synthesis of other cellular components probably occurs. Therefore, the regulation of these events must involve mechanisms dealing with the coordinated syntheses of groups of molecules in contrast to the synthesis of a single molecule which could be specifically regulated.

One of the most appealing classes of molecular candidates for this critical multiregulatory role in proplastid differentiation at the "macroregulation" level is tRNA. Mechanisms for macroregulation by tRNA can be visualized at the transcriptional level as well as the translational level, with controls likely to be operating at both levels. In other systems this has been amply demonstrated. For example, Kaplan et al (1973) reported that in an E. coli mutant in which RNA synthesis was dependent upon protein synthesis (a stringent response) this dependency was due to the absence of charged tRNAs. They showed that the inhibition of protein synthesis by the use of antibiotics which act at the ribosomal level uncoupled the stringent response and

allowed RNA synthesis to proceed in the absence of protein synthesis. They suggested that this uncoupling was a result of the accumulation of charged tRNAs whose use in protein synthesis was spared by the presence of the antibiotics.

At the translational level tRNA was one of the most likely molecules to be involved in macroregulation because of the unique features of its having multiple species for each amino acid and its uniform requirement for the synthesis of proteins. Quantitative and qualitative changes in isoaccepting tRNAs during development have implicated these molecules as possible regulatory agents in a wide variety of biological phenomena and organisms. For example, changes in tRNA profiles have been observed by Sueoka and Sueoka (1969) upon infection of E. coli by T-2 phage. Selective qualitative and quantitative changes in tRNAs have been observed under different growth conditions in Bacillus subtilus by Vold (1972). Taylor et al (1967) found differences in the tRNA patterns of Erlich ascites tumor and mouse sarcoma-1 cells from those of the corresponding normal tissues. Quantitative changes in the levels of tRNA's have been observed by Merrick and Dure (1972) during the developmental stages in cotyledon embryogenesis and germination in cotton. In Euglena, Barnett et al (1969) and Reger et al (1971) have demonstrated the appearance of new tRNA species correlated with the development of the chloroplast in that genus.

In view of these observations, it is conceivable that tRNA plays a significant role in regulating the interactions between

the chloroplast, nucleus, and other cytosol genomes that may be involved in the development of a chloroplast in Euglena gracilis.

With this in mind, a study was undertaken of the characterization of the isoaccepting valyl tRNA species of Euglena gracilis as to localization, specificity, and qualitative and quantitative differences - if any - between aplastidic and plastid-containing cells.

MATERIALS AND METHODS

I. Source and Growth of Organisms

A. Source of Organisms

Euglena gracilis Klebs, variety bacillaris, Pringsheim, strain Z were obtained originally from a culture kindly supplied to Dr. M. I. Selsky by Dr. Harvard Lyman. These cells (light grown) served as a source of chloroplasts or plastid-derived components. The bleached mutant ZUV was derived by Dr. M. I. Selsky from wild type cells by irradiation with ultraviolet light and has been maintained in continuous culture since 1973. No chlorophyll could be detected in ZUV cells by analysis of acetone extracts, and electron micrographs of sections of such cells indicated no plastid structures were present. A streptomycin bleached mutant of the wild type culture, denoted as SM-57 and maintained in continuous culture since 1971 was also used in these studies. There was no evidence of plastid structure by study of electron micrographs of thin sections of these cells nor was chlorophyll detected in acetone extracts of them. Both bleached cell types (ZUV, SM-57) served as sources of non-chloroplast components. In addition, wild type cells grown continuously in the dark and called "dark adapted" were also used in this study.

B. Growth of Organisms

All cell types were grown axenically in the light with

continuous gyratory agitation at 26°-29°C in 2-liter Ehrlenmeyer flasks containing one liter of medium. Continuous illumination was provided by four 40 watt General Electric F72T12OW cool white lamps placed 75 cm above the surface of the shaker where the light intensity, as measured by a YSI (Yellow Springs Instrument) light meter was 3.5×10^5 ergs cm^{-2} sec^{-1}. The medium used was that of Hutner as cited, and modified by Greenblatt and Schiff (1959). The composition of this medium is shown on Table A.

II. Preparation of tRNA

Transfer ribonucleic acid was isolated by a modified version of the method of Holley et al (1965). All procedures described in detail below were carried out at 0°-4° C.

Six liters of Euglena cells in late logarithmic or early stationary phase (pH of culture: 6.8 - 7.2) were filtered through several layers of cheesecloth to remove dead, clumped cells and mucilaginous materials. The filtrate was centrifuged for five minutes at 5,000 x g in a Sorvall centrifuge (RC2B), the supernatant solution discarded, and the pellet washed in buffer A (0.05M Tris-HCl pH 7.6, 0.005M $MgCl_2$). The suspended cells were then centrifuged for ten minutes at 5,000 x g, the supernatant solution discarded, and the entire wash procedure repeated a second time. The resultant pellet was then washed two times in buffer B (0.1M Tris-HCl pH 9, 0.2M NaCl) and weighed. The alkaline pH of buffer B was needed to optimize deacylation of the tRNA and the high salt was added to inhibit nuclease activity. The pellet (average wet weight: 30 gms)

was resuspended in 80 ml of buffer B and the cells were broken in a cooled French Pressure Cell at approximately 10,000 psi. The lysate was collected directly into 80 ml of an aqueous phenol solution (80% redistilled phenol, 20% water) which contained 0.2M NaCl with respect to the aqueous phase. The resultant deproteinized lysate was stirred for 20 minutes at 0° C, followed by 15 minutes of stirring at room temperature (23°-25° C). The mixture was centrifuged for ten minutes at 10,000 x g and the aqueous phase was carefully removed to avoid disturbance of the phenol layer or interface. An equal volume of buffer B was added to the remaining phenol phase and the stirring and centrifugation procedures repeated. The aqueous phases were pooled, additional fresh phenol solution added, and the stirring and centrifugation steps repeated for a third time. The final aqueous layer was collected and to it was added 1/10 volume of 10% NaCl and 2.5 - 3.0 volumes of absolute ethanol (high-salt-ethanol precipitation). Such mixtures, here and elsewhere described, were always vigorously agitated and maintained at -20° C for a minimum of one hour. The precipitate which formed was centrifuged at 10,000 x g for ten minutes. The resultant pellet was dissolved in 0.01M Tris-HCl (pH7.6) and consecutively precipitated and solubilized three more times. The final pellet was extracted with ten times its volume of 1M NaCl for 20 minutes at 0° C followed by 15 minutes at room temperature, in order to solubilize the low molecular weight RNAs, including tRNA. The resultant suspension was centrifuged at top speed in an IEC Clinical Centrifuge (Model CL) for ten minutes. The supernatant

solution was removed, additional 1M NaCl added to the pellet and the extraction procedure repeated once more. The final pellet (1M NaCl insoluble fraction) was redissolved in 0.001M Tris-HCl (pH 7.5) and placed in a freezer for storage, for use in other studies.

The combined 1M NaCl-soluble fractions were precipitated by the high salt-ethanol precipitation method previously described. The resultant pellet was collected by centrifugation at top speed in a clinical centrifuge for ten minutes and dissolved in 0.1M Tris-HCl (pH 7.5). A small aliquot of this solution was removed and its absorbance at 260 nm measured on a Gilford Spectrophotometer (Model 240).

The remaining solution was applied to a DEAE-Cellulose (Whatman DE 52 pre-swollen diethylaminoethyl cellulose) column (50 cm by 2.5 cm) equilibrated with two column volumes of 0.1M NaCl in 0.1M Tris-HCl buffer (pH 7.5). The sample was applied to the column in a few ml of 0.1M Tris-HCl buffer (pH 7.5) and then eluted with 1M NaCl in the same Tris-HCl buffer in order to remove any residual DNA and high molecular weight RNA. Three ml fractions were collected and their optical densities determined at 260 and 280 nm. All fractions with an optical density of 260/280 nm of 1.9 - 2.0 were pooled and precipitated by the high salt-ethanol precipitation method. The precipitate was recovered by centrifugation, dissolved in 0.001M Tris-HCl (pH 7.5), and stored at -20°C as charged bulk tRNA.

In order to discharge the bulk tRNA it was precipitated with

high salt-ethanol, recovered by centrifugation, dissolved in 0.5 Tris-HCl buffer (pH 9) and incubated at 37°C for one hour. The discharged tRNA which was recovered by subsequent high salt-ethanol precipitation was redissolved in 0.001M Tris-HCl buffer (pH 7.5) and stored at -20°C.

III. Preparation of Aminoacyl tRNA Synthetases

Total aminoacyl tRNA synthetases were prepared by a modified version of the method of Barnett et al (1969). All procedures described in the following paragraphs were carried out at 0° - 4° C.

Six liters of Euglena cells in late logarithmic or early stationary phase (p H of culture: 6.8 - 7.2) were harvested and filtered through several layers of cheesecloth. This filtrate was centrifuged for five minutes at 5,000 x g in a Sorvall centrifuge (RC2B). The supernatant solution was discarded and the pellet washed in buffer C (0.2M K_2HPO_4, pH 7.0, 0.01M BME, 10% glycerol). The suspension was centrifuged for five minutes at 5,000 x g, the supernatant solution discarded and the wash procedure repeated a second time. The resultant pellet was weighed and again resuspended in buffer C (4 ml buffer/gm wet weight of cells). The cells were disrupted by passage through a cooled French Pressure Cell at approximately 10,000 psi and the lysate was centrifuged at 10,000 x g for 30 minutes. The supernatant solution was carefully removed and was centrifuged at 100,000 x g (avg.) in a type 30 rotor in a Spinco Ultracentrifuge (model L)

for two hours at 4°C, to pellet ribosomes and other cell particulates. The soluble fraction was collected and applied to a DEAE Cellulose (Whatman DE 52 pre-swollen diethylaminoethyl cellulose) column (50 cm by 2.5 cm) in order to remove nucleic acids. After the column was pre-equilibrated with two column volumes of buffer C, the sample was applied to it dissolved in a few ml of buffer C, and then the column was eluted with the same buffer. Fractions of three ml were collected and optical densities at 260 and 280 nm were determined on a Gilford Spectrophotometer (Model 240). The fractions containing a high 280/260 optical density were pooled and precipitated by the addition of ultra-pure crystalline $(NH_4)_2SO_4$ (Schwarz-Mann) to a final concentration of 3.0M with the pH of the mixture being adjusted to 7.2. After remaining overnight at 4°C, the precipitate was collected by centrifugation at 10,000 x g for 30 minutes. The pellet was resuspended in buffer D (0.01M Tris-HCl pH7.5, 0.1M KCl, 0.01M BME, 20% glycerol) and applied to a column (50 cm by 2.5 cm) of G-25 (coarse) Sephadex (Pharmacia Fine Chemicals) for desalting. The column was pre-equilibrated with two column volumes of buffer D and eluted with the same buffer. Three ml fractions were collected and the optical densities were determined at 280 and 260 nm. Those fractions showing high 280/260 optical densities were pooled, denoted as bulk synthetases and frozen at -60°C in small aliquots of 0.2 - 0.3 ml.

IV. Isolation of Chloroplasts

Chloroplasts were isolated by a modified version of the

method of Preston et al (1972). All procedures described below were carried out at 0° - 4° C.

Six liters of light grown, wild type Euglena in late logarithmic or early stationary phase (pH of culture: 6.8 - 7.2) were passed through several layers of cheesecloth and the filtrate was centrifuged for ten minutes at 2,000 x g in a Sorvall centrifuge (RC2B). The supernatant solution was discarded and the pellet washed in STM buffer (10% sucrose, 0.01M Tris-HCl, pH 7.8, 0.01M $MgCl_2$, 0.001 BME). This suspension was centrifuged for ten minutes at 5,000 x g, the supernatant solution discarded and the entire wash procedure repeated a second time. The resultant pellet was weighed, carefully resuspended in a volume of STM ten times its weight and disrupted by passage through a cooled French Pressure Cell at 2,000 psi directly into a chilled glass flask. Cell breakage was verified by microscopy. The lysate was centrifuged at 500 x g, and the yellow-orange supernatant solution obtained was discarded. The pellet (containing whole cells and chloroplasts) was resuspended in a volume of STM equal to 17 times the initial pellet weight. To obtain a good resuspension of the pellet it was first suspended in a small volume (50 ml) of STM, passed twice through a 17-gauge needle attached to a 50 ml syringe and then mixed well with the additional volume of STM. The resultant suspension was allowed to stand in the cold for 15 minutes to allow cell membrane material to settle out. The upper 2/3 of this suspension was filtered through a double layer of cheesecloth and glass wool and centrifuged at 500 x g. The supernatant solution was discarded and the pellet resuspended in

50 ml of ST buffer (10% sucrose, 0.01M Tris-HCl, pH 7.8). To insure even suspension, the material was passed through a 17-gauge needle four times. The sample was now ready to be centrifuged in the reorienting gradient zonal rotor.

During preparation of the algal material, the zonal rotor was prepared for the sample application as follows: 100 ml of a 60% (w/w) sucrose solution was placed in a cooled Sorvall Reorienting Density Gradient Zonal Rotor (SZ-14) before acceleration, to serve as a cushion. The 1,000 ml gradient of 15% - 50% sucrose dissolved in 0.01M Tris-HCl, pH 8.0, containing 4×10^{-5}M EDTA was generated dynamically (via a Cole-Palmer pump) by addition while the rotor was spinning at 2,000 - 2,500 rpm. After the sucrose was pumped into the rotor, speed was maintained at 2,000 - 2,500 rpm for 20 minutes before the chloroplast suspension was added by syringe injection into the zonal rotor, followed by the injection of an additional 50 ml of ST. The speed of the rotor was increased to 3,000 rpm and maintained at that level for ten minutes. The rate controller on the Sorvall centrifuge (RC2B) was then turned on and the rotor was slowly and uniformly decelerated to a halt. Using a Cole-Palmer Peristaltic Pump (Model #7013-2), 50 ml aliquots were collected from the bottom of the rotor (most dense region) into 50 ml of STM. Each fraction was examined for purity by phase contrast microscopy and only those fractions devoid of whole cell contamination were retained. This material was pooled, centrifuged for ten minutes at 2,000 x g, and the chloroplast-containing pellet stored at -20°C for further use.

V. Preparation of Chloroplast Aminoacyl tRNA Synthetases

Chloroplast aminoacyl tRNA synthetases were isolated by the previously described method that was used to isolate cytoplasmic synthetases. It was necessary, however, to use a number of accumulated batches of frozen purified chloroplasts to insure adequate starting material.

VI. The Aminoacylation Assay

The aminoacylation reaction was carried out according to the method of Barnett et al (1969), with some modifications.

The aminoacylation reaction mixture contained per ml: 50 µmoles HEPES (N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid, Calbiochem Corp.) buffer (pH 7.5); 5 µmoles ATP, disodium (pH 7.0, Sigma Chemical Co.); 10 µmoles magnesium acetate; 5 µmoles 2-mercaptoethanol, and 4.4×10^{-4} µmoles ^{14}C valine * (specific activity 260 mC/mmole) or 1.7×10^{-4} µmoles ^{3}H valine * (specific activity 6.7 C/mmole, Schwarz-Mann); 40-80 µgm tRNA and 100-200 µgm of an appropriate synthetase preparation. Reactions in a total volume of 0.2 ml were initiated by the addition of enzyme and incubated for various times at 30°C. At specified times, aliquots (0.04 - 0.08 ml) were removed and pipetted onto 24 mm filter paper discs (Whatman 3MM) which were then immersed in ice cold 10% TCA (trichloroacetic acid) for at least 15 minutes. The discs were then washed twice for ten

* These values represent concentrations at which valine is limiting.

minutes in ice cold 5% TCA, twice for ten minutes in ice cold absolute ethanol and finally twice for ten minutes in ice cold anhydrous ether. The discs were then dried under an infrared lamp and counted in a Nuclear Chicago or Beckman liquid scintillation counter in 5.0 ml of a toluene-based scintillation mix composed of 0.38 gm POPOP; [1,4-bis(2-(5-phenyloxazolyl))benzene], and 15.2 gm PPO; (2,5 diphenyloxazole, Nuclear Associates Inc.) dissolved in one gallon of toluene (Mallinckrodt, Scintillar).

VII. The Separation of Chloroplast from Cytoplasmic Valyl tRNA Synthetase Activity

Fractions containing chloroplast valyl tRNA synthetase activity were separated from those containing cytoplasmic species by a modification of the method of Hecker et al (1973).

The soluble protein fraction (containing aminoacyl tRNA synthetases and prepared as previously described) from fully greened *Euglena* cells was applied to a 1 x 20 cm column of hydroxylapatite (HTP, Bio Rad Labs), pre-equilibrated with three column volumes of 0.5 mM potassium phosphate (pH 7.0) containing 0.01M 2-mercaptoethanol and 10% glycerol. Three ml fractions were collected during elution with a step gradient consisting of 75 ml each of 0.08, 0.12, 0.15, 0.18, 0.21 and 0.25 M potassium phosphate (pH 7.0) containing 0.01M 2-mercaptoethanol and 10% glycerol. Thirty microliter aliquots of these fractions were assayed against tRNA from bleached or plastid-containing cells, as previously described, in total reaction volume of 0.1 ml.

VIII. Preparation of Large-Scale Reaction Mixtures for RPC-5 Analysis

The large scale reaction mixtures were prepared in a similar manner as the previously described standard mixture except that the reaction size was increased proportionately. After 30 minutes of incubation at 30°C, the reaction was stopped by placing the reaction vessel in ice. The reactions were made 1% with respect to NaCl. One mg of carrier tRNA (Sigma, soluble yeast RNA, type III) was added, followed immediately with an equal volume of acidified aqueous phenol (80% redistilled phenol, 20% 0.2M sodium acetate, pH 4.5). The mixture was stirred vigorously once a minute for twenty minutes, centrifuged in a clinical centrifuge for ten minutes and the aqueous phase removed. The remaining phenol layer was re-extracted with buffer E (0.01M sodium acetate, pH 4.5, 0.01M magnesium chloride, 0.02M 2-mercaptoethanol) and the aqueous phase removed and pooled with the first aqueous phase. The tRNA was precipitated from the pooled aqueous by the high salt-ethanol method and kept at -20°C for one hour. The tRNA precipitate was recovered and redissolved in buffer E. This procedure was repeated several times until the odor of phenol could no longer be detected. At this point the tRNA was again dissolved in buffer E and stored at 20°C until used for RPC-5 analysis.

IX. Reversed Phase Chromatographic (RPC-5) Separation of the Isoaccepting Species of Valyl tRNA

The high pressure liquid chromatographic separation of the

isoaccepting species of valyl tRNA was accomplished by a modified version of the method of Pearson et al (1971). All operations were carried out at $4^{o}C$ and all solutions were degassed prior to use.

The chromatography column was prepared by fitting Swagelock end fittings (Lab Data Control) onto a 100 cm long x 0.6 cm I.D. wide seamless steel tube (Reeve Angel) which had a glass wool plug as a bed support in addition to the Lab Data Control bottom fitting. The column was partially filled with buffer solution type E-1 (0.01M sodium acetate, pH 4.5, 0.01M magnesium chloride, 0.002M mercaptoethanol, and 1M NaCl). A slurried preparation of RPC-5 resin (polychlorotrifluoroethylene resin coated with trialkylammonium chloride, Miles Laboratories) prepared by gently creaming the RPC-5 resin with a mortar and pestle was poured into the column with a Pasteur pipette. Each successive application of RPC-5 resin to the column (until the column was filled to 2 inches from the top) was compacted for three minutes by applying 700 psi pressure using a Milton Roy Corp. positive displacement mini-pump, Model #19-60029-001 with a maximum pressure rating of 5,000 psi. This packing technique reduced the aqueous void volume and improved chromatographic resolution. Before gradient elution each column was equilibrated with several column volumes of equilibration solution (0.01M sodium acetate, pH 4.5, 0.01M magnesium chloride, 0.002M 2-mercaptoethanol, and 0.2M NaCl). Less than one ml of a large-scale ^{14}C or ^{3}H labelled mixture of charged tRNA was then applied to the column, and this was covered with a small amount of RPC-5 resin in buffer. The

column was then washed with at least three times the column volume of equilibration solution. Elution was then performed with a 600 ml linear gradient of 0.33M - 0.46M NaCl dissolved in 0.01M sodium acetate pH 4.5, 0.01M magnesium chloride, and 0.002M 2-mercaptoethanol. The 700 psi pressure which was generated by the pump resulted in a flow rate of one ml per minute. Three minute fractions, equivalent to three ml, were collected using an LKB 7000 Fraction Collector. To each fraction, 200 µgms of carrier tRNA (Sigma, soluble yeast RNA, type III) and TCA to 5.5% were added. The samples were vortexed and allowed to stand for 30 minutes in the cold. Each fraction was then filtered under vacuum through a Schleicher & Schuell nitrocellulose filter (Type B-6, 25 mm, 0.45 µ porosity) on a Millipore 3025 Multiple Sampling Manifold Filtration Apparatus. The filters were washed twice under vacuum with 5 ml of 5% TCA, followed by one rapid wash with one ml of 70% ethanol. The filters were then dried under vacuum (50-100 µ) for 15 minutes at 25°C, equilibrated in air at room temperature (23°-25°C) for 30 minutes, and finally counted in the toluene scintillation mix described earlier.

RESULTS AND DISCUSSION

As described earlier Euglena is well suited to the study of chloroplast development. Utilizing the potential of this system, the initial experiments were designed to determine whether there was a detectable difference in the amount of chargeable valyl tRNA between wild type Euglena and a bleached mutant. Unfractioned tRNA isolated from wild type Euglena (hereafter denoted green tRNA) was charged with ^{3}H valine by bulk aminoacyl tRNA synthetases derived from wild type Euglena (hereafter denoted green enzyme) or from a streptomycin (or ultraviolet) bleached mutant (hereafter denoted bleached or colorless enzyme). No discernable differences in charging characteristics were detected between enzyme isolated from streptomycin or ultraviolet bleached mutants and these enzyme preparations were used interchangeably. In addition, tRNA isolated from the bleached mutant (hereafter denoted bleached or colorless tRNA) was also charged with ^{3}H valine by enzymes derived from green and bleached Euglena cells. The results of these experiments are shown in Table 1. A study of the aminoacylation of valine to green tRNA by green and bleached enzyme shows a decrease of about 11% in the amount of charging by a bleached enzyme compared with that of green enzyme. On the other hand, bleached tRNAs were charged with a greater total amount of valine but to the same level by green or bleached enzyme preparations. These results were in good agreement with those of Kislev et al (1972), and suggested that wild type Euglena might contain additional species of valyl tRNA and valyl tRNA syn-

thetases not found in bleached cells. Since the major differences between the two cell types was the absence of chloroplasts in bleached cells the possibility existed that these additional tRNAs and aminoacyl tRNA synthetases were chloroplast localized.

As previously noted the tRNA derived from bleached cells contained more chargeable valyl tRNA than did the tRNA isolated from wild type cells, regardless of the enzyme source. One possible hypothesis to explain this phenomenon could be based on the observation of Wolken and Palade (1953) who found that there was a great increase in the number of mitochondria in Euglena gracilis under conditions in which the chloroplast content was reduced. Although it is plausible that a great increase in the number of mitochondria present in bleached cells could account for some increase in the amount of chargeable mitochondrial valyl tRNA, it seems unlikely that this could account for an increase of up to 24%. Only a determination of the amount of valyl tRNA contributed per mitochondrion and the relationship of this value to the number of mitochondria in wild type and in bleached cells could clarify this. Additional explanations will be discussed later in the sections describing the aminoacylation reaction itself.

Since there was a possibility that, in Euglena, chloroplast specific valyl tRNAs might exist, a detailed study of the factors affecting the aminoacylation of valine was undertaken. The charging reaction was characterized with respect to a variety of parameters in order to create optimum conditions for the specific

charging of valine to valyl tRNA.

X. The Effect of 2-Mercaptoethanol on the Aminoacylation of Valine to Valyl tRNA

Much of the literature indicates that, as a class, aminoacyl tRNA synthetases contain one or more sulfhydryl groups which are essential for activity. Therefore, most aminoacylation reactions are carried out in the presence of sulfhydryl reagents such as 2-mercaptoethanol, dithiothreitol, etc. It has been reported by Burkhard et al (1970), however, that amounts greater than 2 mM of sulfhydryl reagent inhibit the attachment of valine to valyl tRNA in Phaseolus vulgaris. Therefore, the effect of the presence or absence of added 2-mercaptoethanol was studied in the charging of valine to the tRNAs of Euglena. The results, shown in Table 2, indicate that there were no apparent differences. It should be noted, however, that the aminoacyl tRNA synthetases used here were protected by a sulfhydryl reagent, since (0.001M) 2-mercaptoethanol was present during the isolation and subsequent storage (at -70°C) of the enzymes. Without added 2-mercaptoethanol, the maximum concentration of this sulfhydryl compound that would be present in a reaction mix after the addition of synthetase was about 0.25 μmoles/ml - considerably less than the 10 μmoles/ml added to the mix in these experiments. Therefore, what is actually being measured here is the effect of exogenously added 2-mercaptoethanol. These results indicate that either the valyl tRNA synthetase of Euglena does not require a sulfhydryl reagent or that the 0.25 μmoles/ml

2-mercaptoethanol contained in the enzyme preparation is sufficient to protect the enzyme. Since inclusion of 10 mM 2-mercaptoethanol appeared to have no adverse effect on total aminoacylation level, it was always added.

XI. The Effect of pH on the Aminoacylation of Valine to Valyl tRNA

The effect of pH, in the range of 4.5 - 7.5, on the aminoacylation of valine to valyl tRNA was determined and the results are shown in Table 3. Little if any effect was noted on the extent of charging on the heterologous and homologous systems as the pH was varied from 6.0 - 7.5. Decreases in charging were often observed at pH levels of 5.5 or less. Loss of aminoacylation activity at low pH was probably due to a rapid inactivation of the valyl tRNA synthetases. A rapid decrease in the activity of threonyl tRNA synthetase from rat liver at pH 4.5 had been previously reported by Allende et al (1966). Burkhard et al (1970) reported that the optimal pH for the attachment of 18 different amino acids to their cognate tRNAs were in the range of 6.6 - 7.4 in Phaseolus vulgaris.

The fact that aminoacyl tRNA synthetases catalyze a deacylation reaction in the presence of excess ATP has been elegantly demonstrated by Bonnet and Ebel (1972), Yaniv and Gros (1969), Yarus and Berg (1969), and Lagerkvist et al (1966). The following experiment was therefore performed to demonstrate that the effect of pH was on this enzyme activity. Aminoacylation of ^{3}H valine to valyl tRNA was performed in two separate reactions as

previously described. At the end of 30 minutes the reactions were stopped and the enzyme denatured by the addition of an 80% phenol solution. One sample of charged tRNA was extracted with aqueous buffer at pH 7.5, while the other was extracted with aqueous buffer at pH 4.5. Both extracts of charged tRNA were immediately frozen to minimize the amount of non-enzymic deacylation described by Lagerkvist et al (1966) and Baldwin and Berg (1966). The samples of charged tRNA were thawed and small but equal aliquots of each were incubated at 30°C, under the conditions noted in Table 4. The data summarized in Table 4 indicate that at both pH 4.5 and pH 7.5 the amount of <u>non</u>-enzymic deacylation of val-tRNA^{val} was virtually the same - about 15%. The amount of enzymatically catalyzed deacylation was different, however, at the two pH's. There was little enzymatic deacylation noted above the control level at pH 4.5. However, at pH 7.5 there was an enzymatic deacylation of 21% - a level significantly above that of the control. The results of the reverse reaction, namely the aminoacylation of valine to valyl tRNA, as were shown in Table 3, indicated a decrease in this reaction as the pH was lowered from 6.0 to 4.5. It appears, therefore, that the effect of pH on the synthetase was much more marked on the deacylation reaction than on the forward reaction.

Of particular interest was the dramatic increase noted in the extent of enzymatic deacylation of valine in the presence of 5 mM ATP at pH 7.5. An increase in the level of deacylation also occurred at pH 4.5 but to a much lesser extent.

Bonnet and Ebel (1972) reported very similar findings in yeast with valyl tRNA and valyl synthetase. They described the process of aminoacylation of valine to valyl tRNA as a composite of four simultaneous reactions occurring at different rates (as described below).

1. $val + ATP + tRNA \xrightarrow{\text{valyl synth}} val\text{-}tRNA^{val} + AMP + PPi$

2. $val\text{-}tRNA^{val} \longleftrightarrow val + tRNA^{val}$

3. $val\text{-}tRNA^{val} \xleftrightarrow[\text{synth}]{\text{valyl}} val + tRNA^{val}$

4. (a) $ATP + val + tRNA^{val} \xleftrightarrow[\text{synth}]{\text{valyl}} AMP + PPi + val + tRNA^{val}$

   (b) $AMP + PPi + val + tRNA^{val} + val\text{-}tRNA^{val} \xleftrightarrow[\text{synth}]{\text{valyl}} AMP + PPi + val + tRNA^{val}$

Reaction (1) is the forward reaction, the aminoacylation of valine to valyl tRNA. Reaction (2) represents the non-enzymic deacylation of charged valyl tRNA. Reaction (3) is the enzymatic deacylation of charged valyl tRNA, and finally (4) represents an additional enzymatic degradation of ATP into AMP and PPi which takes place only when high concentrations of valyl synthetase are present. This reaction also requires the presence of uncharged valyl tRNA and valine, leading to an accumulation of AMP and PPi which in turn drives the aminoacylation reaction in the reverse direction.

These findings were essentially in agreement with the results shown in Table 4. One aspect of the experiment described in Table 4 that needed clarification was the origin of uncharged valyl tRNA and valine that would be required for an ATP-enhanced enzymatic deacylation reaction (equation 4). The source of tRNA could have

possibly originated in two ways. (1) As indicated by Bonnet and Ebel (1972) it was virtually impossible to aminoacylate to completion a species of tRNA using bulk enzyme preparations, since the extent of acylation of that species was dependent upon the concentration of the cognate enzyme (to be discussed in greater detail in a following section). Under the conditions of the aminoacylation described in this investigation, a considerable amount of uncharged valyl tRNA was always probably present. (2). Second, the non-enzymic hydrolysis of charged valyl tRNA led to an accumulation of both uncharged valyl tRNA and valine. This situation favored the deacylation of valine, thereby further increasing the amount of free tRNA and amino acid until an equilibrium point was reached.

Thus it appears that in the aminoacylation of valine to valyl tRNA the effect of pH is primarily on the activity of the valyl tRNA synthetase. Low pH decreases the activity of the enzyme with respect to both the aminoacylation as well as the deacylation reaction. Most dramatically, low pH effects the enzyme's ability to hydrolize ATP into AMP and PPi in the presence of valyl tRNA and valine.

XII. The Kinetics of Aminoacylation of Valine to Valyl tRNA

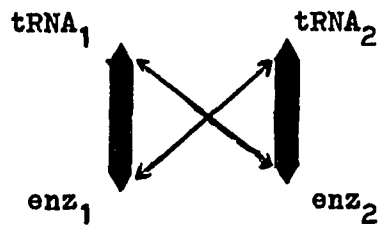
The kinetics of aminoacylation of valine to valyl tRNA are shown in Figure 1. The results indicate that in both the homologous and heterologous systems the aminoacylation reaction reached a plateau in about 15 minutes. Of particular interest

was the biphasic, and in some cases multiphasic, feature of the kinetics of the aminoacylation. As shown in Figure 1, there appeared to be a very rapid rate of acylation in all systems examined during the first one to three minutes. This was followed by a slower rate of charging, until in all four systems the aminoacylation plateaued at about 15 minutes. One possible hypothesis to explain these multiphasic kinetics would be to propose the existence of several different species of valyl tRNA, which were charged with valine at different rates. That this can and does occur in other organisms has been documented by Yegian and Stent (1969). These workers showed that the kinetics of the aminoacylation of isoleucyl tRNA from E. coli were biphasic. They reported a rapid acylation accounting for about one-third of the total isoleucine acceptor activity followed by a second, slower phase during which the remainder of the isoleucyl tRNA was charged. By MAK chromatography of such tRNAs charged for varying time periods they could demonstrate three distinct isoaccepting species of isoleucyl tRNA which were present in varying proportions in each type reaction. They noted that the species of tRNA which charged very rapidly accounted for about one-third of the total isoleucyl tRNA and the remaining two-thirds were accounted for by the two more slowly charging species.

An alternative hypothesis to explain the results shown in Figure 1 was based on the findings of many investigators who have shown that almost any tRNA can be mischarged by one or more non-cognate amino acids. Although tRNAs are very selective in binding strongly only to their cognate amino acid, many may also

bind to a non-cognate amino acid albeit to a much lower level. This has been elegantly demonstrated by Yarus (1973) who showed that isoleucyl tRNA synthetase has the ability to misacylate isoleucyl tRNA with at least 15 different amino acids. He also found that the tRNA species for chemically similar amino acids were more easily misacylated with isoleucine and related amino acids than were those species which normally recognize amino acids chemically unrelated to isoleucine. Based on these findings he categorized tRNAs into families such that a species in one family could be more easily misacylated by an amino acid normally recognized by different tRNA species in the same family.

Since most tRNAs must be aminoacylated by their cognate amino acid to insure fidelity of translation of those proteins necessary for survival and since misacylation appears to be a common event <u>in vitro</u>, how is this situation resolved <u>in vivo</u>? Two different hypotheses have been proposed by Yarus (1972, 1973). The first involved a suppression of misacylation due to marked differences in rate of reaction between tRNAs and their cognate versus non-cognate amino acids. The idea is schematically depicted in the following figure:



Width of line denotes rate of aminoacylation

The results of a series of such competitive interactions were reported by Yarus to lead to a decrease in the rate of aminoacylation of non-cognate tRNA and enzyme by 2 to 3 orders of magnitude in a mixed system. Thus, in vivo, mischarging should be dramatically reduced if this model is correct.

The second mechanism by which an organism can reduce errors is based on the finding that aminoacyl tRNA synthetases can deacylate an amino acid from a misacylated tRNA molecule. For example, Yarus (1972) reported that phenylalanine tRNA misacylated with isoleucine by isoleucyl tRNA synthetase is rapidly deacylated by phenylalanine tRNA synthetase. Thus, the tRNA synthetases may have an additional function to that of aminoacylation, namely that of correcting misacylated tRNAs.

In view of the fact that misacylations can and do occur, the data in Figure 1 may be interpreted in the following manner: In the early rapid aminoacylation reaction observed, valine is probably aminoacylated to both valyl tRNA and a non-cognate tRNA (in this case probably isoleucyl tRNA since valine and isoleucine are structurally very similar, and the corresponding tRNAs belong to the same family as described earlier by Yarus). The misacylated tRNA may be rapidly deacylated by isoleucyl tRNA synthetase, and the opposing reactions may result in the observed decrease in the rate of acylation. Once the deacylations are complete the apparent rate of the aminoacylation reaction should once again increase until equilibrium or a plateau is attained.

This hypothesis was tested as follows: an aminoacylation

reaction in the homologous green system as described in "Materials and Methods", was performed until a plateau level of aminoacylation was attained. At this point a large excess of cold valine (10 times that of the ^{14}C valine present in the reaction mix) was added, aliquots taken at 0.5, 1, 2, 5 and 12 minutes, and the acid precipitable radioactivity determined. In a similar reaction a ten-fold excess of cold isoleucine was added in lieu of cold valine. Since it has been shown here earlier (see Table 4), and by Bonnet and Ebel (1972) that at equilibrium both the aminoacylation and deacylation reactions are occurring concurrently, the cold valine would compete with the ^{14}C valine for the valyl tRNA. Thus one might expect a constant reduction with time of the total radioactive valine bound to the valyl tRNA. This is indeed what one notes in Figure 2. If mischarging, followed by a correction of this mischarging, occurs one would expect that in the second experiment there should initially be a decrease in counts due to the competition between cold isoleucine and the ^{14}C valine, followed by an increase in ^{14}C counts as the misacylations are corrected. The total counts at equilibrium should equal those at the start of the experiment and Figure 2 shows this to be so. The unusual kinetics in Figure 1 could be due, in part, to the mischarging of valine to isoleucyl tRNA followed by a correction by the isoleucyl tRNA synthetase. It is also possible that a combination of both mischarging and differences in rates of aminoacylation of different isoaccepting species of valyl tRNA could account for these results. Since this finding was not considered critical for the main objective (to ascertain when the reaction reached its plateau) the matter

was not pursued any further.

XIII. The Determination of the Optimal Mg:ATP Ratio for the Aminoacylation of Valine to Valyl tRNA

It has been known for some time that, like all or most enzymes utilizing ATP, the aminoacyl tRNA synthetases also require Mg^{++}. However this enzyme is involved in more than one reaction, since in addition to catalyzing the hydrolysis of ATP and acylating an amino acid in the first part of the aminoacylation reaction, it is also involved in the transfer of this acylated amino acid to its cognate tRNA. As previously discussed, the synthetases also have the capability of deacylating both a correctly aminoacylated tRNA as well as a misacylated one. There appears to be no exception to the universal requirement of Mg^{++} in the amino acid activation reaction (ENZ + ATP + AA$\longleftrightarrow$ ENZ·AA·AMP + PPi). The early literature abounds in controversy as to the requirement for Mg^{++} in the transfer reaction (ENZ-AA-AMP + tRNA $\longleftrightarrow$ AA-tRNA + ENZ + AMP). For example, Mg^{++} is not required to form aminoacylated valyl tRNA in yeast (Lagerkvist et al, 1966) isoleucyl tRNA in E. coli (Norris and Berg, 1964), or threonyl tRNA in rat liver (Allende and Allende, 1964), while it is necessary for the formation of charged seryl and threonyl tRNA in Bacillus stearothermophilus (Bluestein et al, 1968). Recently Zimmerman and Robinson (1972) have shown that the requirement for Mg^{++} in the phenylalanyl tRNA synthetase catalyzed transfer reaction can be altered or completely eliminated by simply changing the reaction buffer used. They found

that sodium cacodylate, citrate and succinate buffers were highly active in supporting the transfer reaction in the absence of Mg^{++}. With tris-acetate buffer, however, the synthetase exhibited an absolute requirement for Mg^{++}. They stated that "a greater appreciation of the effect of other assay conditions upon this matter" was necessary. In some systems, such as that reported by Kayne and Cohn (1972), Mg^{++}, where required, can be replaced to varying degrees in the aminoacylation reaction by a variety of trivalent ions such as La^{3+}, Pn^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+} and Gd^{3+}. The polyamine spermidine,has also been used with some success to replace a high Mg^{++} requirement.

It certainly appears from the data shown in Table 4 that Mg^{++} is not required for the enzyme catalyzed deacylation of valine from valyl tRNA in Euglena.

Mg^{++} is also believed to be involved in the stabilization of the tRNA molecule itself. Gartland and Sueoka (1966) have shown that tryptophanyl tRNA of E. coli can exist in two chromatographically separable forms: one active and chargeable by tryptophan, the other inactive and unchargeable. Both are stable in the presence of Mg^{++}, but active species can be converted into the inactive forms by the removal of Mg^{++}. The inactive form can be converted to the active form by exposure to conditions which weaken hydrogen bonds (e.g., low pH) followed by restabilization with Mg^{++}. The tryptophanyl tRNA synthetase apparently can recognize only one of the two species, the chargeable one. These investigators found that similar phenomena were exhibited by 11 other tRNAs. Similar findings have been reported by

Lindahl et al (1966) and Fresco et al (1966), who found that one of three species of leucyl tRNA could not be aminoacylated under ordinary *in vitro* aminoacylation conditions unless the tRNA had been previously heated to 60°C in the presence of 10 mM $MgCl_2$. Ittwas thought that such treatment resulted in the renaturation of the inactive forms of leucyl tRNA and restored its proper tertiary structure.

Later work by Yarus and Rashbaum (1972) appeared to place some doubt on the previously mentioned findings. Utilizing the isoleucyl tRNA synthetase system of *E. coli* from which they scrupulously removed all the Mg^{++} which might contaminate their ligands, they concluded that Mg^{++} (or divalent cations) were required solely for the synthesis of the enzyme-AMP-AA complex. They noted, therefore, that Mg^{++} cannot be an integral part of "the static or dynamic structure of isoleucyl tRNA which is required for recognition and aminoacylation". Additional effects of Mg^{++} on tRNA structure have been reported by Miller and Steiner (1966) who have shown that in the presence of Mg^{++} both pancreatic ribonuclease or snake venom phosphodiesterase liberate only a few nucleotides from tRNA while in its absence these enzymes degrade tRNA much more extensively. In addition, there is a pronounced effect by Mg^{++} on the absorbance melting curve of tRNA, shifting the Tm to considerably higher temperatures.

Divalent cations are persistent contaminants of many of the reagents which are required in reaction mixtures used to assay tRNA activity. Thus, a considerable amount of ambiguity

is introduced in the interpretation of experiments attempting to determine the effect of these divalent cations on aminoacylation parameters.

A survey of the literature by Novelli (1967) indicated that the optimum Mg/ATP ratio must be determined for each different activating enzyme within a species and for similar enzymes between species.

Results of the determination of the optimal Mg/ATP ratios required for the aminoacylation of valine to valyl tRNA in the Euglena systems studied are shown in Figure 3 where the ATP level was constant at 5 mM. It can be noted that when bleached tRNA was charged by either green or bleached enzyme the optimal Mg/ATP ratio was from 1 to 3, with only a slight decrease at ratios greater than 4. When green tRNA was charged by bleached enzyme the optimum ratio was about 2, and decreased slightly at ratios above 6. When green tRNA was charged by green enzyme there was a peak Mg/ATP ratio at 3, followed by a decrease in charging as the ratio increased. As will be shown later, the optimum ratio for the charging of green tRNA by valyl tRNA synthetase from purified chloroplasts was about 3, and this might account for the optimal Mg/ATP ratio of 3 for the green tRNA by green enzyme system. These Mg/ATP optima probably represent an average for all the isoaccepting valyl transfer RNA species. It is possible that each of these species had its own unique Mg/ATP optimum. These results were in good agreement with those of Smith and McNamara (1967) who found that the optimum Mg/ATP ratio for the

aminoacylation of valine to valyl tRNA in rabbit reticulocytes was 1.67 compared with the 1 - 3 range found in this investigation. When these results were compared with the Mg/ATP levels used by Barnett et al (1969) and Reger et al (1970) for the aminoacylation of phenylalanyl, glutamyl and isoleucyl tRNA of Euglena gracilis it seemed that a serious discrepancy existed. They found that a Mg/ATP ratio of 20 was optimal for these tRNAs using 0.5 mM ATP. Although they worked with tRNAs which might require drastically different Mg/ATP optima, the question was raised as to the meaning of their optimal Mg/ATP ratio. One can obtain an almost infinite number of identical Mg to ATP ratios by proportionately varying both the Mg and ATP concentrations. For example 10 mM Mg and 1 mM ATP gives the same Mg/ATP ratio as does 20 mM Mg and 2 mM ATP. In most systems described in the literature, and in the experiment described in Figure 3, the ATP concentration was kept constant while the Mg^{++} concentrations were varied. Is it the absolute Mg^{++} concentration, the absolute ATP concentration or the ratio of both that is critical for optimizing the aminoacylation reaction?

An experiment similar to that in Figure 3 was performed, except that the uniform ATP concentration was changed from 5 μM to 0.5 μM per ml reaction.

As can be seen in Figure 4 the optimum Mg/ATP ratio now appears to be 20 compared with the value of 2 found in the previous experiment. However, in both cases the optimal absolute Mg^{++} concentration was about 10 mM. The 15 mM Mg^{++} optimum

seen at a 5 mM ATP concentration for green tRNA by green enzyme (Figure 3) seemed to be present here too, although not as sharply defined. Also, at 0.5 mM ATP and 10 mM Mg^{++}, the extent of aminoacylation at the plateau appears to be slightly higher than in the previous experiment carried out at 5 mM ATP and 10 mM Mg^{++}. The implication of this will be discussed in the next section.

It appeared from these experiments that, in the case of the aminoacylation of valine to valyl tRNA in Euglena gracilis, the absolute Mg^{++} concentration was the most critical factor in the Mg to ATP ratio, within the limits tested. Whether this finding will apply to all of the tRNA systems in Euglena remains to be determined.

XIV. The Effect of ATP Concentration on the Aminoacylation of Valine to Valyl tRNA

Reducing the ATP concentration in the aminoacylation mix from 5 µmoles to 0.5 µmoles/ml resulted in the attainment of a higher level of aminoacylation. Based on the work of Bonnet and Ebel (1972) and the work reported in Table 4, it appeared that the valyl tRNA synthetase could catalyze a breakdown of ATP into AMP and PPi, leading to a reversal of the aminoacylation reaction. Thus in the presence of decreasing amounts of ATP, less AMP and PPi would be formed and the reversal of the aminoacylation would be reduced. This would result in a greater degree of total ^{14}C valine charging to its tRNA at equilibrium. The need for uncharged valyl tRNA and valine required further

clarification. Ogata (1961) and Hele (1964) found that the isoleucine-dependent ATP$\longleftrightarrow$PPi exchange reaction catalyzed by isoleucyl tRNA synthetase was greatly enhanced by the presence of isoleucyl tRNA. The binding of the tRNA to the synthetase molecule may cause a conformational change which may be required for the subsequent enzymatic recognition of ATP. The requirement of tRNA for the ATP$\longleftrightarrow$PPi exchange reaction is, however, not universal as has been amply demonstrated by Ravel (1965) and Lazzarini and Mehler (1966). These workers showed the opposite effect, namely a sharp reduction in the ATP$\longleftrightarrow$ PPi exchange reaction in the presence of glutamic acid and its cognate tRNA. In the case of aspartyl tRNA synthetase as reported by Norton (1963) the presence of the aspartyl tRNA appeared to have no effect whatever. To quote Novelli (1967), "these results, unfortunately, add a little more confusion to an already highly confused situation".

XV. Effect of tRNA and Enzyme Concentration on the Aminoacylation of Valine to Valyl tRNA

Experiments were performed to determine the effect of tRNA concentration on the extent of aminoacylation of valine to valyl tRNA in Euglena. The results (Table 5) indicated that increasing the tRNA concentration when a constant level of enzyme was used did not result in an increase in the extent of aminoacylation per µgm tRNA. The specific level of valyl charging activity, per unit tRNA, was found to be identical in the range of

5 - 10 µgm of tRNA studied here, and therefore enzyme at the level normally used should not have been limiting. The constant level of CPM/µgm tRNA observed was to be expected since the factor CPM/µgm tRNA should be the equilibrium constant in the following simplified expression of the aminoacylation reaction:

$$tRNA + AA \longleftrightarrow tRNA \cdot AA$$

$$\frac{[tRNA \cdot AA]}{[tRNA]\,[AA]} = \text{Kequil} = \text{CPM}/\mu\text{gm tRNA}$$

The effect of a range of enzyme concentrations on the extent of aminoacylation of ^{3}H valine to a fixed level of green tRNA in the homologous wild type (green) system was next tested. These results (Table 6) showed that at a low concentration of enzyme (12 µgm protein/reaction) there was a decreased rate and level of total valine aminoacylation when compared to the higher enzyme concentrations. Enzyme in this investigation has been routinely used at a level of approximately 120 µgm protein/reaction. These results are in general agreement with those obtained by Bonnet and Ebel (1972) and Yegian and Scott (1968), who showed that at low concentrations of synthetase the extent of aminoacylation of valine in yeast, and of isoleucine in E. coli, was almost directly proportional to the concentration of the cognate aminoacyl tRNA synthetases. At higher concentrations of enzyme they found the rate of aminoacylation decreased rapidly. At extremely high enzyme concentrations there was a rapid acylation until a plateau was reached ("equilibrium") and this was followed

by a deacylation reaction which increased in rapidity with increasing concentration of enzyme.

It was difficult to explain the finding that the extent of aminoacylation was dependent upon the synthetase concentration (i.e., 12 µgm protein vs. 120 µgm protein). One can speculate at this time that in the green enzyme preparations used here there is just not enough activity or availability of certain valyl synthetase species to aminoacylate completely all of the tRNA species in the time, and under the conditions, in which the reactions are monitored. The findings of Calender and Berg (1966) and Yarus and Berg (1969) indicated that in vivo synthetases are present in almost equimolar concentrations with their cognate tRNAs and suggest that the synthetases may be saturated with their respective tRNAs at all times. Whether this applies to the Euglena system remains to be determined.

XVI. Effect of CTP on the Aminoacylation of Valine to Valyl tRNA

Studies by Ingram et al (1963), Apgar et al (1966) and Makman et al (1966) indicated that a large number of isolated yeast tRNAs lacked a 3' terminal adenosine, Purified nucleotidyl transferase has been shown by Furth et al (1961) and Anthony et al (1963) to catalyze the addition of AMP and CMP onto the 3' end of tRNA molecules from ATP and CTP respectively, releasing PPi. In addition, crude aminoacyl tRNA synthetase preparations have been found to contain the nucleotidyl transferases and could repair those tRNAs lacking in a terminal adenosine or -CCA so that they

could be subsequently aminoacylated normally. In this investigation, in the presence of added CTP there was a decrease noted in the amount of valyl tRNA chargeable by ^{14}C valine, as shown in Table 7. Two alternatives are offered to explain these findings. First, in the presence of nucleotidyl transferases the addition of CTP results in the formation of CMP and PPi and, as was discussed earlier, increasing concentrations of PPi would tend to drive the reaction in the reverse direction. This would be manifested by a decrease in the level of aminoacylation. The increased level of PPi would possibly have been due to the repair of any species of "incomplete" tRNA, but the effect of high levels of PPi would only be measurable here as a decrease in the level of ^{14}C valine charged to tRNA.

An alternative but less likely explanation would be that during the aminoacylation reaction a cytosine could be added in place of the normal adenosine by the nucleotidyl transferase. This would result in a tRNA molecule incapable of being charged. It was also noted that the bleached enzyme preparation was less efficient with respect to nucleotidyl transferase activity than was the green preparation. Since CTP had an adverse effect on the level of charging of valine to valyl tRNA it was not added in any of the subsequent experiments.

In all of the previously discussed experiments the concentration of valine was in considerable excess to insure the attainment of a maximum level of charging. However, in view of the evidence presented by Yarus and Mertes (1973) in which they showed that isoleucyl tRNA synthetase could misacylate isoleucyl tRNA

with a large number of different amino acids, it was necessary to determine if mischarging occurred under the conditions of excess valine used in these studies. Those amino acids shown by Ritter and Jacobson (1972) and Bergman et al (1961) to be most competitive for valyl tRNA synthetase, namely phenylalanine and isoleucine, were selected for study. In addition, two amino acids were included which were classified with valine as to structural similarity, namely leucine and alanine. The results of a series of experiments designed to determine whether mischarging of valyl tRNA occurred with these amino acids are shown in Table 8. Addition of each of the competitive amino acids in concentrations up to 10 times that of the ^{14}C valine resulted in no detectable change in the amount of ^{14}C valine charged to valyl tRNA. If mischarging did occur, as was shown for the isoleucine system, it was probably rapidly corrected by the valyl tRNA synthetase, so that at equilibrium it appeared that ^{14}C valine was charged only to valyl tRNA.

Most investigators studying aminoacylation reactions add to their mixtures specified, but equal, amounts of 19 cold amino acids in order to bind the respective cognate tRNAs. This supposedly prevents mischarging to these non-cognate tRNAs by the specific isotopically labelled amino acid being studied. The amount of each tRNA species in a cell can vary considerably, as shown by Smith and McNamara (1971). Thus, this method of adding uniform amounts of 19 cold amino acids could still lead to mischarging since the added quantity of one cold amino acid which

is sufficient to completely acylate its cognate species of tRNA might be in great excess for another cold amino acid or be limiting for others. It would appear that if this method is to be employed with confidence then the amounts of each tRNA (or at least those tRNAs known to be capable of being mischarged by the amino acid used in the investigation) should be determined and the appropriately calculated amount of each cold amino acid added. To avoid this cumbersome and time-consuming procedure, levels of limiting valine were used to minimize the possibility of mischarging. This was merely precautionary since it had been shown that little or no mischarging occurred even under conditions of non-limiting valine.

These findings corroborate the work of Yarus described earlier in which in vitro mischarging in homologous systems could be detected primarily under conditions where the only entities present were non-cognate components. Such in vitro conditions were present only when purified tRNAs and synthetases were employed. There was, however, a decreasing amount of misacylation reported as the cognate amino acid, cognate tRNA and cognate tRNA synthetase were added. This situation approached that found when bulk tRNA and tRNA synthetases were used and closely resembled the conditions found in vivo.

Having determined the optimal parameters for the specific aminoacylation of valine to valyl tRNA using bulk tRNA and bulk synthetases, all additional experiments were carried out under these conditions.

XVII. Aminoacylation of Valine to Valyl tRNA from Green and Bleached Euglena by Chloroplast Enzymes

The extent of aminoacylation of ^{3}H valine to valyl tRNA from green and bleached Euglena by chloroplast and green enzyme is shown in Table 9. These results indicated that chloroplast valyl tRNA synthetase (obtained by two methods) was capable of charging tRNA derived from green Euglena cells but could not charge valyl tRNA isolated from bleached cells. In addition, it should be noted that valyl tRNA from green cells was charged by chloroplast enzyme to a level of about 10% that it was charged by green enzyme. This was in good agreement with the earlier results where colorless synthetase charged green tRNA about 10% - 14% less than did green enzyme. These findings also suggested that there were valyl tRNA species present in tRNA from green cells which were not present in bleached cells lacking chloroplasts.

XVIII. Effect of Mg^{++} on the Aminoacylation of Valine to Green tRNA by Enzyme Derived from Purified Chloroplasts

It was previously observed that the Mg^{++} optimum for charging of green tRNA by green enzyme was 15 mM in contrast to 10 mM for the bleached systems. A separate experiment was therefore carried out to determine the optimal Mg^{++} concentration for the activity of the chloroplast valyl tRNA synthetase. The results are shown in Figure 5. First, the chloroplast valyl synthetase was most active at 15 mM Mg^{++}. Second, the chloroplast enzyme was much more sensitive to low Mg^{++} concentrations

than were the cytoplasmic enzymes. Although there appeared to be little change in the enzyme activity at Mg^{++} concentrations of 10 - 20 mM, there was an extremely sharp drop in extent of aminoacylation at Mg^{++} concentrations less than 10 mM. At 20 mM Mg^{++} or higher the chloroplast enzyme could aminoacylate valine less effectively although the decline was not as marked as it was at lower Mg^{++} concentrations. In addition, the length of time required to reach an aminoacylation plateau was considerably longer for the chloroplast enzyme than it was for whole cell synthetase. This might reflect a low valyl synthetase activity in the enzyme preparation used, the presence of very small quantities of chloroplast valyl synthetase, the loss of valyl synthetase activity in the isolation procedure, or the very slow rate of aminoacylation to chloroplast valyl tRNA.

Chloroplast valyl tRNA synthetase activity was also separated in total green cell extracts from its cytoplasmic counterpart according to the method of Hecker et al (1974) as described in "Materials and Methods". This chloroplast synthetase was utilized in charging both green and colorless tRNA. The results of this experiment are shown in Table 9, and are in excellent agreement with those obtained when the chloroplast synthetase came from isolated chloroplasts. The extremely small amount of charging seen with colorless tRNA (7 CPM/µgm tRNA) can be attributed to the fact that the fraction utilized (0.06M KH_2PO_4 fraction) contained a small quantity of cytoplasmic synthetase contamination. A slightly lower KH_2PO_4 concentration would have probably eliminated this small amount of contamination.

XIX. Aminoacylation of Valine to Valyl tRNA from Dark Adapted Wild Type Euglena Cells by Chloroplast Enzymes

The extent of aminoacylation of ^{3}H valine to valyl tRNA from dark adapted wild type Euglena cells is shown in Table 10. These results indicated that chloroplast valyl tRNA synthetase (obtained by two methods) was capable of charging tRNA derived from dark adapted wild type cells to the level of about 10% of that of green cells charged by green enzymes. In addition, chloroplast synthetase charged tRNA from dark adapted wild type cells to the same extent it did tRNA from wild type green cells. The significance and implications of these findings will be discussed later.

Prior to the final preparation of large scale reactions for the the chromatographic separation of the valyl tRNA species, aliquots of green and bleached bulk tRNA were subjected to gel filtration chromatography on G-100 Sephadex. The results are shown in Figures 6 and 7. Gel filtration showed that the tRNAs existed in monomeric form as evidenced by the single symmetrical elution peak from G-100 Sephadex which could separate monomers from dimers. The width of the peak can probably be attributed to the fact that bulk tRNA contained all or almost all of the major tRNA species. These tRNAs vary somewhat in molecular weight, the range being about 24,000 - 28,000. It was therefore assumed that the peaks obtained by chromatographic separation of the valyl tRNA into isoaccepting species would not be a result of artifacts of molecular aggregation.

XX. The Separation of Isoaccepting Species of tRNA by Reverse Phase Chromatography

Barnett et al (1969) have demonstrated that new chromatographically resolvable species of phenylalanine, glutamic acid and isoleucine tRNAs were formed in dark adapted Euglena cells upon chloroplast induction in these cells by exposure to light. These light induced species were not found in wild type cells grown heterotrophically in the dark. These experiments were also repeated with light grown, ultraviolet bleached mutants which had permanently lost their ability to form chloroplasts and contained no detectable chloroplast DNA or other chloroplast components. These cells contained the same tRNA species for the amino acids discussed above as did wild type cells grown in the dark, and the authors concluded that "the alterations in the tRNA patterns were directly related to chloroplast development and not to some other light related phenomenon".

Reger et al (1970) also demonstrated that light grown, wild type Euglena gracilis contained tRNA species for phenylalanine and isoleucine not detectable in dark grown wild type cells or in cells of an ultraviolet bleached mutant. In addition, these authors found that each of these light induced species of tRNA was aminoacylable only by its corresponding chloroplast synthetase. The isoleucyl tRNA synthetase, like its cognate chloroplast tRNA, was light inducible and was not found in either dark grown wild type or bleached mutant cells. The chloroplast specific phenylalanine tRNA synthetase, however, was not found to be light

inducible since it was present in bleached cells as well as in wild type cells.

The appearance of new isoaccepting species of tRNA correlated with the presence of chloroplasts was not uniquely associated with Euglena gracilis. Burkhard et al (1970) reported that in Phaseolus vulgaris (bean) there are three species of leucyl tRNA found only in the chloroplast, and such species could only be charged by chloroplast enzyme. In addition, they found two chloroplast localized valyl tRNA's, one of which was recognized only by the cognate chloroplast enzyme.

XXI. The Reversed Phase (RPC-5) Chromatographic Separation of the Isoaccepting Species of Valyl tRNA from Green Cells Charged by Green Enzyme

The elution profile of the valyl tRNA species obtained by the charging of green tRNA by green enzyme is shown on Figure 8. The details of the chromatographic procedure are described in "Materials and Methods".

It was found that the isoaccepting valyl tRNA species were resolved into four peaks. Peaks I and II eluted very close to one another, and p eak II was about 1/3 the size of peak III.

XXII. RPC-5 Separation of the Isoaccepting Species of Valyl tRNA from Green Cells Charged by Bleached Enzyme

When tRNA isolated from green cells was aminoacylated with valine by enzyme derived from bleached cells (denoted

"colorless" on the graphs) and chromatographically resolved, the profile obtained (Figure 9) contained only three distinct peaks. Peak I was no longer present - a feature consistently seen in subsequent elutions of similar material. Peak I was presumed to be of chloroplast origin based on the following lines of additional evidence.

1. It appeared in chromatographic profiles of green tRNA but only when this tRNA had been charged with valine by green enzyme.

2. Aminoacyl tRNA synthetases isolated from purified chloroplasts charged valine to green but not to bleached tRNA.

3. Chloroplast valyl tRNA synthetase activity separated from cytoplasmic valyl tRNA synthetase activity on hydroxylapatite columns charged green tRNA but not bleached tRNA.

4. The decrease in the level of charging of green tRNA by bleached versus green enzyme was found to be about 8% - 12% (chloroplast enzyme charged green tRNA about 10% the level that green enzyme charged it). These findings are summarized in Table 11.

XXIII. RPC-5 Separation of the Isoaccepting Species of Valyl tRNA from Bleached Cells Charged by Green Enzyme

The chromatographic resolution of the valyl tRNA species derived from bleached cells that were charged by green enzyme is shown in Figure 10. This profile showed the same three peaks which eluted at the same salt molarities as those seen with green

tRNA (Figure 9) with the exception that there was a dramatic shift in the relative size of peaks II and III. Whereas peak II was about 1/3 the size of peak III in green tRNA, it was now about three times the size of peak III. Peak I, found in green tRNA, was missing, suggesting again that the first peak was of chloroplast origin.

XXIV. RPC-5 Separation of the Isoaccepting Species of Valyl tRNA from Bleached Cells Charged by Bleached Enzyme

When the elution profile of bleached tRNA charged by bleached enzyme was analyzed it was seen (Figure 11) that there was virtually no difference in the elution profile from when bleached tRNA was charged by green enzyme.

XXV. RPC-5 Separation of the Isoaccepting Species of Valyl tRNA from Dark Adapted Cells Charged by Green Enzyme

The chromatographic separation of the valyl tRNAs which were derived from dark adapted Euglena cells and charged by green enzyme is shown in Figure 12. The elution profile very closely resembled that of bleached tRNA charged by either green or bleached enzyme, with one major exception. Peak I attributed to chloroplast origin was present. This finding was not expected in view of the earlier discussed reports concerning other Euglena chloroplast tRNAs. In each of the cases examined thus far in Euglena the chloroplast localized species was reported absent in dark adapted cells. Here it appeared the chloroplast localized and specific valyl tRNA species was not light inducible,

but was present in cells containing the chloroplast precursor bodies, proplastids. Valyl tRNA derived from dark adapted cells was then charged by chloroplast enzyme in order to determine whether or not this peak could be of chloroplast origin. The results shown in Table 10 indicated that valyl tRNA derived from dark adapted cells was indeed chargeable by the chloroplast valyl tRNA synthetase.

CONCLUSIONS AND GENERAL DISCUSSION

The study of chloroplast development has been impeded by three major problems (none of which has been completely resolved to date) namely, (1) the extent of genetic autonomy possessed by the chloroplast, (2) the molecular level at which such autonomy, if it existed, would be expressed (transcriptional, translational), and (3) the nature of the regulatory mechanism(s) involved.

It is quite clear from the aforementioned material that the chloroplast has all the necessary prerequisites for self-perpetuation: its own unique DNA, a mechanism for the replication and transcription of this DNA, and a functional protein synthesizing system. However, what has become apparent is the existence of many bi- or multi-directional interactions between the chloroplast and other cell genomes. A clarification of such interplay is certainly necessary. Considerable information is available concerning structural and chemical changes which accompany chloroplast development, yet little is known about the control and regulatory mechanisms involved. Summarized below are some of the major findings of the present investigation which will be discussed with respect to the unresolved issues.

1. Aplastidic mutants of Euglena contain higher levels of aminoacylatable valyl tRNA species than do wild type cells.

2. Wild type, light grown cells contain four isoaccepting valyl tRNA species as determined by reversed phase chromatography

(RPC-5) at 6°C.

3. One of these isoaccepting species of valyl tRNA is unique to the chloroplast.

4. This chloroplast valyl tRNA species appears to be constitutive since it is also found in dark grown wild type cells.

5. Wild type light grown Euglena, or chloroplasts isolated from them, contain a valyl tRNA synthetase which shows specificity for the chloroplast localized valyl tRNA species.

6. Major quantitative differences exist in two non-chloroplast valyl tRNA species between green cells and dark adapted or bleached cells.

The observation that the aplastidic mutants used in this investigation contained considerably more chargeable valyl tRNA than did wild type cells was very puzzling. Some increase in valyl tRNA could be accounted for by the increased number of mitochondria in aplastidic cells (Wolken and Palade, 1953). However, it was hard to believe that an increase of 24% could be due to the additional mitochondria. Until more is known about the number of mitochondria per cell and the amount and characteristics of the valyl tRNA per mitochondrion, this hypothesis remains unproven.

Another possible explanation for the increased levels of valyl tRNA in aplastidic cells could be based on the findings of Zeldin and Schiff (1967 and 1968). They reported that mutants lacking detectable chloroplasts incorporated considerably more P^{32} labelled inorganic phosphorus into phenol-extractable RNA

than did wild type cells. They also noted that aplastidic cells showed considerable levels of RNA labelling in the dark, and an even greater extent of labelling when these cells were exposed to light. Dark grown resting cells, on the other hand, showed no RNA labelling in the dark at all. These findings were interpreted to indicate that aplastidic mutants lacked the ability to produce regulatory signals required to repress the labelling of RNA in the dark, and that the proplastid might be the source of such regulatory signals.

A similar but related explanation could also account for the apparent light induced superinduction of RNA labelling noted in these mutants. This would require the existence of a cytoplasmic photoreceptor whose activation would result in the production of the necessary cytoplasmic components for the developing chloroplast. However, since these mutants contain no developing chloroplast, regulation exerted by the developing chloroplast was lost, thereby leading to a superinduction of the RNA components required for the chloroplast. The distribution of labelled RNA components was not determined in that study; thus one can only speculate as to whether this model could explain the increased levels of chargeable tRNA found in aplastidic cells. An experiment to clarify this matter should be the comparison of the levels of chargeable valyl tRNA species between aplastidic and wild type cells grown both in the dark and in the light.

Reversed phase chromatographic separation of the isoaccepting valyl tRNAs of Euglena resulted in the resolution of four distinct species. One of these species, denoted as peak I on

Figure 8 was present only when tRNAs isolated from wild type or dark adapted cells were charged by synthetases from chloroplast containing cells (green enzyme). As stated earlier, the origin of this species was attributed to the chloroplast on the basis of the following additional evidence:

1. Synthetase fractions isolated from purified chloroplasts, or those separated from the cytoplasmic valyl synthetase activity by hydroxylapatite chromatography, charged green or dark adapted tRNA but not tRNA derived from the bleached mutant.

2. When the charging of green tRNA by enzyme from green and bleached cells was compared (Table 1) it was noted that bleached enzyme charged about 10% less than did green enzyme. When green tRNA was charged with synthetases from chloroplasts the value obtained was about 10% that noted when it was charged with green enzyme. The high degree of specificity noted by the cytoplasmic and chloroplast synthetases for their homologous tRNAs permitted an estimation of the distribution of valyl tRNAs, namely 90% from the cytoplasm, 10% from the chloroplast. Cytoplasmic and chloroplast enzyme activities were additive with green tRNA and equalled the level of aminoacylation of total cell valyl tRNA activity noted when synthetases from green cells were used.

A review of the literature indicated that as one goes from lower to higher organisms the number of isoaccepting species of valyl tRNA increased. For example, in E. coli, Roy et al (1971) found two isoaccepting valyl tRNAs, and Takemoto et al (1973) found yeast had three. As reported here, plastidic Euglena have four isoaccepting tRNAs. In the higher plant Phaseolus vulgaris,

Burkhard et al (1972) reported that the number of valyl tRNA species was five. The significance of these findings is still obscure and whether this evolutionary trend reflects a greater need for tRNA variability in organisms of greater complexity remains to be clarified.

That the chloroplast of Euglena has a unique species of valyl tRNA was not surprising since similar findings for isoleucyl, phenylalanyl and glutamyl tRNA had been reported by Barnett et al (1969) and Reger et al (1971). These experiments indicate, however, that Euglena contains only one chloroplast specific valyl tRNA species, in contrast to the five chloroplast localized valyl tRNA species reported by Burkhard et al (1972) for Phaseolus vulgaris. In this later report, only one of the chloroplast localized valyl tRNAs could be solely charged by chloroplast synthetase - a finding which may be quite significant.

The fact that the chloroplast specific valyl tRNA species in Euglena is constitutive (as indicated by its presence in dark adapted cells) is interesting in view of the findings of Barnett et al (1969) and Reger et al (1971) who found that the chloroplast specific tRNAs examined in Euglena appeared to be induced by light, with two exceptions. The exceptions were noted in the report of Goins et al (1973) which demonstrated that in an ultraviolet induced cytoplasmic pale mutant (G_1BU) the chloroplast localized isoleucyl and methionyl tRNAs were present in dark adapted cells to the same extent as in light grown wild type cells. The results presented here represent the first report of a constitutive chlo-

roplast specific tRNA in wild type Euglena Z strain. Preliminary experiments of Selsky (personal communication) indicated that a similar situation exists for the chloroplast specific isoleucyl tRNA species of Euglena Z strain. His data indicates that tRNAs from dark adapted cells could be charged with isoleucine by chloroplast enzyme to a level of about 1/3 that of tRNA from wild type green cells. tRNA derived from bleached cells, on the other hand, showed no significant charging with isoleucine with the same chloroplast enzyme preparation.

Another interesting observation concerning chloroplast specific valyl tRNA was the fact that this particular tRNA was present to the same level in dark adapted cells as that found in light induced wild type cells. This finding was somewhat surprising in view of other reports, where it was noted that even in cases where detectable amounts of chloroplast specific valyl tRNAs were present in the dark, light nevertheless stimulated a considerable increase in these species. Burkhard et al (1972), for example, have reported significant quantitative increases in the levels of chloroplast specific valyl tRNA in Phaseolus vulgaris as they studied the system from etioplast to fully developed chloroplast. A similar situation has been reported by Merrick and Dure (1972) in cotton.

The current finding of a chloroplast specific tRNA in dark adapted Euglena is not unexpected in view of the fact that dark adapted cells contain proplastids. These precursor bodies possess some structural complexity and reduced levels of several

chloroplast components such as 70S ribosomes, RuDP carboxylase, and NADP triosephosphate dehydrogenase. In addition, the proplastids probably contain chloroplast DNA which, in turn, is presumably responsible for their replication. Therefore the proplastid, in many ways, resembles a "miniaturized" chloroplast and is capable of reproducing itself and its normal complement of components in the dark. It would not be difficult to visualize similar types of bidirectional interactions between the proplastid genome and other cell genomes in maintaining and regulating the proplastid in the cell cycles. These interactions may require the presence of some chloroplast specific tRNAs in a manner similar to those required by the mature chloroplast itself. Supporting this speculation are the findings of Barnett et al (1969) which indicate that at least one of the chloroplast specific tRNA synthetases (phenylalanyl) is not light induced and is present in dark adapted cells. Additional, but more ambiguous, findings are those of Parthier et al (1973). They reported that although light appeared to induce large increases in the activity of chloroplast specific synthetases (among them valyl synthetase), these synthetases were nevertheless present to a much lesser extent in dark adapted cells. The ambiguity in their results arose from the fact that they based the level of chloroplast synthetase activity on its ability to charge tRNA from the blue-green alga Anacystis nidulans. They did not prove that Anacystis could only be charged by chloroplast synthetase.

Although a large number of chloroplast localized aminoacyl tRNA synthetases have been detected in a wide variety of photo-

synthetic organisms, the specificities of many of these enzymes differ markedly. For example, Burkhard et al (1970) reported that in Phaseolus vulgaris the chloroplast leucyl tRNA synthetase could aminoacylate both chloroplast and cytoplasmic localized leucyl tRNAs. A similar situation was found for the chloroplast localized valyl tRNA synthetase. In both cases there was virtually no difference in activity between the chloroplast and cytoplasmic synthetase in the level of aminoacylation obtained with the cognate cytoplasmic tRNAs. In fact, the data indicated that only one chloroplast localized synthetase, that for alanyl tRNA, could not charge cytoplasmic tRNA. All of the other chloroplast synthetases tested could charge their cytoplasmic tRNA counterparts to a significant level.

In the case of Euglena however, a different situation exists. Kislev et al (1972), using high purity chloroplast preparations, reported that only about 1/2 of the chloroplast localized aminoacyl synthetases studied could charge the tRNAs of the cytoplasm.

It appears that "chloroplast localized" need not infer "chloroplast specific". The possibility exists that the chloroplast and cytoplasmic localized synthetases are one and the same, being compartmentalized at different sites within the cells.

The results presented here show a much clearer picture for the case of Euglena valyl tRNA synthetases. Not only is the chloroplast valyl tRNA synthetase localized within that organelle but it appears to have an identity different from that of its cytoplasmic counterpart. This was demonstrated by the fact that

its activity could be physically separated from that of the cytoplasmic species by hydroxylapatite chromatography. In addition, this chloroplast specific valyl tRNA synthetase was capable of charging only its cognate chloroplast localized tRNA.

Hecker et al (1974) have shown that the chloroplast valyl tRNA synthetase was present in both dark adapted and aplastidic mutants of Euglena gracilis. Their data support a nuclear genome and an 80S ribosome synthetic site for it. Whether the presence of the chloroplast valyl tRNA synthetase as demonstrated by Hecker et al (1974) and the presence of chloroplast valyl tRNA in dark adapted cells (as demonstrated here) implies functionality in vivo is still obscure. This situation needs further clarification since, in the case of the chloroplast phenylalanyl system, tRNA appears to be missing from dark adapted cells while its cognate chloroplast synthetase is present.

One of the most intriguing findings of this investigation was the observation that there were large quantitative differences between two of the cytoplasmic isoaccepting valyl tRNA species seen when tRNA derived from light grown, wild type and bleached cells were compared (peaks II and III on Figures 8 and 10). Approximations (cut and weigh paper method) indicated that peak II occupied about 1/3 the area of peak III when green tRNA was analyzed and about three times the area of peak III when colorless tRNA was studied. That these differences were a function of the tRNA rather than the enzyme used was apparent from the fact that there were no changes in proportions of these two peaks in green

tRNA regardless of the enzyme used in charging it (Figure 9). The same situation held for bleached tRNA (Figure 11).

It would be interesting at this point to speculate as to the possible significance of the major quantitative differences in the elution profiles noted between green and bleached tRNA. Among the major events that take place upon greening are significant changes in both cytoplasmic and chloroplast RNA and protein synthesis. It is as if the cell were retooling for the production of the necessary components for the developing chloroplast. It would not be difficult to visualize a control mechanism wherein the availability of one or a few species of tRNA could regulate the translation of a group of mRNAs.

tRNA regulatory mechanisms of this type have been reported for other organisms. For example, Sueoka and Sueoka (1969) have shown that of 17 tRNAs examined in E. coli only leucyl tRNA showed a clear alteration of elution pattern upon infection of the cell by T-2 phage. E. coli normally has five resolvable isoaccepting leucyl tRNA species. Upon T-2 infection, peak I was reduced to about 50% of its original amount, while there was an increase in peak V, and no alteration in the other three peaks. Ribosome binding experiments indicated that peak I responded to the codon CUG, while other studies using in vitro protein synthesizing systems indicated that T-2 mRNA rarely contained the CUG codon. On the basis of these findings the authors proposed the following: "Upon infection, phage T-2 induces a specific ribonuclease that cleaves leucyl $tRNA_1$ at the site of the translation

of E. coli mRNA on ribosomes; this in turn leads to the cessation of host protein synthesis. Since phage mRNA contains little or no CUG codons corresponding to leucyl $tRNA_1$ its potential for protein synthesis remains unimpaired". It should be noted however, that new findings by Sueoka and co-workers (Neidhardt, personal communication, 1975) have caused that laboratory to modify the original idea and suggest that perhaps more than leucyl tRNA species was involved.

In another report by Vold (1973) differences between the tRNA species of spores and exponentially growing cells of Bacillus subtilus were compared by reversed phase chromatography. Three patterns of elution profile for vegetative cell and spore tRNAs were observed for different aminoacyl tRNAs, namely (1) no change; (2) change in the ratios of existing peaks; and (3) the appearance and disappearance of some unique peaks. Here again, the changes noted in tRNA profile at different developmental phases indicate a possible regulatory role.

On the basis of the previously discussed findings and the results reported earlier in this paper, it was postulated that the non-chloroplast valyl tRNAs play a regulatory role in chloroplast development in Euglena. With this in mind, the isoaccepting valyl tRNA species of dark adapted wild type cells were chromatographically resolved by the RPC-5 method. It was of interest to know if the elution profile would resemble that of wild type green cells, bleached cells or some intermediate situation. If the hypothesis that non-chloroplast valyl tRNAs should play a

regulatory role in chloroplast development in Euglena had any merit, then one would expect the elution profile of these species in dark adapted cells to resemble those noted for bleached cells. The elution profile shown in Figure 12 did indeed resemble that obtained from bleached cells, with one exception. Peak I, attributed to valyl tRNA of chloroplast origin, was present. This finding was expected, since it had been shown that the chloroplast synthetase could charge tRNAs from dark adapted cells to the same level as those from light grown green cells. The differences in elution profiles between green and bleached cells could be explained by major alterations in nucleotide sequence and/or major changes in secondary or tertiary structure of such tRNAs. It was, however, tempting to speculate that the differences seen were due to the relatively minor conversion of one species into the other. This could be accomplished by modification of one or a few nucleotides in those tRNA molecules which, in turn, might result in differences in the elution profile of such tRNAs as well as differences in their ability to function in vivo. A vast literature exists pertaining to the role of modified nucleotides and their effect on tRNA function. In general, it appears that modifications result in an alteration of the ability of the tRNAs to bind to the ribosome, but not to their capacity to be aminoacylated.

One illustrative example was described in the work of Gefter and Russell (1969). These workers isolated three forms of the amber suppressor tyrosyl tRNA of E. coli, each of which differed in degree of modification at the base adjacent to the 3' nucleo-

tide of the anticodon. The degree of modification represented successive reactions in the biosynthesis of such tRNA molecules. The differences did not affect the ability of tRNAs to accept an amino acid but did alter their ribosomal binding capacity. The fully modified form was found to be most active, in terms of binding, while the partially modified form showed 54% of this activity and the completely unmodified form only 14% of this binding capacity.

Whether or not the quantitative differences in the non-chloroplast valyl tRNAs represent the conversion of one form into the other will only be determined by base sequence analysis of these two tRNA species.

The conversion hypothesis is also in good agreement with the previously discussed work of Zeldin and Schiff (1968), who showed that light induced labelling of non-chloroplast species appeared to be due to a turnover, rather than a net increase, in labelled RNA. This could be determined in the system investigated in this study by performing an experiment with double labelling. Cells could be grown in the dark in the presence of ^{14}C uridine and just prior to turning on the lights ^{3}H uridine would be added. The tRNAs could then be isolated and chromatographically analyzed. If the quantitative differences represented a conversion of one species into the other, then one peak (peak II) might contain a greater ratio of $^{14}C/^{3}H$. This type of experiment remains to be done.

To help clarify many of the unresolved issues discussed in

this work it is proposed that the following investigations be undertaken in the future:

1. The determination of codon recognition by the various isoaccepting valyl tRNAs via ribosome binding studies and by incorporation of valine in the presence of various tri and polynucleotides. Of prime interest are the non-chloroplast specific species. A stronger argument for a regulatory role of tRNAs could be established if one could isolate newly transcribed mRNAs during the chloroplast development process and correlate their codon sequence with the increase or decrease in level of specific tRNAs, a formidable task indeed.

2. The determination of whether there are chloroplast specific tRNAs and tRNA synthetases for other amino acids and the investigation of whether they are light inducible or constitutive.

3. The determination of whether the non-chloroplast tRNAs for other amino acids show quantitative differences in going from light to dark, using both dark adapted and bleached cells.

These investigations may shed light on the relative role of the proplastid and cytoplasm in chloroplast development in Euglena. Most importantly, one should avoid taking the "thermodynamic" approach (namely being concerned with the initial and final state) for the determination of the presence or absence of specific tRNA molecules. It is of major importance to follow the changes in tRNA species from a developmental standpoint and attempt to correlate such quantitative and qualitative differences with the appearance of new proteins or with the increase of those already present as the chloroplast develops.

TABLE A

EUGLENA MEDIUM (pH 3.5)*

Constituent	Quantity per liter
KH_2PO_4	0.4 gms
$MgSO_4 \cdot 7H_2O$	0.5 gms
$CaCO_3$	0.2 gms
L-Glutamic acid	5.0 gms
DL-Malic acid	2.0 gms
$(NH_4)_2HPO_4$	0.2 gms
Metals mix #49** (see below)	130.0 mgs
$FeCl_3 \cdot 6H_2O$**	5.0 mgs
Thiamine HCl**	1.0 mgs
Cyanocobalamin** (B-12)	0.2 µgms

Hutner's trace metals mix #49**	gms/100 liters basal medium
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	70
$MnSO_4 \cdot 4H_2O$	31
$ZnSO_4 \cdot 7H_2O$	22
$CuSO_4 \cdot 5H_2O$	4 (2.5 for anhydrous)
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.72
$CoSO_4 \cdot 7H_2O$	2.4
$Na_3VO_4 \cdot 16H_2O$	0.46
H_3BO_3	0.57

The above metals mix was used at 13 mg%; i.e., 130 mg/1000 ml of growth medium

* Greenblatt, C., and Schiff, J., Protozool., 6:23 (1959)

** Added from separate stock

TABLE B

Chloroplast Components Affected and Not Affected in Mixotrophic *Chlamydomonas reinhardi* (ac-20)

Affected	Not Affected
1. RuDP carboxylase	1. Phosphoribosomerase
2. Cytochrome 559	2. Phosphoribulokinase
3. Q (the quencher of fluorescence PSII)	3. PGA-kinase
4. Membrane organization	4. G-3-P-dehydrogenase (NAD)
5. Pyrenoid formation	5. G-3-P-dehydrogenase (NADP)
	6. Triosephosphate isomerase
	7. FDP aldolase
	8. Total quinone
	9. Plastocyanin
	10. Cytochrome 553
	11. Cytochrome 564
	12. P700
	13. Ferredoxin
	14. Ferredoxin NADP reductase
	15. Chlorophyll (reduced at most by half)
	16. Carotenoid (reduced by half)
	17. Membrane formation (reduced by half)
	18. Eyespot formation
	19. Starch synthesis

TABLE 1

Aminoacylation of ^{3}H valine to tRNAs from Green and Bleached Euglena by the Homologous or Heterologous Aminoacyl tRNA Synthetases

Source of tRNA	Source of Enzyme	CPM/µg tRNA
Wild type (green)	Wild type (green)	125
Wild type (green)	Bleached	111
Bleached	Wild type (green)	154
Bleached	Bleached	155

Standard reaction conditions as described in "Materials and Methods" were followed.

TABLE 2

Effect of 2-Mercaptoethanol on the Aminoacylation of ^{3}H Valine to tRNA from Green and Bleached Euglena Cells

Source of tRNA	Source of Enzyme	CPM/µg tRNA +BME	CPM/µg tRNA -BME
Wild type (green)	Wild type (green)	92	93
Wild type (green)	Bleached	84	83
Bleached	Wild type (green)	113	111
Bleached	Bleached	112	113

Standard reaction conditions as described in "Materials and Methods" were followed. BME when present was added at 10 µM/ml

TABLE 3

Effect of pH on the Aminoacylation of H^3 Valine to Valyl tRNA*

Source of tRNA	Source of Enzyme	pH	4.5	5.0	5.5	6.0	6.2	6.4	6.6	6.8	7.5
Wild type (green)	Wild type (green)		77	91	103	102	103	103	103	102	100
Wild type (green)	Bleached		70	71	76	85	86	86	86	85	86
Bleached	Wild type (green)		91	121	129	137	141	137	137	139	142
Bleached	Bleached		95	110	129	135	135	135	137	136	142

Standard reaction conditions as described in "Materials and Methods" were followed except that the pH of the reaction mix was varied as indicated above.

*expressed as CPM/µg tRNA

TABLE 4

Effect of pH on the Enzymatic Deacylation of ^{3}H Valine from tRNA in the Homologous Wild Type Green System

		^{3}H valyl tRNA	ATP*	Enzyme**	Incubation Time (Min)	CPM	% Deacylation
A.	pH 7.5						
	1.	+	-	-	0	2460	-
	2.	+	-	-	30	2117	14
	3.	+	+	-	30	2157	12
	4.	+	-	+	30	1952	21
	5.	+	+	+	30	800	67
B.	pH 4.5						
	1.	+	-	-	0	2000	-
	2.	+	-	-	30	1700	15
	3.	+	+	-	30	1676	16
	4.	+	-	+	30	1690	15
	5.	+	+	+	30	1500	25

* ATP added as in standard reaction mix.

** Enzyme added as in standard reaction mix.

TABLE 5

Effect of Increasing Green tRNA Concentration on the Level of Aminoacylation with Homologous and Heterologous Enzyme

Source of tRNA	Source of Enzyme	Amount of tRNA (µg/reaction)	CPM/µg tRNA
Wild type (green)	Wild type (green)	5	99
Wild type (green)	Bleached	5	90
Wild type (green)	Wild type (green)	10	100
Wild type (green)	Bleached	10	92

Standard reaction conditions as described in "Materials and Methods" were followed.

TABLE 6

Effect of Enzyme Concentration on the Extent of Aminoacylation of ^{3}H Valine to tRNA in the Wild Type (Green) System

Amount of Enzyme (µg protein/reaction)	CPM/µg tRNA Incubation Time (min) 10	20	40
12	50	73	78
120	102	103	102
240	102	101	102
480	96	98	96

Standard reaction conditions as described in "Materials and Methods" were followed except that enzyme concentration was varied as indicated.

TABLE 7

Effect of the Addition of CTP on the Aminoacylation of ^{14}C Valine to tRNA from Green and Bleached Euglena by the Homologous and Heterologous Aminoacyl tRNA Synthetases

Source of tRNA	Source of Enzyme	CTP*	CPM/µg tRNA	% change
Wild type (green)	Wild type (green)	-	72	-
Wild type (green)	Wild type (green)	+	47	-34
Wild type (green)	Bleached	-	65	
Wild type (green)	Bleached	+	48	-26
Bleached	Wild type (green)	-	92	
Bleached	Wild type (green)	+	62	-32
Bleached	Bleached	-	91	
Bleached	Bleached	+	67	-26

Standard reaction conditions as described in "Materials and Methods" were followed.

* When CTP was present, it was added at a concentration of 5µM/ml

TABLE 8

Effect of Addition of Unlabelled Amino Acids on the Aminoacylation of ^{14}C Valine to Valyl tRNA in Homologous Systems Under Conditions of Non-Limiting Valine

Unlabelled Amino Acid Added	^{14}C Valine Added	System Studied: Green tRNA x Green Enzyme (CPM/µg tRNA)	System Studied: Bleached tRNA x Bleached Enzyme (CPM/µg tRNA)
ISOLEUCINE			
0	$5x10^{-5}M$	99	146
$5x10^{-5}M$	$5x10^{-5}M$	100	148
$1x10^{-4}M$	$5x10^{-5}M$	101	145
$1x10^{-3}M$	$5x10^{-5}M$	99	145
PHENYLALANINE			
$4.2x10^{-5}M$	$5x10^{-5}M$	103	140
$8.4x10^{-5}M$	$5x10^{-5}M$	98	148
$8.4x10^{-4}$ M	$5x10^{-5}M$	100	137
LEUCINE			
$5x10^{-5}M$	$5x10^{-5}M$	101	143
$1x10^{-4}M$	$5x10^{-5}M$	99	147
$1x10^{-3}M$	$5x10^{-5}M$	98	139
ALANINE			
$5x10^{-5}M$	$5x10^{-5}M$	101	149
$1x10^{-4}M$	$5x10^{-5}M$	101	144
$1x10^{-3}M$	$5x10^{-5}M$	101	143

TABLE 9

Aminoacylation of ^{3}H Valine to tRNA from Green and Bleached Euglena by Enzyme Obtained from Green Cells or Chloroplasts

Source of tRNA	Source of Enzyme	CPM/µg tRNA
Wild type (green)	Wild type (green)	427
Wild type (green)	Chloroplast (a)	41
Bleached	Chloroplast (a)	0.7
Wild type (green)	Wild type (green)	427
Wild type (green)	Chloroplast (b)	41
Bleached	Chloroplast (b)	7

Reaction mix contained per ml: 50 µM Hepes, pH 7.5; 0.5 µM ATP, pH 7.0; 10 µM magnesium acetate; 5 µM BME; 1.2 µc ^{3}H valine sp. act. 6.7 C/mmole; 61.5 µg green tRNA or 103.5 µg colorless tRNA; 190 µg enzyme protein (green enzyme) or 36 µg enzyme protein (chloroplast enzyme). Reaction time was 60 minutes.

(a) enzyme isolated from purified chloroplasts

(b) chloroplast enzyme activity separated from cytoplasmic activity by hydroxylapatite chromatography of whole green cell extracts

TABLE 10

Aminoacylation of ^{3}H Valine to tRNA Derived from Dark Adapted or Wild Type Euglena by Enzyme Obtained from Green Cells or from Isolated Chloroplasts

Source of tRNA	Source of Synthetase	CPM/ug tRNA
Wild type (green)	Wild type (green)	410
Wild type (dark adapted)	Chloroplast (a)	40
Wild type (dark adapted)	Chloroplast (b)	38

Reaction conditions were the same as those described in Table 9.

(a) enzyme isolated from purified chloroplasts

(b) chloroplast enzyme activity separated from cytoplasmic activity by hydroxylapatite chromatography of whole green cell extracts

TABLE 11

Aminoacylation of ^{14}C Valine to tRNA Derived from Wild Type (Green) Cells by Enzymes Obtained from Wild Type or Bleached Euglena or Purified Chloroplasts

Source of tRNA	Source of Enzyme	Relative % Aminoacylation
Wild type (green)	Wild type (green)	100
Wild type (green)	Bleached	90-93
Wild type (green)	Purified chloroplasts	8-10

Standard reaction conditions as described in "Materials and Methods" were followed.

Figure 1. Kinetics of charging of valine to valyl tRNA

● Green tRNA x green enzyme

O Green tRNA x bleached enzyme

△ Bleached tRNA x bleached enzyme

▲ Bleached tRNA x green enzyme

FIGURE 1.

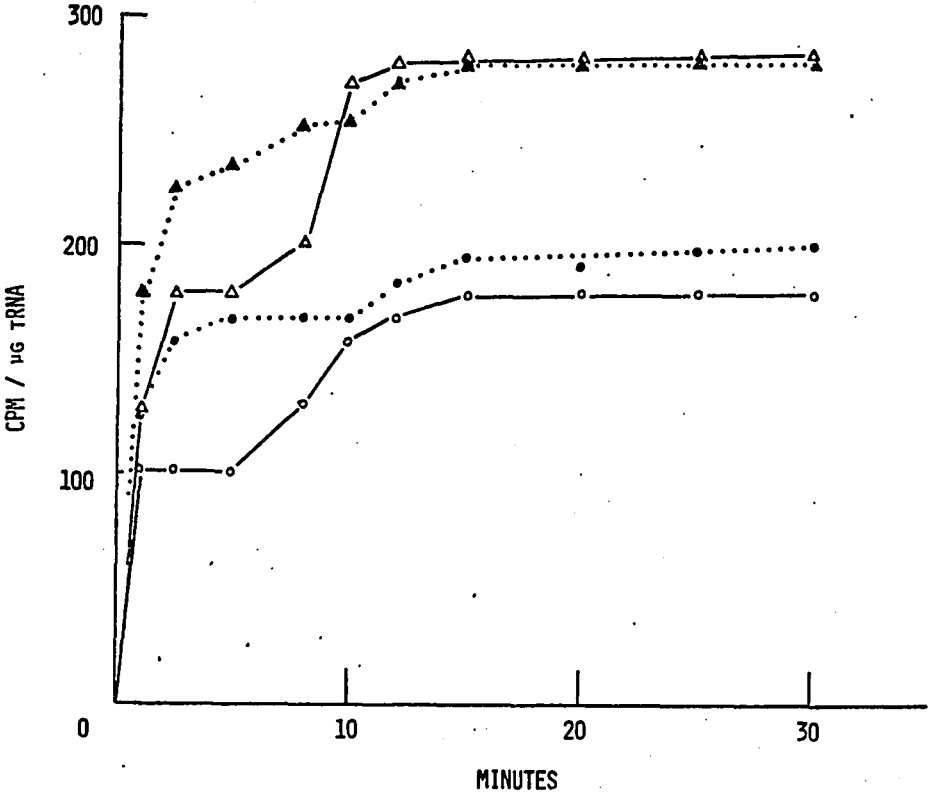


Figure 2. Effect on the aminoacylation of ^{14}C valine to valyl tRNA in the homologous green system by the addition of a large excess of cold valine or isoleucine

o addition of 10x excess cold isoleucine

● additionnof 10x excess cold valine

FIGURE 2.

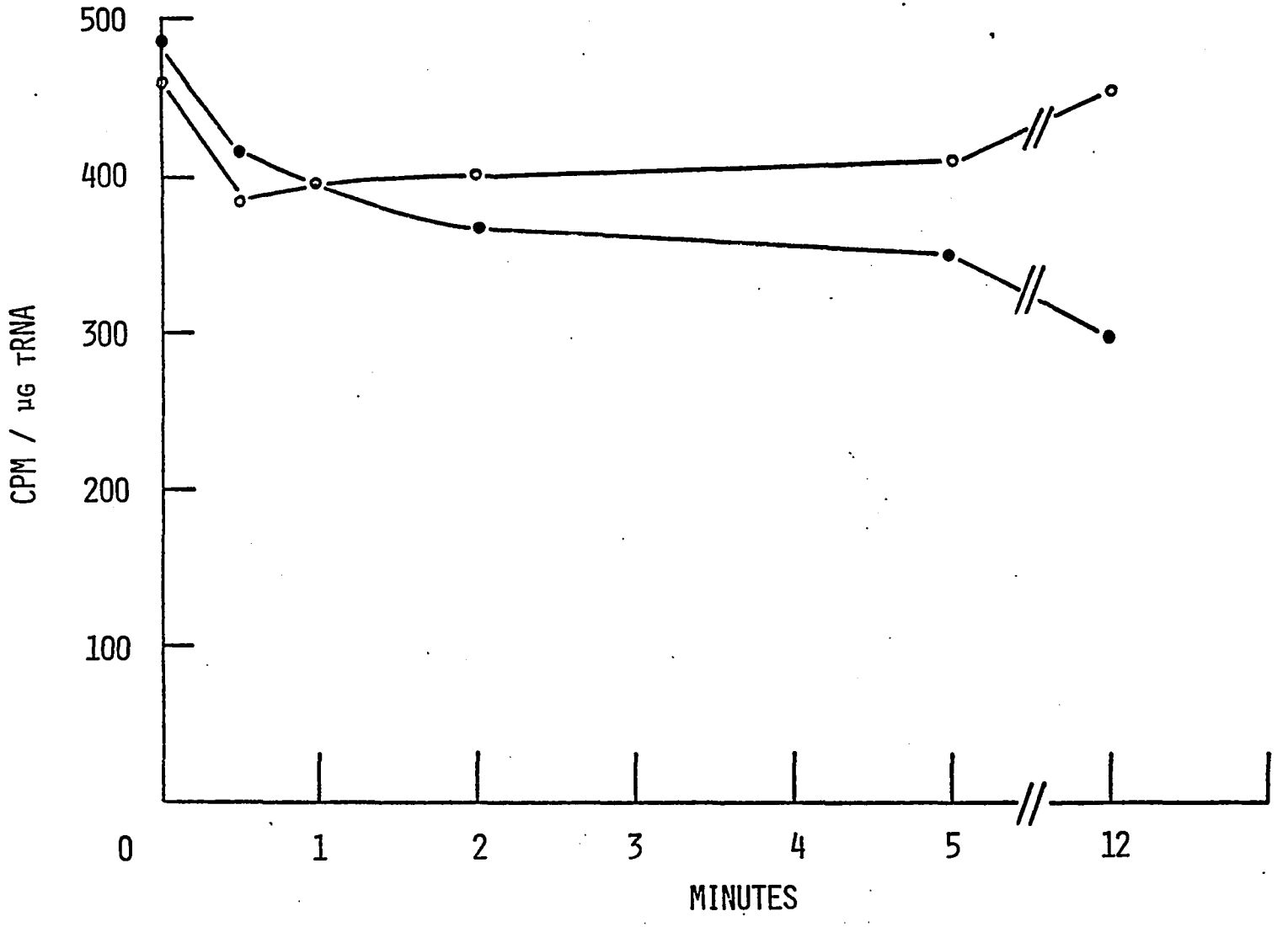


Figure 3. Effect of Mg^{++}/ATP and Mg^{++} on the aminoacylation of valine to valyl tRNA at 5 mM ATP

- ● Green tRNA x green enzyme
- △ Green tRNA x bleached enzyme
- ○ Bleached tRNA x bleached enzyme
- ▲ Bleached tRNA x green enzyme

FIGURE 3.

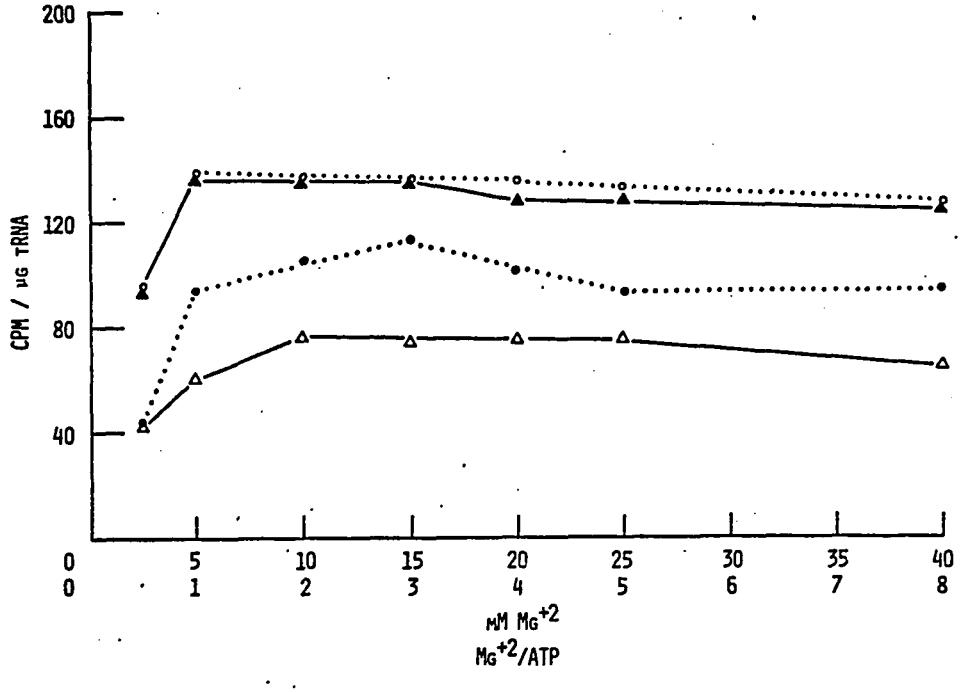

200
160
120
80
40
0
CPM / µG tRNA
0
5
1
10
2
15
3
20
4
25
5
30
6
35
7
40
8
mM Mg+2
Mg+2/ATP

Figure 4. Effect of Mg^{++}/ATP and Mg^{++} on the aminoacylation of valine to valyl tRNA at 0.5 mM ATP

● Green tRNA x green enzyme

○ Green tRNA x bleached enzyme

△ Bleached tRNA x bleached enzyme

▲ Bleached tRNA x green enzyme

FIGURE 4.

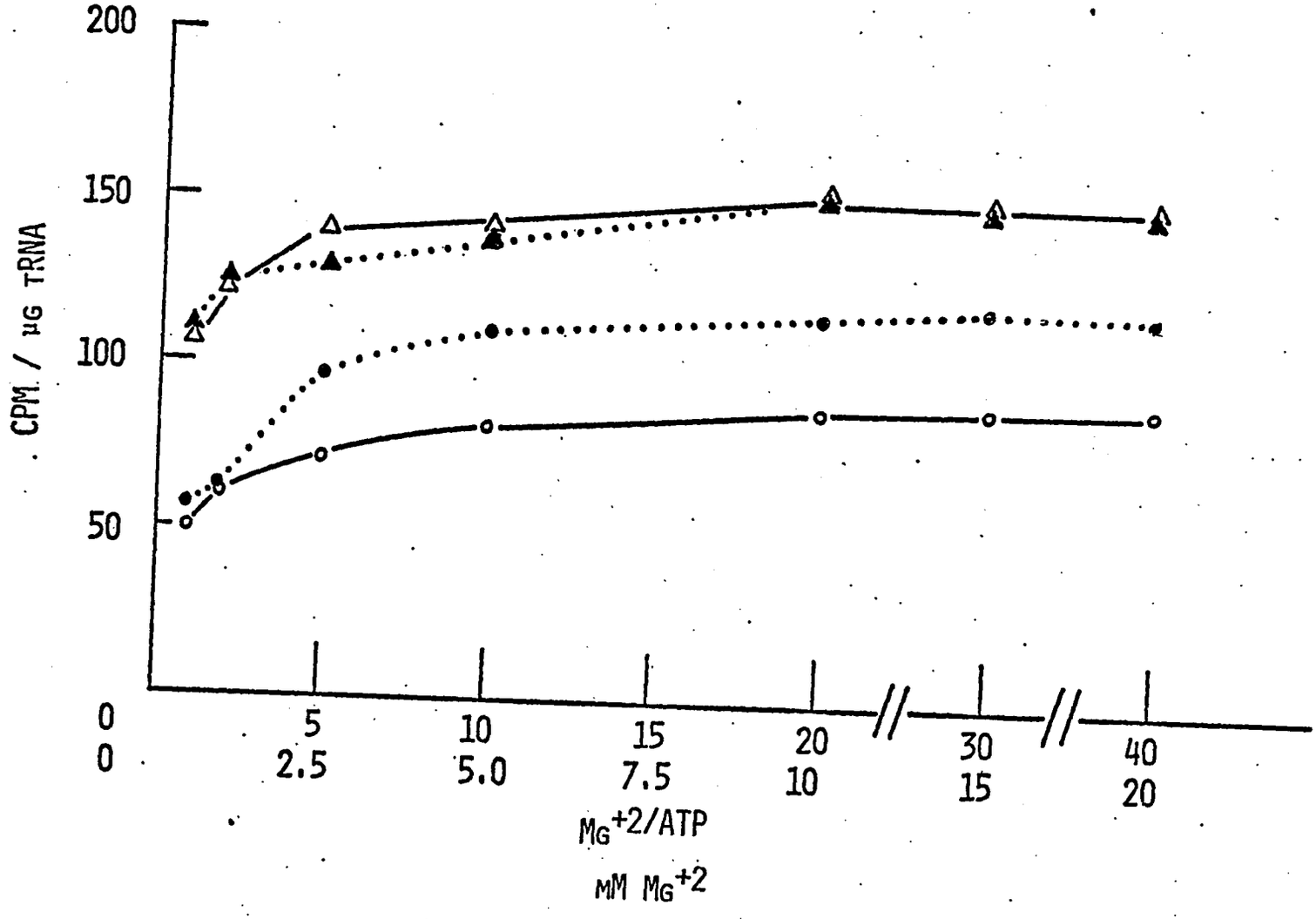

200
150
100
50
0
CPM / µG tRNA
0
0
5
2.5
10
5.0
15
7.5
20
10
30
15
40
20
Mg+2/ATP
mM Mg+2

Figure 5. The effect of Mg^{++} on the aminoacylation of ^{3}H valine to green and bleached tRNA by enzyme from isolated chloroplasts

□ Colorless tRNA, 20'

○ Colorless tRNA, 40'

△ Colorless tRNA, 60'

■ Green tRNA, 20'

● Green tRNA, 40'

▲ Green tRNA, 60'

FIGURE 5.

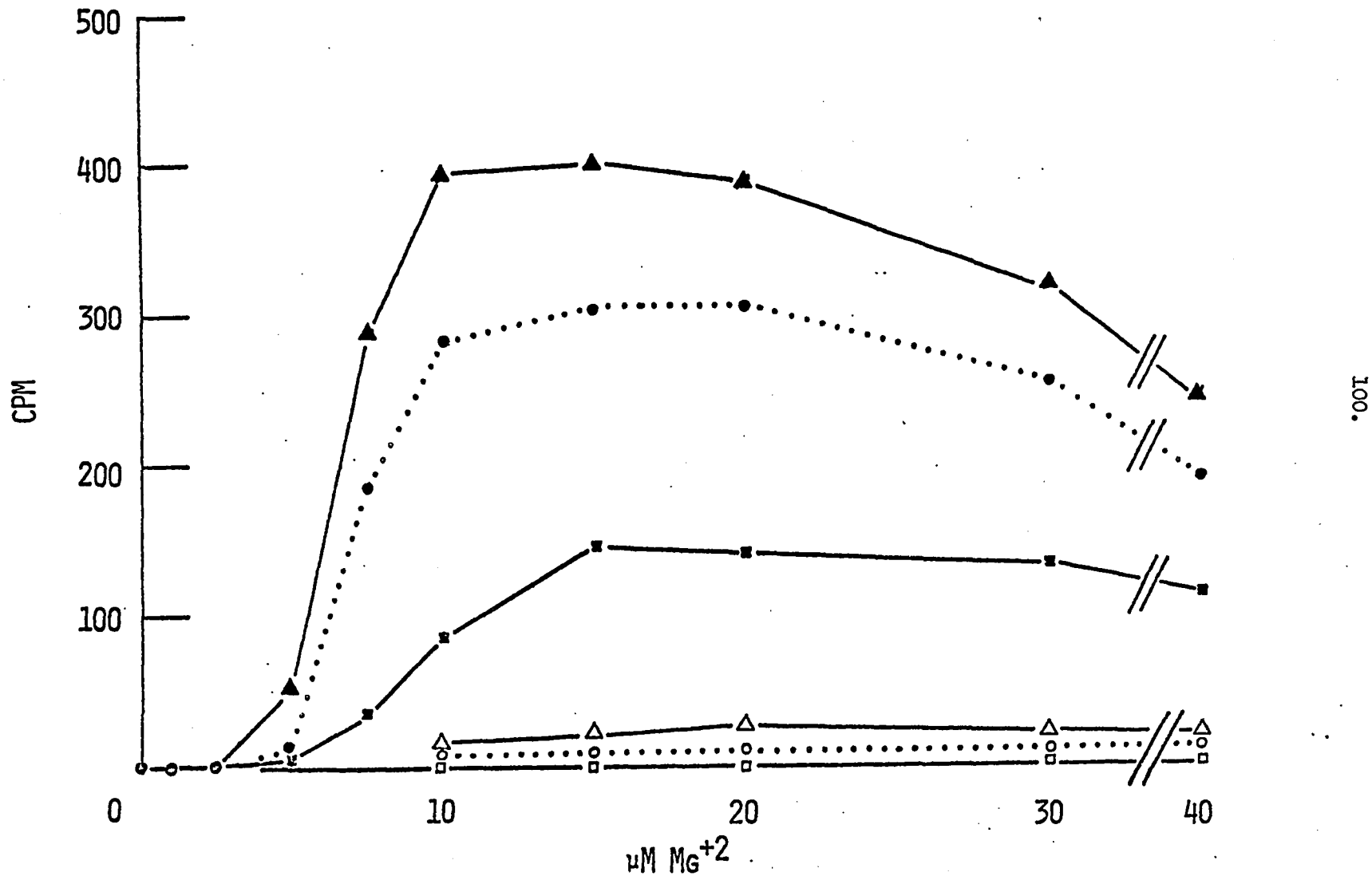


Figure 6. G-100 Sephadex chromatography of bulk tRNA isolated from green cells.

FIGURE 6

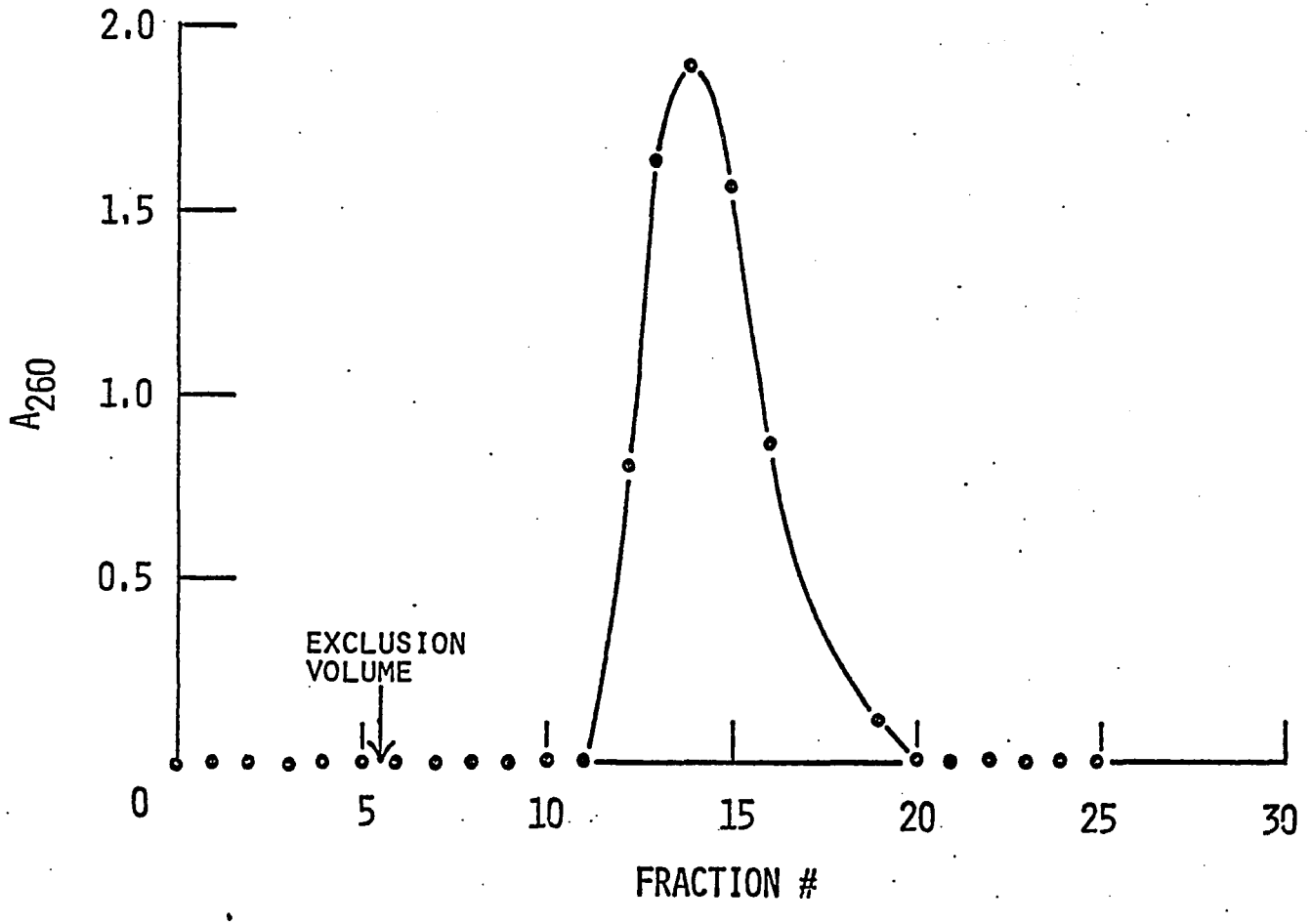


Figure 7. G-100 Sephadex chromatography of bulk tRNA isolated from bleached cells.

FIGURE 7.

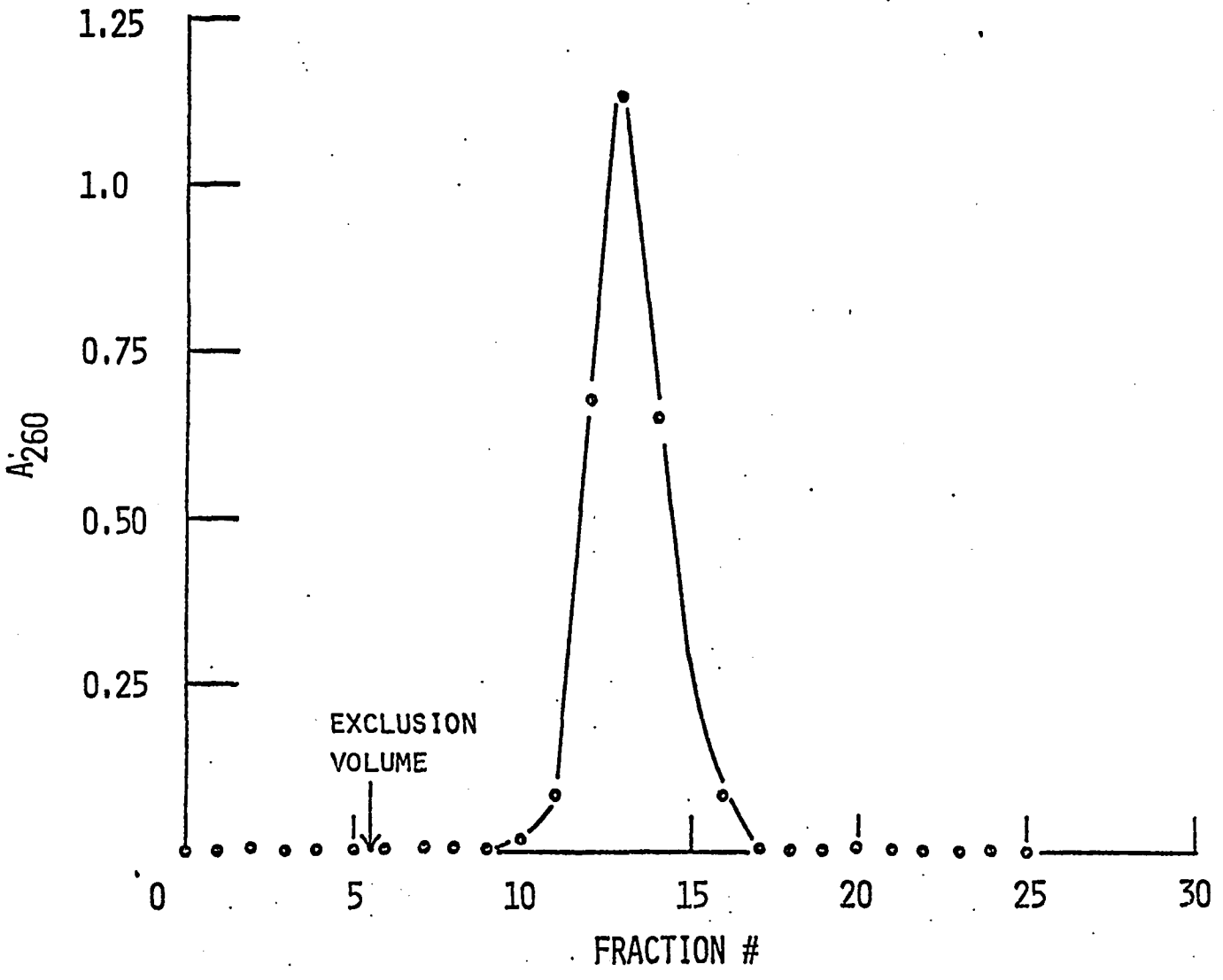

1.25
1.0
0.75
0.50
0.25
A_{260}
EXCLUSION
VOLUME
0
5
10
15
20
25
30
FRACTION #

Figure 8. The Reversed Phase (RPC-5) Chromatographic separation of the isoaccepting species of valyl tRNA from green cells charged by green enzyme.

FIGURE 8.

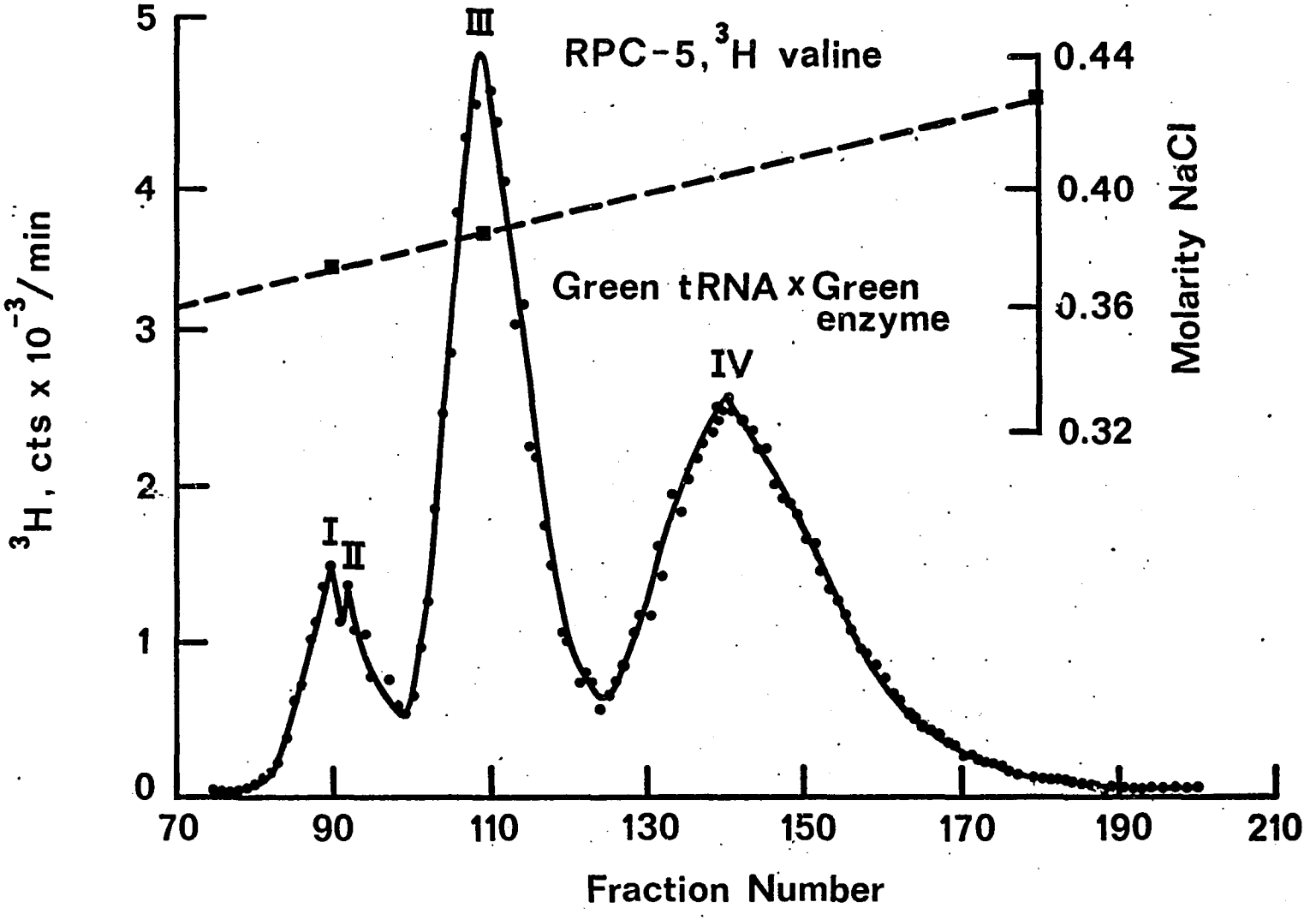

RPC-5, ^{3}H valine
Green tRNA x Green enzyme
^{3}H, cts x 10^{-3}/min
Molarity NaCl
0.44
0.40
0.36
0.32
I
II
III
IV
0
1
2
3
4
5
70
90
110
130
150
170
190
210
Fraction Number

Figure 9. The Reversed Phase (RPC-5) Chromatographic separation of the isoaccepting species of valyl tRNA from green cells charged by bleached enzyme.

FIGURE 9.

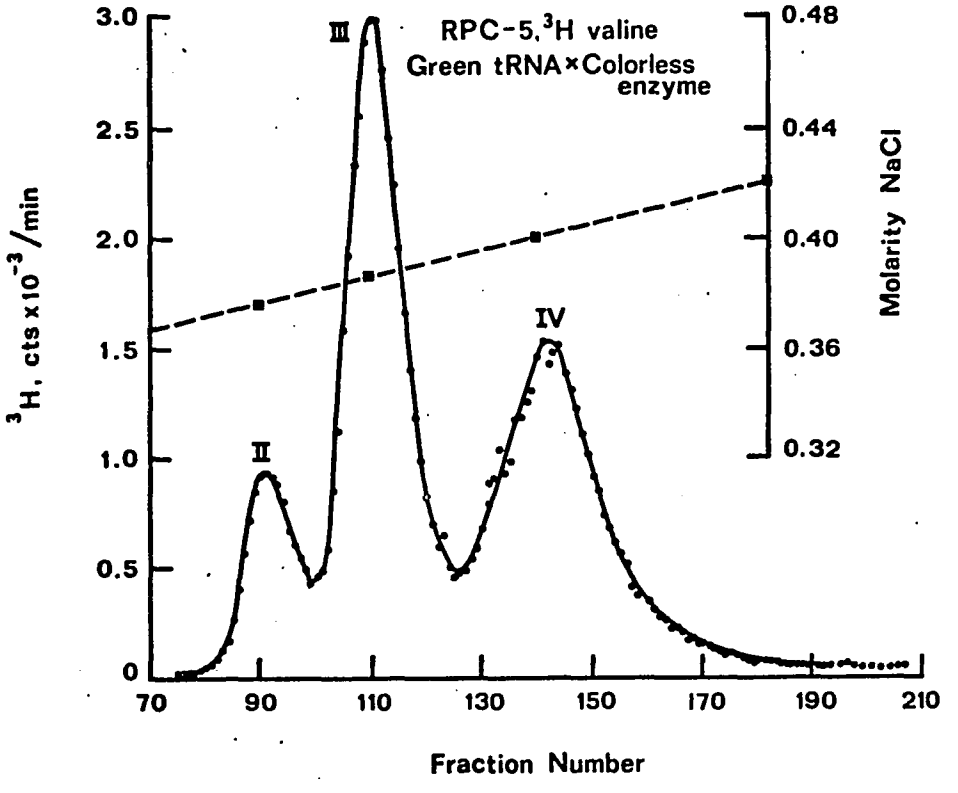

RPC-5, ^{3}H valine
Green tRNA×Colorless enzyme
II
III
IV
^{3}H, cts x10^{-3}/min
Molarity NaCl
Fraction Number
3.0
2.5
2.0
1.5
1.0
0.5
0
0.48
0.44
0.40
0.36
0.32
70
90
110
130
150
170
190
210

Figure 10. Reversed Phase (RPC-5) Chromatographic separation of the isoaccepting species of valyl tRNA from bleached cells charged by green enzyme.

FIGURE 10.

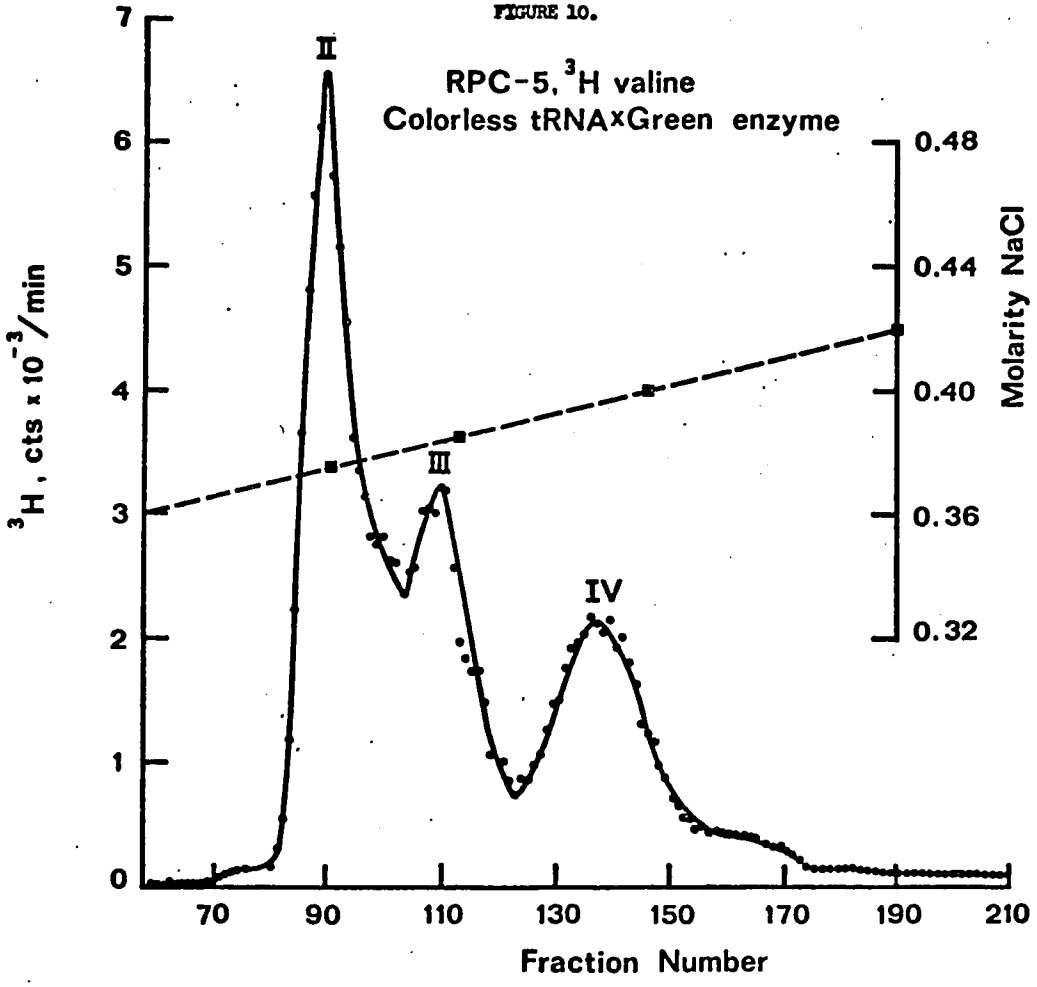

RPC-5, ^{3}H valine
Colorless tRNA×Green enzyme
II
III
IV
^{3}H, cts x 10^{-3}/min
Molarity NaCl
0.48
0.44
0.40
0.36
0.32
0
1
2
3
4
5
6
7
70
90
110
130
150
170
190
210
Fraction Number

Figure 11. Reversed Phase (RPC-5) Chromatographic separation of the isoaccepting species of valyl tRNA from bleached cells charged by bleached enzyme.

FIGURE 11.

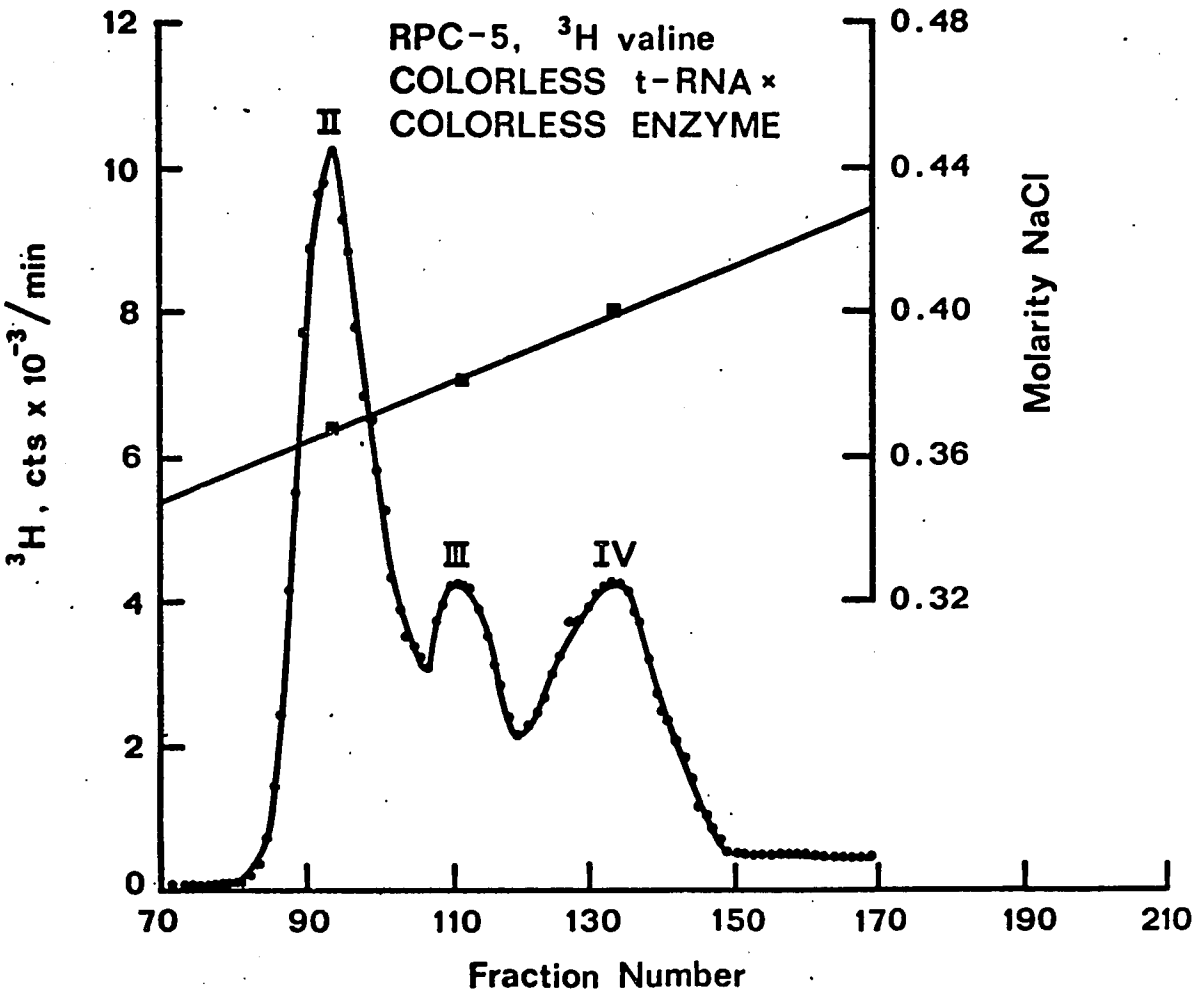
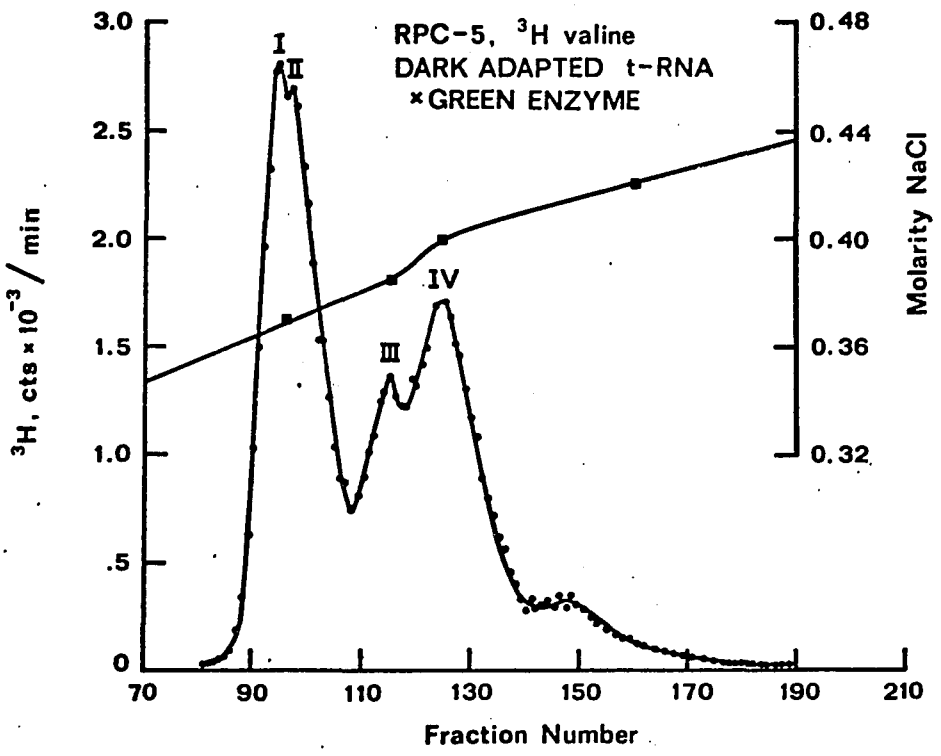

RPC-5, ³H valine
COLORLESS t-RNA ×
COLORLESS ENZYME
II
III
IV
³H, cts x 10⁻³/min
Molarity NaCl
Fraction Number
0
2
4
6
8
10
12
0.32
0.36
0.40
0.44
0.48
70
90
110
130
150
170
190
210

Figure 12. Reversed Phase (RPC-5) Chromatographic separation of the isoaccepting species of valyl tRNA from dark adapted cells charged by green enzyme.

FIGURE 12.


RPC-5, ^{3}H valine
DARK ADAPTED t-RNA
×GREEN ENZYME
I
II
III
IV
^{3}H, cts × 10^{-3} / min
Molarity NaCl
Fraction Number
3.0
2.5
2.0
1.5
1.0
.5
0
0.48
0.44
0.40
0.36
0.32
70
90
110
130
150
170
190
210

LITERATURE CITED

Allende, C.C. and Allende, J.E. (1964) Purification and substrate specificity of arginyl ribonucleic acid synthetase from rat liver. J. BIOL. CHEM. 239: 1102-1106

Allende, C.C., Allende, J.E., Gattica, M., Celis, J., Mora, G., and Matamola, M. (1966) The aminoacyl ribonucleic acid synthetases. I. Properties of threonyl adenylate enzyme complex. J. BIOL. CHEM. 241: 2245-51

Anthony, D.D., Starr, J.L., Kerr, D.S. and Goldwaith, D.A., (1963) The incorporation of nucleotides into amino acid tRNA. II. Evidence for the separate enzymatic sites for incorporation of adenosine 5'-monophosphate and cytidine 5'-monophosphate, J. BIOL. CHEM. 238: 690-96

Apgar, J., Everett, G.A., and Holley, R.W. (1966) Analyses of large oligonucleotide fragments obtained from a yeast alanine tRNA by partial digestion with ribonuclease T_1. J. BIOL. CHEM. 241: 1206-1211

Baldwin, A.N., and Berg, P. (1966) tRNA induced hydrolysis of valyl adenylate bound to isoleucyl ribonucleic acid synthesis. J. BIOL. CHEM. 241: 839

Barnett, W.E., Pennington, C.J., and Fairfield, S.A. (1969) Induction of Euglena transfer RNAs by light. PROC. NATL. ACAD. SCI. 63: 1261-1269

Ben Shaul, Y., Schiff, J.A. and Epstein, J.T. (1964) Studies of chloroplast development in Euglena. VII. Fine structure of the developing plastid. PLANT PHYSIOL. 39: 231-240

Bergman, F.H., Berg, P. and Diekman (1961) The enzymatic synthesis of aminoacyl derivatives of RNA. II. The preparation of leucyl, valyl, isoleucyl, and methionyl RNA synthetases from E. coli. J. BIOL. CHEM. 236: 1735-40

Bishop, N.I. (1966) Partial reactions of photosynthesis and photoreduction. ANN, REV. PLANT PHYSIOL. 17: 185

Blamire, J., Flechtner, V.R. and Sager, R. (1974) Regulation of nuclear DNA replication by the chloroplast in Chlamydomonas. PROC. NATL. ACAD. SCI. 71: 2867-2871

Bleustein, H.G., Allende, C.C., Allende, J.E. and Cantoni, G.L. (1968) Seryl transfer ribonucleic acid synthetase from Baker's Yeast. J. BIOL. CHEM. 243: 4693-4699

Bottomley, W., Smith, H.J., and Bogorod, L. (1971) RNA polymerases of maize: partial purification and properties of the chloroplast enzyme. PROC. NATL. ACAD. SCI. 68: 2412

Bottomley, W., Whitfield, P.R. and Spencer, D. (1972) Stimulation of chloroplast DNA-dependent RNA polymerase by exogenous DNA. ARCH. BIOCHEM. BIOPHYS. 151: 35

Bonnet, J., and Ebel, J. (1972) Interpretation of incomplete reactions in tRNA aminoacylation. EUR. J. BIOCHEM. 31: 335-344

Bourque, D.P. and Wildman, S.G. (1973) Evidence that nuclear genes code for several chloroplast ribosomal proteins. BIOCHEM. BIOPHYS. RES. COMMUN. 50: 532-537

Brawerman, G. and Eisenstadt, J.M. (1964) DNA from chloroplasts of Euglena gracilis. BIOCHEM. BIOPHYS. ACTA 91: 477-485

Burkhard, G., Guillemaut, P. and Weil, J.H. (1970) Comparative studies of the tRNAs and the aminoacyl tRNA synthetases from the cytoplasm and the chloroplasts of Phaseolus vulgaris. BIOCH EM. BIOPHYS. ACTA 224: 184-198

Burkhard, G., Vaultier, J.P. and Weil, J.H. (1972) Differences in the level of plastid-specific tRNAs in chloroplasts and etioplasts of Phaseolus vulgaris. PHOTOCHEM. 11: 1351-1353

Britten, R.J., and Kohne, D.E. (1966) Nucleotide sequence repetition in DNA. CARNEGIE INSTITUTE WASHINGTON YEARB. 65: 78-106

Calendar, R. and Berg, P. (1966) Purification and physical characterization of tyrosol ribonucleic acid synthetases from E. coli and Bacillus subtilus. BIOCHEM. 5: 1681

Chan, P.H. and Wildman, S.G. (1972) Chloroplast DNA codes for the primary structure of the large subunit of Fraction I protein. BIOCHEM. BIOPHYS. ACTA 277: 677-680

Chun, E.H.L., Vaughn, M.H. and Rich, A. (1963) The isolation and characteristics of DNA associated with chloroplast preparations. J. MOL. BIOL. 7: 130-141

Clark, M.F., Matthews, R.E.F. and Ralph, R.K. (1964) Ribosomes and polyribosomes in Brassica pekinensis. BIOCHEM. BIOPHYS. ACTA 91: 289

Eisenstadt, J.M. and Brawerman, G. (1964) The protein synthesizing systems from the cytoplasm and the chloroplasts of Euglena gracilis. J. MOL. BIOL. 10: 392-402

Fresco. J., Adams, A., Ascione, R., Henley, D., and Lindoh, T. (1966) Tertiary structure in transfer RNAs. COLD SPRING HARBOR SYMPOSIUM QUANTITATIVE BIOLOGY 31: 527

Fuller, R.C. and Gibbs, M. (1959) Intracellular and phylogenic distribution of ribulose 1,5 diphosphate carboxylase and D- glyceraldehyde 3-phosphate dehydrogenase. PLANT PHYSIOL. 34: 324

Furth, J.J., Hurwitz, J., Krug, R. and Alexander, M. (1961) The incorporation of adenylic and cytidylic acids into ribonucleic acid. J. BIOL. CHEM. 236: 3317-22

Gartland, W.J. and Sueoka, N. (1966) Two incontrovertible forms of tryptophanyl sRNA in E. coli. PROC. NATL. ACAD. SCI. 55: 948-955

Gefter, M.L. and Russel, R.L. (1969) Role of modifications in tyrosine tRNA: A modified base affecting ribosome binding J. MOL. BIOL. 39: 145-157

Gibor, A. and Izawa, M. (1963) The DNA content of the chloroplasts of Acetabularia. PROC. NATL. ACAD. SCI. 50: 1164-1169

Gibor, A. and Granick, S. (1962) The plastid system of normal and bleached Euglena gracilis. J. PROTOZOOL. 9: 327-334

Gibor, A. (1967) DNA synthesis in "Biochemistry of Chloroplasts". (T.W. Goodwin, ed.) II ACADEMY PRESS (New York and London) pp. 321-328

Goffeau, A. and Brachet, J. (1965) DNA-dependent incorporation of amino acids into the proteins of chloroplasts isolated from anucleate Acetabularia fragments. BIOCHEM. BIOPHYS. 95: 302-313

Goins, D.J., Reynolds, R.D., Schiff, J.A. and Barnett, W.E. (1973) A cytoplasmic regulatory mutant of Euglena: Constitutivity for the light inducible chloroplast transfer RNAs. PROC. NATL. ACAD. SCI. 70: 1749-1752

Goodenough, U.W., Togasaki, R.K., Paszewski, A. and Levine, R.P. (1971) Inhibition of chloroplast ribosome formation by gene mutation in Chlamydomonas reinhardi. In "Autonomy and Biogenesis of Mitochondria and Chloroplasts". (N.K. Boardman, A.W. Linnane and R.M. Smillie, eds.) NORTH-HOLLAND/AMERICAN ELSEVIER PUBL. pp. 224-234

Goodwin, T.W. (1965) In "Chemistry and Biochemistry of Plant Pigments". (T.W. Goodwin, ed.) ACAD. PRESS (London and New York) pp. 175-196

Greenblatt, C. and Schiff, J. (1959) A pheophytin-like pigment in dark adapted Euglena gracilis. J. PROTOZOOL. 6: 23-28

Guderian, R.H., Pulliam, R.L. and Gordon, M.P. (1972) Characterization and fractionation of tobacco leaf tRNA. BIOCHEM. BIOPHYS. ACTA 262: 50

Holowinsky, A. and Schiff, J.A. (1968) Potentiation of chloroplast development in Euglena by preillumination. PLANT PHYSIOL. 43: 5-7

Harris, E.H., Preston, J.F. and Eisenstadt, J.M. (1973) Amino acid incorporation and products of protein synthesis in isolated chloroplasts of Euglena gracilis. BIOCHEM. 12: 1227-1233

Hecker, L.I., Egan, J., Reynolds, R.J., Nix, C.E., Schiff, J.A. and Barnett, W.E. (1974) The sites of transcription and translation for Euglena chloroplastic aminoacyl tRNA synthetases. PROC. NATL. ACAD. SCI. 71: 1910-1914

Hele, P. (1964) Evidence suggesting the participation of a polyribonucleotide "allosteric effector" in the control of amino acid dependent pyrophosphate exchange reactions. BIOCHEM. J. 90:9F

Holley, R.W., Apgar, J. Doctor, B.P., Farrow, J., Marini, M.A. and Merrill, S.H. (1961) A simplified procedure for the preparation of tyrosine and valine acceptor reactions of yeast soluble ribonucleic acid. J. BIOL. CHEM. 236: 200

Hoober, J,K. and Stegman, W.J. (1973) Control of the synthesis of a major polypeptide of chlorop last membranes in Chlamydomonas reinhardi. J. CELL BIOL. 56: 1-12

Hoober, J.K. and Blobel, G. (1969) Characterization of the chloroplastic and cytoplasmic ribosomes of Chlamydomonas reinhardi. J. MOL. BIOL. 41: 122

Hudock, G.A. and Fuller, R.C. (1965) Control of triosephosphate dehydrogenase in photosynthesis. PLANT PHYSIOL. 40: 1205

Hutner, S.J. As cited in Greenblatt, C. and Schiff, J. (1959) A pheophytin-like pigment in dark adapted Euglena gracilis J. PROTOZOOL. 6: 23-28

Ingles, J., Wells, R., Possingham, J.V. and Leaver, C.J. (1971) The origins of chloroplast rRNAs. In "Autonomy and Biogenesis of Mitochondria and Chloroplasts". (N.K. Boardman, A.W. Linnane and R.M. Smillie, eds.) NORTH-HOLLAND/AMERICAN ELSEVIER PUBL. pp. 393-401

Ingram, V.M. and Sjoquist, J.A. (1963) Studies on the structure of purified alanine and valine transfer RNA from yeast. COLD SPRING HARBOR SYMPOSIUM QUANTITATIVE BIOLOGY 28: 133-138

Kaplan, S., Atherly, A,G. and Barrett, A. (1973) Synthesis of stable RNA in stringent E. coli cells in the absence of charged tRNA. PROC. NATL. ACAD. SCI. 70: 689-692

Kawashima, N. and Wildman, S.G. (1972) Studies of Fraction I protein. IV. Mode of inheritance of primary structure in relation to whether chloroplast or nuclear DNA contain the code for a chloroplast protein. BIOCHEM. BIOPHYS. ACTA 262: 42-49

Kayne, M.S. and Cohn, M. (1972) Cation requirements of isoleucyl tRNA synthetases from E. coli. BIOCHEM. BIOPHYS. RES. COMMUN. 46: 1285-1291

Kirk, J.T.O. (1964) Studies on RNA synthesis in chloroplast preparations. BIOCHEM. BIOPHYS. RES. COMMUN. 16: 233-238

Kirk, J.T.O. (1963) The deoxyribonucleic acid of broad bean chloroplasts. BIOCHEM. BIOPHYS. ACTA 76: 417-424

Kirk, J.T.O. and Tilney-Bassett, R.A.E. (1967) "The Plastids". W. H. FREEMAN & CO. (London and San Francisco)

Kislev, N., Selsky, M.I., Norton, C. and Eisenstadt, J.M. (1972) tRNA and tRNA aminoacyl synthetases of chloroplasts, mitochondria and cytoplasm from Euglena gracilis. BIOCHEM. BIOPHYS. ACTA 287: 256-269

Kislev, N., Swift, H. and Bogorod, L. (1965) Nucleic acids of chloroplasts and mitochondria in Swiss chard. J. CELL BIOL. 25: 327-344

Klein, S., Schiff, J.A. and Holowinsky, A.W. (1972) Events surrounding the early development of Euglena chloroplasts. II. Normal development of fine structure and the consequences of pre-illumination. DEVEL. BIOL. 28: 253-273

Kung, S.D., Thornber, J.P. and Wildman, S.G. (1972) Nuclear DNA codes for the photosystem II chlorophyll-protein of chloroplast membranes. FEBS LETTERS 24: 185-188

Lagerkvist, U., Ryno, L. and Waldenstrom, J. (1966) Structure and function of tRNA. II. Enzyme substrate complexes with valyl tRNA synthetase from yeast. J. BIOL. CHEM. 241: 5391

Lazzarine, R.A. and Mehler, A.H. (1966) In "Procedures in Nucleic Acid Research". (G.L. Cantoni and D.R. Davis, eds.) HARPER & ROW, N.Y. p. 409

Lazzarine, R.A. and Woodruff, A. (1964) Photoinduction of transhydrogenase in Euglena. BIOCHEM. BIOPHYS. ACTA 79: 412

Lewis, S.C., Schiff, J.A., and Epstein, H.T. (1965) Studies of chloroplast development in Euglena. IX. Chloroplast antigens and their appearance during chloroplast development. J. PROTOZOOL. 12: 281

Lindahl, T., Adams, A., and Fresco, J. (1966) Renaturation of tRNAs through site binding of magnesium. PROC. NATL. ACAD. SCI. 55: 941-948

Loening, V.E. and Ingle, J. (1967) Diversity of RNA components in green plant tissues. NATURE (London) 215: 363

Lyman, H., Epstein, H.T. and Schiff, J.A. (1959) Ultraviolet inactivation and photoreactivation of chloroplast development in Euglena without cell death. J. PROTOZOOL. 6: 264-266

Lyman, H., Epstein, H.T. and Schiff, J.A. (1961) Studies of chloroplast development in Euglena. I. Inactivation of green colony formation by UV light. BIOCHEM. BIOPHYS. ACTA 50: 301-309

Lyttleton, J.W. (1962) Isolation of ribosomes from spinach chloroplasts. EXP. CELL. RES. 26: 312

Makman, M.H. and Cantoni, G.L. (1966) Studies concerning the interaction of serine soluble ribonucleic acid with seryl soluble ribonucleic acid synthetase from Baker's yeast. BIOCHEM. 5: 2246-2254

Manning, J.E., Wolstenholme, D.R., Ryan, R.S., Hunter, J.A. and Richards, O.C. (1971) Circular chloroplast DNA from Euglena gracilis. PROC. NATL. ACAD. SCI. 68: 1169-1179

Merrick, W.C. and Dure, L.S. (1972) The developmental biochemistry of cotton seed embryogenesis and germination. J. BIOL. CHEM. 247: 7988-7999

Mets, L. and Bogorod, L. (1972) Altered chloroplast ribosomal proteins associated with erythromycin resistant mutants in two genetic systems of Chlamydomonas reinhardi. PROC. NATL. ACAD. SCI. 69: 3779-3783

Millar, D.B. and Steiner, R.F. (1966) The effect of environment on the structure and helix-ciol transition of soluble ribonucleic acid. BIOCHEM. 5: 2289-2301

Norris, A.T. and Berg, P. (1964) Mechanism of aminoacyl RNA synthesis: Studies with isolated aminoacyl adenylate complexes of isoleucyl RNA synthetases. PROC. NATL. ACAD. SCI. 52: 330-336

Norton, S,J,, Ravel, J.M., Lee, C. and Shive, J.W. (1963) Purification and properties of the aspartyl ribonucleic acid synthetase of Lactobacillus arabinosus. J. BIOL CHEM. 238: 269-274

Novelli, D.G. (1967) Amino acid activation for protein synthesis. ANN. REV. BIOCHEM. 36: 444-481

Ogata, K., Nohara, H., Ishikawa, K., Morita, T., and Asaoka, H. (1961) In "Protein Biochemistry". (R.J.C. Harris, ed.) ACAD. PRESS, N.Y. p. 163

Parthier, B., Krauspe, R. and Santleben, S. (1972) Light stimulated synthesis of aminoacyl tRNA synthetase in greening Euglena gracilis. BIOCHEM. BIOPHYS. ACTA 277: 335-341

Pearson, R.L., Weiss, J.F. and Kelmers, A.D. (1971) Improved separation of transfer RNAs on polychlorotrifluoroethylene-supported reversed phase chromatography columns. BIOCHEM. BIOPHYS. ACTA 228: 770-774

Polya, G.M. and Jagendorf, A.T. (1971) Wheat leaf RNA polymerases. II. Kinetic characterization and template specificities of nuclear, chloroplast and soluble enzymes. ARCH. BIOCHEM. BIOPHYS. 146: 649

Perini, F., Schiff, J.A. and Kamen, M.D. (1964) Iron containing proteins in Euglena. II. Functional location. BIOCHEM. BIOPH YS. ACTA 88: 91

Preston, J.F., Parenti, F. and Eisenstadt, J.M. (1972) Studies on the isolation and purification of chloroplasts from Euglena gracilis. PLANTA 107: 351-367

Pringsheim, F. and Pringsheim, O. (1952) Experimental elimination of chromatophores and eyespot in Euglena gracilis. NEW PHYTOL. 51: 65-76

Provasoli, L., Hutner, S.H. and Schatz, A. (1948) Streptomycin induced chlorophyll-less races of Euglena. PROC. SOC. EXP. BIOL. MED. 69: 279-282

Ravel, J.M., Wang, S.F., Heinmeyer, C., Shive, W.J. (1965) Glutamyl and glutaminyl ribonucleic acid synthetases of E. coli. J. BIOL. CHEM. 240: 432

Rawson, J.R. and Stutz, E. (1969) Isolation and characterization of Euglena gracilis cytoplasmic and chloroplast ribosomes and their RNA components. BIOCHEM. BIOPHYS. ACTA 190: 368

Ray, D.S. and Hanawalt, P.C. (1965) Satellite DNA components in Euglena gracilis cells lacking chloroplasts. J. MOL. BIOL. 11: 760-768

Reger, B.J., Fairfield, S.A., Epler, J.L. and Barnett, W.E. (1970) Identification and origin of some chloroplast aminoacyl tRNA synthetases and tRNAs. PROC. NATL. ACAD. SCI. 67: 1207-1213

Ris, H. and Plaut, M. (1962) Ultrastructure of DNA containing areas in the chloroplast of Chlamydomonas. J. CELL BIOL. 13: 383-291

Ritter, P.O. and Jacobson, K.B..(1972) Interactions of phenylalanyl transfer ribonucleic acid synthetase of Neurospora crassa with valyl tRNA of E. coli. J. BIOL. CHEM. 247: 7603-7608

Roy, K.L., Bloom, A. and Soll, D. (1971) tRNA separation using benzoylated DEAE-cellulose in "Procedures in Nucleic Acid Research" II. (G.L. Cantoni and D.R. Davis, eds.) HARPER & ROW, N.Y. pp. 524-541

Sager, R. (1972) Cytoplasmic DNAs in "Cytoplasmic Genes and Organelles". ACAD. PRESS (London and New York) p. 5

Sager, R. (1972) Cytoplasmic DNAs in "Cytoplasmic Genes and Organelles". ACAD. PRESS ((London and New York) p.30

Sager, R. (1972) Cytoplasmic DNAs in "Cytoplasmic Genes and Organelles". ACAD. PRESS (London and New York) p. 301

Schiff, J.A. (1971) The informational and nutritional requirements of cellular organelles. STADLER SYMP. 3 Univ. of Missouri 89-114

Schiff, J.A. (1963) Oxygen exchange by Euglena cells undergoing chloroplast development. CARNEGIE INSTITUTE WASHINGTON YEARB. 62: 375

Schiff, J.A. and Epstein, H.T. (1966) The replicative aspect of chloroplast continuity in Euglena. In "Biochemistry of Chloroplasts" I. (T.W. Goodwin, ed.) ACAD. PRESS, New York pp. 341-353

Schiff, J.A., Lyman, H. and Epstein, H.T. (1961) Studies of chloroplast development in Euglena. II. Photoreversal of the UV inhibition of green colony formation. BIOCHEM. BIOPHYS. ACTA 50: 310-318

Schiff, J.A., and Zeldin, M.H. (1968) The developmental aspect of chloroplast continuity in Euglena. J. CELL PHYSIOL. 72: 103

Schweiger, H.G., Dillard, W.L., Gibor, A. and Berger, S. (1967) RNA synthesis in Acetabularia. I. RNA synthesis in enucleated cells. PROTOPLASMA 64: 1

Scott, N.S., Shah, V.C. and Smillie, R.M. (1968) Synthesis of chloroplast DNA in isolated chloroplasts. J. CELL BIOL. 38: 151-57

Scott, N.S. and Smillie, R.M. (1967) Evidence for the direction of chloroplast ribosomal RNA synthesis by chloroplast DNA. BIOCHEM. BIOPHYS. RES. COMMUN. 28: 598

Semal, J., Spencer, D., Kim, Y.T. and Wildman, S.G. (1964) Properties of a ribonucleic acid synthesizing system in cell-free extracts of tobacco leaves. BIOCHEM. BIOPHYS. ACTA 91: 205

Shah, V.C., and Lyman, H. (1966) DNA dependent RNA synthesis in chloroplasts of Euglena gracilis. J. CELL BIOL. 29: 174-176

Smillie, R.M., Graham, D., Dwyer, M.R., Grieve, A. and Tobin, N.F. (1967) Evidence for the synthesis in vivo of proteins of the Calvin cycle and of the photosynthetic electron transfer pathway on chloroplast ribosomes. BIOCHEM BIOPHYS. RES. COMMUN. 28: 604-610

Smith, D.W.E. and McNamara, A.L. (1967) Specialization of rabbit reticulocytes tRNA content for hemoglobin synthesis. SCIENCE 171: 577-579

Spencer, D. and Whitfield, P.R. (1967) Ribonucleic acid synthesizing activity of spinach chloroplasts and nuclei. ARCH. BIOCHEM. BIOPHYS. 121: 336

Spencer, D. and Whitfield, P.R. (1969) The characterization of spinach chloroplast DNA polymerase. ARCH. BIOCHEM. BIOPHYS. 132: 477-488

Spencer, D., Whitfield, P.R., Bottomley, W. and Wheeler, A.M. (1971) The nature of the proteins and nucleic acids synthesized by isolated chloroplasts. In "Autonomy and Biogenesis of Mitochondria and Chloroplasts". AUST. ACAD. SCI. SYMP. (N.K. Boardman, A.W. Linnane and R.M. Smillie, eds.) North Holland Publ. Amsterdam pp. 372-382

Stephenson, D.M. and Sheridan, W.F. (1965) Incorporation of ^{3}H thymidine into chloroplast DNA of marine algae. J. CELL BIOL. 25: 619-626

Stern, A.I., Schiff, J.A. and Epstein, H.T. (1964) Studies of chloroplast development in Euglena. V. Pigment biosynthesis, photosynthetic oxygen evolution and CO_2 fixation during chloroplast development. PLANT PHYSIOL. 39: 220

Stutz, E. and Noll, H. (1967) Characterization of cytoplasmic and chloroplast polysomes in plants. PROC. NATL. ACAD. SCI. 57: 774

Stutz, E. (1970) The kinetic complexity of Euglena gracilis chloroplast DNA. FEBS LETTERS 8: 25-28

Stutz, E. and Rawson, J.R. (1970) Characterization of Euglena gracilis chloroplast single strand DNA. BIOCHEM BIOPHYS. ACTA 209: 16-23

Stutz, E. and Vandrey, J.B. (1971) Ribosomal DNA satellite of Euglena gracilis chloroplast DNA. FEBS LETTERS 17: 277-280

Sueoka, K.T. and Sueoka, N. (1969) Leucine tRNA and cessation of E. coli protein synthesis upon phage T-2 infection. PROC. NATL. ACAD. SCI. 62: 1229-1236

Surzycki, S.J. (1969) Genetic functions of the chloroplast of Chlamydomonas reinhardi: Effect of rifampin on chloroplast DNA-dependent RNA polymerase. PROC. NATL. ACAD. SCI. 63: 1327

Taylor, M.W., Granger, G.A., Buck, C.A. and Halland, J.J. (1967) Similarities and differences among specific tRNAs in mammalian tissue. PROC. NATL. ACAD. SCI. 57: 1712-19

Tewari, K.K. (1971) Genetic autonomy of extranuclear organelles. ANN. REV. OF PLANT PHYSIOL. 22: 141-168

Tewari, K.K. and Wildman, S.G. (1967) DNA polymerase in isolated tobacco chloroplasts and the nature of the polymerized product. PROC. NATL. ACAD. SCI. 58: 689-96

Tewari, K.K. and Wildman, S.G. (1970) Information content in the chloroplast DNA. In "Control of Organelle Development". SYMP. SOC. EXP. BIOL. 24: 147-180 (P.L. Miller, ed.) Cambridge Univ. Press, London

Thomas, J.R. and Tewari, K.K. (1974) Conservation of 70S ribosomal RNA genes in the chloroplast DNAs of higher plants. PROC. NATL. ACAD. SCI. 71: 3147-3151

Tokimoto, T., Takeishi, K., Nishimura, S. and Ukita, T. (1973) Transfer of valine into rabbit hemoglobin from various isoaccepting species of valyl tRNA differing in codon recognition. EUR. J. OF BIOCHEM. 38: 489-496.

Vold, B. (1973) Analysis of isoaccepting tRNA species of Bacillus subtilus: Chromatographic differences between tRNA from spores and cells in exponential growth. J. OF BACT. 113: 825-833

Wells, R. and Sager, R. (1971) Denaturation and the renaturation kinetics of chloroplast DNA from Chlamydomonas reinhardi. J. MOL. BIOL. 58: 611-622

Wells, R. and Birnsteil, M. (1969) Kinetic complexity of chloroplastal DNA and mitochondrial DNA from higher plants. BIOCHEM. J. 112: 777-786

Whitfield, P.R. (1973) Chloroplast RNA. In "The Ribonucleic Acids". (P.R. Stewart and D.S. Letham, eds.) Springer-Verlag (New York, Berlin, Heidelberg) pp. 179-206

Williams, G.R. and Williams, A.S. (1970) Hybridization of bean leaf leucyl tRNA with nuclear DNA and with chloroplast DNA. BIOCHEM. BIOPHYS. RES. COMMUN. 39: 858

Wolken, J.J. and Palade, G.E. (1953) An electron microscope study of two flagellates. Chloroplast structure and variation. ANN. N.Y. ACAD. SCI. 56: 873

Wollgiehn, R. and Mothes, K. (1964) Uber die incorporation ^{3}H-thymiden in die chloroplasten-DNS von Nicotiana rustica. EXP. CELL RES. 35: 52-57

Woodcock, C.L.F. and Bogorod, L. (1971) Nucleic acids and information processing in chloroplasts. In "Structure and Function of Chloroplasts". (M. Gibbs, ed) Springer-Verlag, New York pp. 89-120

Yaniv, M. and Gros, F. (1969) Studies on valyl tRNA synthetases and $tRNA^{val}$ from E. coli. II. Interaction between valyl tRNA synthetases and valine acceptor tRNA. J. MOL. BIOL. 44: 17-30

Yarus, M. (1972) Intrinsic precision of aminoacyl tRNA synthesis enhanced through parallel systems of ligands. NATURE NEW BIOL. 239: 106-108

Yarus, M. and Berg, P. (1969) Recognition of tRNA by isoleucyl tRNA synthetase. Effect of substrates on the dynamics of tRNA enzyme reaction. J. MOL. BIOL. 42: 171-189

Yarus, M. and Rashbaum, S. (1972) Divalent cations in tRNA and aminoacyl tRNA synthetase function and structure. BIOCHEM. 11: 2043-2049

Yarus, M. and Mertes, M. (1973) The variety of intraspecific misacylations carried out by isoleucyl tRNA synthetases of E. coli. J. BIOL. CHEM. 248: 6744-6749

Yarus, M. (1973) Pseudoverification. Hydrolysis of aminoacyl tRNA catalyzed by an aminoacyl tRNA synthetase in mixed solvents. J. BIOL. CHEM. 248: 6750-6754

Yegian, C.D. and Stent, G.S. (1969) Differential aminoacylation of three species of isoleucine transfer RNA from E. coli. J. MOL. BIOL. 39: 59-71

Zeldin, M.H. and Schiff, J.A. (1967) RNA metabolism during light induced chloroplast development in Euglena. PLANT PHYSIOL. 42: 922

Zeldin, M.H. and Schiff, J.A. (1968) A comparison of light dependent RNA metabolism in wild type Euglena with that of mutants impaired for chloroplast development. PLANTA 81: 1-15

Zimmerman, T.P. and Robinson, B. (1972) Effect of assay conditions on the magnesium requirement of the transfer reaction catalyzed by phenylalanyl tRNA synthetase from Baker's yeast. BIOCHEM. BIOPHYS. RES. COMMUN. 47: 1138-1148